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Strathclyde
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**Does Protease-activated Receptor
2 (PAR2) Modulate Human
Astrocyte Reactivity?**

By

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**A thesis submitted in fulfilment of the requirements for the
degree of Doctor of Philosophy**

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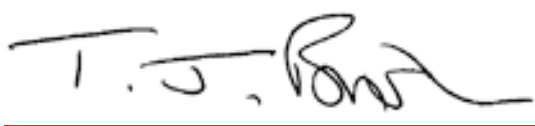
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Disclaimer

English is not my first language. This thesis has been written with the assistance of Perplexity AI for grammar correction, sentence structure improvement, and language polishing to ensure clarity and academic standards. All scientific content, experimental data, analyses, interpretations, and conclusions represent my original research and intellectual contributions. The use of AI was limited strictly to enhancing language expression and does not affect the authenticity or validity of the research findings.

As her PhD supervisor, I can confirm that I provided feedback on the original chapters written by Aparna. Her use of Perplexity AI has not altered the scientific content but was used to solely improve the written language.

A handwritten signature in black ink, appearing to read 'T. J. Bushell', is written above a solid red horizontal line. To the left of the signature, there is a short vertical black line.

Dr Trevor Bushell

01/12/2025

Abstract

Introduction: Astrocytes are the most abundant glial cells in the central nervous system, playing critical roles in maintaining normal function as well as contributing to disease processes. In many neurodegenerative diseases, astrocytes undergo activation and transition into a reactive phenotype, which is proposed to exacerbate disease progression. Hence, these findings have fuelled investigations into whether reducing astrocytic reactivity is a novel pathway to develop therapies for diseases like Alzheimer's. In a 5X FAD, mouse model of amyloid pathology, we have confirmed that astrocytic reactivity is increased and following protease-activated receptor 2 (PAR2) activation, astrocytic reactivity is significantly reduced leading to reduced amyloid pathology. However, whether modulation of astrocytic reactivity occurs in human astrocytes remains unknown. Hence, in the present study, we hypothesise that PAR2 activation will impair astrocytic reactivity when examined in commercially available human astrocytes.

Methods:

Ca²⁺ imaging and western blotting were undertaken in commercially available human astrocytes (cHAs) to examine the consequence of PAR2 activation in naïve cHAs. Astrocytic reactivity was assessed using immunocytochemistry using markers for reactivity, including GFAP, C3 and Gbp2.

Results: PAR1 and PAR2 activation resulted in significant increases in intracellular Ca^{2+} levels in cHAs, which were inhibited by the PLC inhibitor U73122. However, in contrast to previous studies, PAR1 or PAR2 activation did not affect MAPK signalling in cHAs. Similarly, no differences in astrocytic reactivity were observed in cHAs, quantified using astrocytic reactivity markers, when cultured in either serum or serum-free media, as has been reported previously. In addition, PAR1 and PAR2 activation did not alter astrocytic reactivity in cHAs under either media condition. Moreover, these experiments were repeated in human iPSC-derived astrocytes to make a direct comparison with cHAs and determine whether the type of human astrocytes markedly alters their reactivity and potential modulation. TIC stimulation did not induce reactivity in iPSC-derived astrocytes although increased reactive marker transcripts levels were observed from transcriptomic analysis. However, TIC stimulated iPSC-derived astrocytes exhibited increased phagocytic function. In contrast, phagocytosis was unchanged in cHAs upon TIC stimulation.

Conclusion: These findings confirm, for the first time, the presence of functional PARs in human astrocytes. However, neither serum-containing nor serum-free media, nor PAR activation, altered the reactivity or phagocytic activity of cHAs, underscoring the complex and heterogeneous nature of astrocytic reactivity.

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List of abbreviations

Abbreviation	Full Form
AC264613	2-amino-4-(5-chloro-2-[[3-(4-methylpiperazin-1-yl)propylamino]carbonyl]phenyl)-6-[4-(trifluoromethyl)phenyl]pyridine-3-carbonitrile
A β ₁₋₄₂	Amyloid-beta peptides
AD	Alzheimer's disease
ADP	Adenosine diphosphate
AGS	Astrocyte growth supplement
ALDH1L1	Aldehyde dehydrogenase 1 family member L1
ANOVA	Analysis of Variance
AP	Activating peptide
APS	Ammonium persulfate
APQ4	Aquaporin-4
a.u.	Arbitrary units
AYPGKF	PAR4 activating peptide
BCA	Bicinchoninic acid
BMP4	Bone morphogenetic protein 4
BSA	Bovine serum albumin

C1q	Complement Component 1q
C3	Complement component 3
[Ca ²⁺] _i	Intracellular calcium concentration
CCD	Charge-coupled device
cHA	Commercial human astrocytes
CNS	Central nervous system
DAG	Diacylglycerol
DHPG	(S)-3,5-dihydroxyphenylalanine
DIV	Days <i>in vitro</i>
DMEM	Dulbecco's modified eagle medium
DMEM/F12	Dulbecco's modified eagle medium/nutrient mixture f-12
DMSO	Dimethyl sulfoxide
DPP	Days post-plating
DTT	Dithiothreitol
EAAT	Excitatory amino acid transporters
EC ₅₀	Half-maximal effective concentration
EDTA	Ethylenediaminetetraacetic acid
ERK1/2	Extracellular signal-regulated kinase 1/2

FBS	Fetal bovine serum
FGF2	Fibroblast growth factor 2
Gbp2	Guanylate-binding protein 2
GFAP	Glial fibrillary acidic protein
GPC	Glial precursor cell
GPCR	G protein-coupled receptor
HA	Human astrocytes
HBSS	Hank's balanced salt solution
HBS	Hepes-buffered saline
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IFN- γ	Interferon-gamma
IL-1 β	Interleukin-1 beta
IL-6	Interleukin-6
IP ₃	Inositol 1,4,5-trisphosphate
iPSC	Induced pluripotent stem cell
KCl	Potassium chloride
LIF	Leukemia inhibitory factor
LRRK2	Leucine-rich repeat kinase 2

LPS	Lipopolysaccharide
MAP-2	Microtubule-associated protein 2
MAPK	Mitogen-activated protein kinase
MEGF10	Multiple egf-like domains 10
MERTK	Mer proto-oncogene tyrosine kinase
MET	Mesenchymal-to-epithelial transition
mGlu	Metabotropic glutamate receptor
N2B27	N2 and B27 Supplement Medium
NEAA	Non-essential amino acids
NF- κ B	Nuclear factor kappa b
OHSC	Organotypic hippocampal slice cultures
P/S	Penicillin/streptomycin
PAR	Protease-activated receptor
PAR1	Protease-activated receptor 1
PAR2	Protease-activated receptor 2
PAR3	Protease-activated receptor 3
PAR4	Protease-activated receptor 4
PBS	Phosphate-buffered saline

PBS-T (PBST)	Phosphate-Buffered Saline with Tween-20
PD	Parkinson's disease
PDL	Poly-d-lysine
PDGF	Platelet-derived growth factor
PIP ₂	Phosphatidylinositol 4,5-bisphosphate
PLC	Phospholipase C
PLC/IP ₃	Phospholipase c/inositol triphosphate
PLL	Poly-l-lysine
PSEN1	Presenilin 1
qRT-PCR	Quantitative real-time PCR
RIPA	Radioimmunoprecipitation assay buffer
ROI	Region of Interest
RPM	Revolutions per minute
SeV	Sendai virus
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Standard Error of the Mean

SLIGKV-NH ₂	PAR2 activating peptide
SLIGRL-NH ₂	PAR2 activating peptide
SOD1	Superoxide dismutase 1
SOX-9	Sry-box transcription factor 9
STAT3	Signal Transducer and Activator of Transcription 3
TEMED	Tetramethylethylenediamine
TFLLR-NH ₂	PAR1 activating peptide
TIC	TNF- α , IL-1 β , and C1q (Inflammatory Cytokine Cocktail)
TNF- α	Tumor necrosis factor alpha
TGF- β	Transforming growth factor beta
TRPV4	Transient receptor potential vanilloid 4
U73122	1-(6-((17 β -3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione
WT	Wild type

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CHAPTER 1

Introduction

1. Introduction

1.1 The central nervous system

The central nervous system (CNS) is a critical component of the human nervous system, comprising the brain and spinal cord. It serves as the primary control centre for processing sensory information, coordinating bodily functions, and facilitating complex processes such as thought, memory, and emotion (Sharon et al., 2016). The CNS integrates sensory inputs, generates responses, and maintains homeostasis by communicating with the peripheral nervous system (PNS), which includes nerves and ganglia outside the CNS (Georgopoulos & Carpenter, 2020). The brain, consists of specialized regions, including the cerebrum, cerebellum, and brainstem, each responsible for distinct functions. The cerebrum governs higher cognitive functions, such as reasoning and language, while the cerebellum coordinates movement and balance, along with contributing to higher cognitive functions, emotions and behaviour (Kim et al., 2024). The brainstem regulates vital autonomic functions like heart rate and breathing (Georgopoulos & Carpenter, 2020; Leonard, 1993). The spinal cord, encased in the vertebral column, acts as a conduit for signals between the brain and the body, mediating reflexes and motor control (Suzuki & Dickenson, 2000).

Until recently, the CNS was regarded as an immune-privileged site, characterized by limited interaction with the peripheral immune system to protect its delicate neural environment. This concept posited that CNS immune activities were largely independent of systemic immune responses as it is

tightly regulated by the blood-brain barrier (BBB). The BBB consists primarily of specialized endothelial cells that line the luminal surface of CNS blood vessels, reinforced by pericytes and an encircling basal lamina. In contrast to peripheral endothelial cells, CNS endothelial cells are characterized by tight, unfenestrated junctions that effectively limit paracellular diffusion of water, ions, and large molecules (Kadry et al., 2020; Rust et al., 2025). The BBB's functional integrity depends not only on these endothelial cells but also on the basal lamina, pericytes, and astrocytes. Together, these components collaborate to uphold the barrier's selective permeability, tightly regulating molecular exchange to safeguard the CNS (Anderson et al., 2011; Kadry et al., 2020; Rust et al., 2025). Furthermore, within the CNS, resident immune cells, notably microglia, maintain constant surveillance of the neural microenvironment (Kadry et al., 2020; Paolicelli et al., 2022; Rust et al., 2025). These cells play a pivotal role in clearing cellular debris and defending against pathogenic threats. Upon detection of a potential danger, such as invading pathogens, microglia initiate an immune response, triggering the activation and recruitment of peripheral immune cells. This process involves the transient modulation of BBB permeability, allowing activated immune cells to cross into the CNS to assist in neutralizing the threat (Arima et al., 2013; Ballabh et al., 2004; Rust et al., 2025; Yang et al., 2010). Thus, BBB is dynamic barrier rather than just a static and unfenestrated barrier, and it selectively permits molecules such as glucose, amino acids, hormones, vitamins and gases such as oxygen and restricts any infiltration of peripheral immune cells and pathogens, (**Figure 1**).

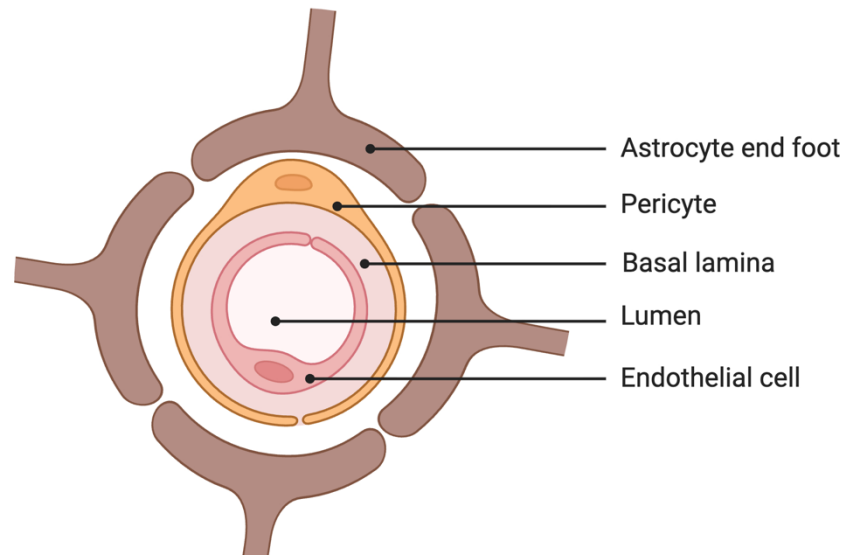


Figure 1: An overview of the blood brain barrier (BBB): Schematic representation of transverse section of the BBB showing endothelial cells forming the tight junctions that act as maintain the integrity by acting as a physical, metabolic and transport barrier. These are surrounded by the basal lamina, pericytes, and astrocytic endfeet which also contribute to the BBB integrity. Created in <https://BioRender.com>.

1.2 Cells types of the CNS

1.2.1 Neurons

Neurons are the fundamental units of the nervous system, specialized for processing and transmitting information through electrochemical signals. These highly specialized cells underpin the functionality of the central and peripheral nervous systems, enabling sensory processing, motor coordination, and cognitive functions such as learning and memory. Structurally, neurons consist of a cell body (soma), dendrites, and an axon. The soma houses the nucleus and organelles, dendrites receive incoming signals, and the axon conducts electrical impulses away from the cell body to other neurons or effector cells (Albright et al., 2000; Purves, 2022).

1.2.2 Microglia

The term “microglia” was coined by Del Rio-Hortega in the 20th century (Lannes et al., 2017). They are the brain-resident myeloid cells and are the main immunocompetent cells within the CNS, comprising of almost 0.5%-16% of the total cells within the CNS. They have the capacity to regenerate and play a major role in brain development and maintenance of proper neuronal function throughout life is widely recognized (Askew et al., 2017; Thion et al., 2018). They mediate key functions to support the CNS, and utilize their specific receptor repertoire to dynamically monitor the microenvironment in the brain (Arcuri et al., 2017; Paolicelli et al., 2022; Woodburn et al., 2021). The heterogeneous nature and the complexity of microglial cells can lead to different functions and morphological adaptations depending on the

microenvironment (Zhang et al., 2025). Under normal physiological conditions they are considered to be in a resting state (Sun et al., 2022; Woodburn et al., 2021), and conduct continuous immunosurveillance and detect any change in the microenvironment through their processes (Duggan & Parikh, 2021; Nimmerjahn et al., 2005), while studies have shown that they undergo a morphological change and become ramified with increased cell body volume (Green & Rowe, 2024; Lannes et al., 2017; Loh et al., 2024) and release cytokines upon detection of environmental changes (Guo et al., 2022; Madry et al., 2018; Wang et al., 2021). However, microglia release proinflammatory mediators upon prolonged activation (Lyman et al., 2014; Madry et al., 2018) that play a major role in contributing to neuroinflammation, inducing significant changes in the function and structure of the CNS (Bhusal et al., 2022; Khan & Chang, 2023; Lannes et al., 2017). Consequently, microglia are critical component for immune responses within the CNS, but their ability to maintain CNS homeostasis and their response to injury and pathogens is also linked with astrocytic function. Prolonged microglial activation has an impact on the broader cellular milieu, causing phenotypic changes in astrocytes as a response to inflammatory signals, playing a pivotal role in determining the outcome of tissue repair or neurodegeneration (Hurley & Tizabi, 2013; Lee et al., 2023; Lyman et al., 2014). Without considering astrocytes the whole concept of neuroinflammation remains incomplete, as they are the most abundant of the glial population and engage in extensive bidirectional crosstalk with microglia and neurons to orchestrate both neuroinflammatory and

neuroprotective functionalities within the CNS (Beurel et al., 2020; M. Sun et al., 2022; Woodburn et al., 2021).

1.2.3 Astrocytes

1.2.3.1 *Physiology and functions.*

Astrocytes were first described by Rudolf Virchow (Virchow et al., 1860) as non-neuronal cells termed the “neuroglial” component in the CNS. This concept of neuroglia was conceived as neural glue to help maintain the structural integrity of the brain. However, this concept was later changed by Santiago Ramón y Cajal, where he provided morphologically distinct subtypes of astrocytes, with perivascular endfeet and peri-synaptic processes (R. Cajal, 1913; S. Cajal, 1897; Ramón y Cajal, 1895, 1899).

As discussed above, microglia comprise almost 5% - 10 % of the total CNS while astrocytes comprise 50% of the brain and spinal cord cells in humans and mice (Allen & Lyons, 2018; Zhang et al., 2016). They provide trophic support to the neurons and aid in synapse formation, maturation and maintenance (Zuchero & Barres, 2015). Their fine processes are connected with others through gap junctions, and communicate with each other via calcium signalling (Hasel & Liddelow, 2021). They are derived in the subventricular zone from neural progenitor cells and they migrate along the radial glia processes during the late embryonic and early postnatal development. The main purpose of them is to maintain CNS homeostasis, synaptic transmission, synaptogenesis modulation and maintain the BBB (Vasile et al., 2017). They form a thin astrocytic end-feet barrier that is a major

component of the BBB and surrounds endothelial cells, pericytes, and the basal lamina and respond to molecules produced by peripheral immune cells – cytokines and potential pathogens (Liddelw & Barres, 2017; Sofroniew & Vinters, 2010; Zhou et al., 2019).

Another interesting role of astrocytes is their participation in the formation of tripartite synapses (Perea & Araque, 2005; Endo et al., 2022) where they are involved in the modulation of synaptic transmission and plasticity, and for the extensive communication between astrocytes and neurons is through ion channels, neurotransmitters and through metabolites (Endo et al., 2022; Hasel & Liddelw, 2021). For instance, astrocytes regulate extracellular potassium levels in the CNS through specialized K^+ channels and spatial buffering mechanisms. (Walz, 2000), to maintain neuronal membrane potential and excitability. However a downregulation of this mechanism can lead to neuronal hyperexcitability of neurons which is often associated with neurological disorders (Endo et al., 2022). Finally, astrocytes are responsible for the uptake of excess glutamate from the synapse via glutamate transporters and dysfunction of these transporters can lead to excessive glutamate in the synapse causing neuronal death via excitotoxicity (Hasel & Liddelw, 2021). Notably, recent studies involving single cell transcriptomics and molecular profiling has revealed regional heterogeneity among astrocytes. It is shown that they exhibit specific molecular signatures, specialized functions across various regions of the brain (Bocchi et al., 2025a; Hennes et al., 2025). For example, forebrain astrocytes show proliferative activity which is absent in the

cerebellar white matter astrocytes (Bocchi et al., 2025b). There are region specific gene expression profiles reported which underpin the various specialised role of astrocytes in various regions of the CNS. These differences contribute to synaptic plasticity and to the overall CNS health, underscoring their importance in studying their functions and their roles in pathophysiology (Clayton & Liddelow, 2025; Matias et al., 2019; Torres-Ceja & Olsen, 2022). It is important to understand this heterogeneity to develop model systems to investigate astrocytic biology. Induced pluripotent stem cells have emerged as a potential and a powerful tool to replicate human astrocyte heterogeneity *in vitro* to understand its mechanisms under health and disease conditions.

1.3 Introduction to induced pluripotent stem cells (iPSCs)

The development of iPSC technology for *in vitro* modelling of human biology created vast opportunities for cell therapy and therapeutic interventions for disease modelling. iPSCs are reprogrammed somatic cells, that attained pluripotency, giving them the capacity to differentiate into virtually any cell type, while the ability to unlimited self-renewal is maintained (Cerneckis et al., 2024a; Rowe & Daley, 2019; Yu et al., 2007a). The first reports of mouse and human iPSC from somatic cell programming was reported in 2006 and 2007 (Shi et al., 2017; Yu et al., 2007b), since then iPSCs have been extensively used in drug screening assays, and in *in vitro* human disease modelling.

iPSC-based cellular models mimic human-specific phenotype and molecular mechanisms which do not necessarily exist in animal models (DeFronzo & Dai, 2025; Matiukhova et al., 2025). Studies have shown that iPSCs can be modified and differentiated into many cell types, providing a platform to develop autologous and allogenic cell therapies and this technology holds a great promise for developing novel therapeutic strategies for many diseases (DeFronzo & Dai, 2025; Matiukhova et al., 2025; Yamanaka, 2020). For example, autologous iPSC-derived retinal pigment epithelium was transplanted in the patients with macular degeneration demonstrating its feasibility and safety of ocular-matched patients (Dehghan et al., 2022; Madrid et al., 2021). Also, Kyoto trial was the first human-trial in Japan which involved transplantation of allogeneic iPSC-derived CORIN-sorted dopaminergic progenitors into putamen of patients with Parkinson Disease (Sawamoto et al., 2025; R. Takahashi et al., 2025).

1.3.1 Reprogramming somatic cells – molecular mechanisms and maintenance

Reprogramming of somatic cells gives them the capacity for pluripotency. This reprogramming causing extensive cellular remodelling (Matiukhova et al., 2025; Zhu et al., 2013). This occurs in two states classified as early stochastic phase wherein the somatic genes are silenced and early pluripotent genes are activated, and then the late phase, which activates the core pluripotency factors (Apostolou & Stadtfeld, 2018; Matiukhova et al., 2025; Simic et al., 2019; Y. Wu et al., 2019). Overall, reprogramming includes, changes to

chromatin structure, cellular processes and epigenome (Pei et al., 2019; Simic et al., 2019; Wu et al., 2019). Furthermore, iPSC's are derived from somatic cells namely fibroblasts, and via the process that involves mesenchymal-to-epithelial transition (MET) they acquire pluripotency. This occurs through the conversion of fibroblasts which exhibit mesenchymal properties, to epithelial like cells, which possess differentiation and proliferative properties (Cerneckis et al., 2024a; Pei et al., 2019; Shi et al., 2017).

The three main modern reprogramming strategies include mRNA transfection, use of Sendai virus (SeV), and the use of chemical reprogramming with small molecules. mRNA transfections includes the delivery of OCT4, SOX2, KLF4, and c-MYC via the transient expression, and reduces mutation risks and genomic alterations, at the same time provides faster reprogramming kinetics (Di Giammartino et al., 2019; García-Gutiérrez et al., 2019; Roberts et al., 2021; Vanzan et al., 2021). SeV is a non-pathogenic virus that helps in efficient transport of reprogramming factors without its integration into the host genome (Chao et al., 2022; Feng et al., 2020). Chemical reprogramming employs the use of small molecule combinations for the regulation of molecular pathways (Apostolou & Stadtfeld, 2018; Matiukhova et al., 2025).

The tightly regulated system to maintain iPSC pluripotency depends on cell cycle control, metabolism, signalling pathways and epigenetics. Pluripotent stem cells have a short G1 phase in their cell cycle which helps maintaining genomic stability and pluripotency (DeFronzo & Dai, 2025; Matiukhova et al., 2025; Shi et al., 2017; Yamanaka, 2020). To maintain these cells an optimised

cultural environment is needed with defined concentrations of growth factors including FGF2, LIF, and other cytokines essential for optimal maintenance and expansion (Chen et al., 2024; Kamińska & Kochański, 2024). For example, FGF2 helps in reducing the frequency of media changes as it has reduced degradation rates, which then reduces heterogeneity and thereby induces long term pluripotency (**Figure 2**) (Apostolou & Stadtfeld, 2018; Y. Chen et al., 2024; Matiukhova et al., 2025).

1.3.2 Differentiation into glial progenitor cells (GPCs)

The first critical step towards generating astrocytes requires recapitulating early neural development, which involves the differentiation of iPSCs into NPCs. This employs dual-SMAD inhibition combined with Wnt/ β -Catenin inhibition which selectively induces the formation of cortical NPCs (Monguió-Tortajada et al., 2019; Neaverson et al., 2023). It is this widely employed process that generates NPCs in 10-14 days with initially the neural induction phase (days 0-10) and finally the neural maintenance phase (days 10-14) (Chen et al., 2024; Feng et al., 2020). Upon establishment of this stable NPC population, it can further be directed towards more specific neural lineages, and among these is the generation of glial progenitor cells. This is a crucial intermediate stage, marking the transition from multipotent NPC to restricted glial lineages that give rise to astrocytes and oligodendrocytes (Matiukhova et al., 2025; Santos et al., 2017a, 2022).

Generation of astrocytes from GPCs and their derivation from NPCs lays an efficient foundation to astrocyte production. It is a 3-4 week protocol to differentiate NPCs to GPCs which utilises specific growth factors, including basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), for astrocyte induction and maturation (O'Toole et al., 2024; Shi et al., 2017; Takahashi et al., 2007; Yamanaka, 2020). Cells are cultured in astrocyte-promoting medium which contains PDGF, essential for activating JAK/STAT3 signalling pathway, promoting gliogenesis and suppressing neurogenesis (Llorente et al., 2021; Neaverson et al., 2023; Santos et al., 2017a). BMP antagonist is also added to promote glial fate via the modulation of the SMAD pathway (Llorente et al., 2021). GPC-based protocols are advantageous as the cells can be cryopreserved in liquid nitrogen and later thawed for rapid astrocyte differentiation, allowing batch standardisation and overall astrocyte production timeline is also reduced (Bose et al., 2025; O'Toole et al., 2024; Savchenko et al., 2019).

1.3.3 Differentiation of GPCs into astrocytes

Terminal differentiation of GPCs into matured and functional astrocytes need activation of specific signalling pathways, to recapitulate the development of astrocyte maturation. GPCs are maintained in a medium containing leukemia inhibitory factor (LIF) and bone morphogenetic protein (BMP4) which together activate the JAK-STAT3 signalling pathway (Nakashima et al., 1999), that is required for the induction of astrocyte differentiation. Additionally LIF promotes MAPK pathway activation particularly extracellular signal-regulated

kinase (ERK1/2) which is important for promoting astrocyte specification and maturation. BMP4 signals via SMAD pathway activating JAK-STAT3 inducing astrocyte specific gene expression and induce functional maturity (Nakashima et al., 1999; Pistollato et al., 2017; Scholze et al., 2014; TCW et al., 2017a; Yu et al., 2007a). The differentiation of GPCs involves 3-week of proliferative stage and a subsequent of 3-week maturation phase, which enhances the expression of astrocyte-specific genes and helps in reducing reactive phenotypes (Hong & Song, 2014; Schuurmans et al., 2024; TCW et al., 2017a).

1.3.3.1 *Molecular characterisation of mature astrocytes*

Molecular characterisation is important to confirm their identity, purity, maturity, of iPSC-derived astrocytes to differentiate from GPCs. Immunofluorescence, qRT-PCR and RNA sequencing, are used to identify and verify astrocyte-specific populations (Lendemeijer et al., 2024; Leventoux et al., 2020). The maturation status of iPSC-derived astrocytes can be assessed by examining the expression of stage-specific astrocytic markers. The late developmental astrocyte markers include GFAP, S100 β , and AQP4 (Lendemeijer et al., 2024; Santos et al., 2017b). While, the early markers to identify astrocytes include ALDH1L1 (Leventoux et al., 2020; Santos et al., 2017b; Szabo et al., 2021). Immunocytochemical validation at 4–6 weeks of culture confirmed high expression of key astrocytic markers, including GFAP, S100 β , and AQP4, with enrichment levels exceeding 90% GFAP-positive

cells and 99% S100 β -positive cells. (Llorente et al., 2021; Neaverson et al., 2023; Santos et al., 2017b; Szabo et al., 2021).

Finally, establishing well-characterised protocols of deriving astrocyte populations from iPSCs, makes it feasible to model disease conditions *in vitro* including neuroinflammation. Astrocytes play a pivotal role not only in maintaining CNS homeostasis, but also in disease initiation and progression. Their functional dysregulation can contribute to neurodegeneration, and in modulation of inflammatory responses. Thus, iPSC-derived astrocyte, provide an interesting platform to investigate complex cellular mechanisms in astrocyte-mediated disease onset, progression and therapeutic interventions including neuroinflammation associated with many CNS diseases (Qiu & Caiazzo, 2025; Rowland et al., 2025; Stöberl et al., 2023; TCW et al., 2017b)

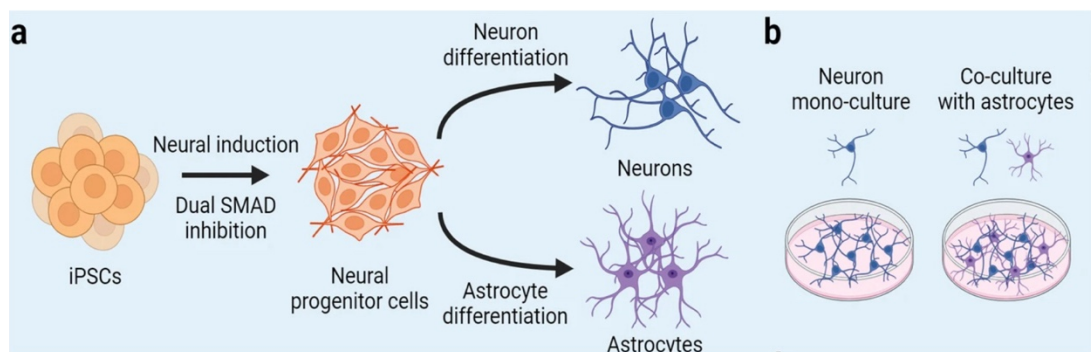


Figure 2: iPSC-derived Models. This technology can be applied to obtain various cellular models ranging from two-dimensional to three dimensional models. a. shows the differentiation of NPCs from iPSCs. This is achieved via dual SMAD inhibition by promoting neuroectoderm specification. Furthermore NPCs are differentiated into

neural lineages, such as neurons and astrocytes. B. these cells can be cultured as a mono-culture or as a co-culture. (Cerneckis et al., 2024b).

1.4 Neuroinflammation

Neuroinflammation is a cellular milieu of reactions within the CNS, that occurs in response to certain stimuli like trauma, infection or injury and is body's normal reaction to pathogens (Adamu et al., 2024; Kwon & Koh, 2020; Loh et al., 2024). However, tissue repair and pathogen clearance is a protective aspect under acute inflammatory conditions (Adamu et al., 2024; Kim & Lee, 2024;. Lee et al., 2023b), but under chronic inflammation significantly exacerbates pathology and the progression of neurodegenerative disorders like Parkinson's disease (Wang et al., 2021; Wang et al., 2023; Yang et al., 2022; Yun et al., 2018), Alzheimer's disease (Baldwin et al., 2024; Liao et al., 2021; Liddelow, 2019a; Patani et al., 2023; Wilkinson & El Khoury, 2012), multiple sclerosis (Adamu et al., 2024; Choi et al., 2011; Ghirotto et al., 2022;. Lee et al., 2023b; Liddelow et al., 2024; Ponath et al., 2018) and various other neurodegenerative disorders. Recent paradigm shifts have positioned chronic neuroinflammation as a key driver of pathogenesis, causing the activation of glial cells leading to cytokines, reactive oxygen and nitrogen species production (Adamu et al., 2024; Kwon & Koh, 2020; Sharma et al., 2021). At a molecular level neuroinflammation involves intricate signalling cascades involving NF- κ B (Alhadidi & Shah, 2018; Anilkumar & Wright-Jin, 2024; Kim & Lee, 2024), JAK-STAT (Alhadidi & Shah, 2018; Yan et al., 2024), the NLRP3 inflammasome (Kim & Lee, 2024; Ślusarczyk et al., 2018), and MAPK pathways (Almasoudi et al., 2025; Viorel et al., 2024), which exacerbate

neuronal damage by amplifying the inflammatory responses. Recent studies also indicated the role of non-coding RNAs , epigenetic modifications (Chen et al., 2024; Giallongo et al., 2022; Liu et al., 2022; Yang et al., 2023), and the gut-brain axis (Adamu et al., 2024; Loh et al., 2024) in playing a role in neuroinflammation. Reactive astrocytes add more complexity and orchestrate these neuroinflammatory responses and serve as critical mediators by bridging immune signalling and neuronal homeostasis (Liddel et al., 2024; Linnerbauer et al., 2020; Sofroniew, 2020; Sofroniew & Vinters, 2010).

Astrocytes constantly interact with microglia, and immune cells and shape neuroinflammatory responses through the production of cytokines, chemokines (Choi et al., 2014), and complement components (Hong et al., 2016; Negro-Demontel et al., 2024; Stephan et al., 2012; van Erp et al., 2023) , establishing bidirectional communication with neurons, endothelial cells, and other glial cells (Clayton & Liddel, 2025; Matyash & Kettenmann, 2010). The heterogeneity exhibited by astrocytes can either exacerbate neurodegeneration through the release of pro-inflammatory factors and neurotoxic factors (Borsini et al., 2015; Kobayashi et al., 2025; Tak & Firestein, 2001), or can also provide neuroprotection via tissue repair through tropic support (Cameron et al., 2024; Clayton & Liddel, 2025; Pertusa et al., 2007; Spampinato et al., 2018), highlighting the importance of reactive astrocytes in CNS pathology and repair.

1.5 Astrocyte Reactivity

When astrocytes are subjected to stress, inflammation or injury, they undergo a process known as astrocyte reactivity, inducing cellular heterogeneity which is beyond baseline diversity (Clayton & Liddelw, 2025; Liddelw et al., 2024; Patani et al., 2023). Reactive astrocytes show significant alterations in cellular morphology (Baldwin et al., 2024; Delgado-García et al., 2024; Green & Rowe, 2024; Matyash & Kettenmann, 2010), gene expression profiles (Bernard et al., 2011; Clayton & Liddelw, 2025; Gammie, 2022; Matyash & Kettenmann, 2010), and protein and lipid complements (Clayton & Liddelw, 2025; H. G. Lee et al., 2022; H.-G. Lee et al., 2023a; Liddelw et al., 2024), which ultimately alter their functionality leading to disrupted brain homeostasis.

Various clinical and preclinical studies have indicated the role of astrocytes in response to peripheral inflammation (Hasel & Liddelw, 2021; Liddelw et al., 2017; Liddelw & Barres, 2017) and their involvement in various neurological diseases such as Alzheimer's disease (Furman et al., 2012; Han et al., 2025; Liao et al., 2021; Liddelw, 2019a; Liddelw et al., 2024), multiple sclerosis (Ghirotto et al., 2022; Lassmann, 2020; Liddelw et al., 2024; Ponath et al., 2018), Parkinson's disease (Liddelw et al., 2024; Wang et al., 2021; Wang et al., 2023) and affective disorders such as major depressive disorder (Lee et al., 2022; Medina-Rodriguez & Beurel, 2022). Reactive astrocytes are known to express a wide variety of receptors which include cytokine receptors and pattern recognition receptors and communicate with infiltrating immune cells into the CNS due to compromised BBB and therefore contributing to the critical role of developing tissue pathology (Liddelw & Barres, 2017; Linnerbauer et

al., 2020; Lee et al., 2022). This necessitates the need of understanding the underlying molecular mechanisms that drive reactivity.

1.5.1 Molecular mechanisms of astrocytic reactivity

Understanding the mechanistic basis of these diverse functional outcomes requires systematic characterization of the cellular changes that define astrocyte reactivity. Classical morphological analysis has provided the fundamental framework for identifying reactive astrocytes across disease contexts, revealing the structural transformations that accompany their altered transcriptional and functional profiles.

1.5.2 Morphological characterization of astrocyte reactivity

For many years heterogeneity in astrocytes was described using morphological analysis (Amlerova et al., 2024; Lanjakornsiripan et al., 2018; Matyash & Kettenmann, 2010; Verkhratsky & Nedergaard, 2018). However, modern research has provided new dimensions to study morphology in reactive astrocytes (Clayton & Liddelow, 2025; Guttenplan et al., 2020; S. A. Liddelow, 2019b; Liddelow & Barres, 2017). In general, reactive astrocytes exhibit, hypertrophy, increased size of the cell body and processes (Amlerova et al., 2024; Baldwin et al., 2024; Matyash & Kettenmann, 2010), which is also accompanied by increased expression of GFAP and vimentin both known cytoskeletal components (de Reus et al., 2024; Sánchez-Juan et al., 2024; Wilhelmsson et al., 2004). These variations in cytoskeletal components and

morphology can influence astrocytic interactions with the surrounding environment.

However, while morphological changes provides valuable insights about reactivity in astrocytes, it is limited in its ability to differentiate between functionally distinct reactive states. To address this issue, the field has increasingly used molecular markers that complement morphological assessments and enable more precise characterization of reactive astrocyte subtypes and their context-dependent functional roles.

1.5.3 Identifying molecular markers in astrocyte reactivity

Over the years of research GFAP has been the most widely used marker in identifying and characterising reactive astrocyte across various pathological conditions (Araya-Callís et al., 2012; de Reus et al., 2024; Phillips et al., 2024; Sánchez-Juan et al., 2024). It is an intermediate filament protein, with it being accepted that an increased expression of this marker correlates with the degree of astrocyte reactivity (Bennett et al., 2016; Sofroniew & Vinters, 2010; Zamanian et al., 2012). However, GFAP expression cannot always correlate or distinguish between various subtypes of reactive astrocytes (Amlerova et al., 2024; Sofroniew, 2020; Sofroniew & Vinters, 2010) as it varies regionally and also on the disease type (Leipp et al., 2024).

Vimentin is another cytoskeletal filament protein, which is generally co-expressed with GFAP in reactive astrocytes (O'Leary et al., 2020a), and

provides insight into cytoskeletal remodelling during CNS pathologies (O'Leary et al., 2020b). It is generally expressed in immature astrocytes but predominantly increases in astrocytes when subjected to CNS injury or pathology (O'Leary et al., 2020a; Wilhelmsson et al., 2004, 2006). Hence, changes in vimentin and GFAP (Galou et al., 1996) allows the use of cytoskeletal remodelling, to help characterise reactive astrocytes (Escartin et al., 2021a; Pekny & Pekna, 2014).

In addition to cytoskeletal markers, transcriptomic studies have identified distinct markers associated with reactive astrocytes. Wherein subjecting astrocytes to neuroinflammatory signals such as IL-1 α , TNF- α , and C1q, researchers have identified the activation of complement cascade system (Liddelow & Barres, 2017), including upregulation of C3, where astrocytes lose their ability to support neuronal survival and synapse formation (Hasel & Liddelow, 2021; Liddelow, 2019b; Liddelow & Barres, 2017). Another marker of reactivity identified is Lipocalin-2 (LCN2). In various neuroinflammatory and neurodegenerative conditions like Alzheimer's disease and multiple sclerosis, LCN2 is significantly upregulated in reactive astrocytes (Amlerova et al., 2024; Liddelow et al., 2024; Matyash & Kettenmann, 2010; Yuan et al., 2025). It not only serves as a marker for reactivity but also modulates neuroinflammation and neuronal damage (Jurga et al., 2021; Stoklund Dittlau & Freude, 2024; Yuan et al., 2025).

Gbp2, is an interferon-inducible GTPase, and has been identified as a marker of neuroinflammatory reactive astrocytes, particularly those exhibiting neurotoxic phenotypes ((S. A. Liddelow, 2019a; S. A. Liddelow & Barres, 2017; Liddelow et al., 2017; Zamanian et al., 2012). Its expression reflects transcriptional change and interferon-responsive in reactive astrocytes induced by microglial-derived inflammatory cytokines IL-1 α , TNF- α , and C1q (Hasel et al., 2021; Hasel & Liddelow, 2021; Sadick et al., 2022). Studies have reported that elevated expression of Gbp2 in astrocytes across various neurodegenerative diseases, including AD, PD, amyotrophic lateral sclerosis (ALS), and multiple sclerosis (Clayton et al., 2024; Jurga et al., 2021; Santiago-Balmaseda et al., 2024; Stoklund Dittlau & Freude, 2024; Yang et al., 2024). Unlike GFAP its expression provides information on distinct inflammatory pathways activated within reactive astrocytes, which is strongly induced by interferon-gamma (IFN- γ) and other pro-inflammatory cytokines, such as tumour necrosis factor (TNF) and interleukin-1 beta (IL-1 β) via the activation of JAK-STAT signalling pathway (D. Han et al., 2025; Kirkby et al., 2023; Pandey et al., 2025).

Aquaporin-4 (AQP4) which is a prominent water channel expressed in astrocytes, shows altered polarisation and expression in reactive astrocytes (Gildea & Liddelow, 2025a; Mohamadi & Borhani-Haghighi, 2023). However, it isn't an exclusive marker of reactivity, but changes in its distribution has been shown to be linked with alterations in astrocyte-vascular interactions, which is

relevant in conditions such as BBB dysfunction and oedema (Haj-Yasein et al., 2011; Papadopoulos & Verkman, 2013; Thormann et al., 2024).

Even though these diverse markers underlines the heterogeneity of astrocyte reactivity, there is a need for a multi-marker approach to characterise astrocytic phenotypes in various disease-contexts. Importantly, these markers provide information regarding the identification and classification of reactive astrocytes, they also help identifying downstream signalling causing the reactivity. (Ben Haim, Carrillo-de Sauvage, et al., 2015; Escartin et al., 2021a; Hasel & Liddelow, 2021; Liddelow & Barres, 2017). To understand and to therapeutically modulate various reactive astrocyte phenotypes, it is important to acknowledge that they express a spectrum of reactivity and do not conform to discrete categories. This facilitates to understand disease-dependent responses of astrocytes in health and disease. Finally, studying reactive astrocytes involves unravelling multitude of pathological stimuli and the signalling pathways that regulate these distinct phenotypes (Endo, 2025; Escartin et al., 2021b).

1.5.4 Signalling pathway driving reactivity

Various downstream transcription factors and pathways control, initiate and modulate the reactivity in astrocytes. These include Janus kinase/signal transducer and activator of transcription 3 (JAK/STAT3) and nuclear factor- κ B (NF- κ B) (Escartin et al., 2021b) play pivotal role in mediating reactive phenotypes. NF- κ B is an important regulator of neuroinflammation within

astrocytes; its activation and nuclear translocation is triggered by proinflammatory cytokines, TNF- α , IL-1 β and IL-17 (Linnerbauer et al., 2020; Escartin et al., 2021; Lee et al., 2022), reactive oxygen species (ROS) and environmental factors (Wheeler et al., 2019; Linnerbauer et al., 2020), amplify NF- κ B signalling reinforcing responses of reactive astrocyte.

Similarly, JAK/STAT3 is a highly conserved inducer of reactive astrocyte in neurodegeneration or injury conditions. When triggered by cytokines such as IL-6, (JAKs) phosphorylate STAT3, leading to translocation to the nucleus and activates transcription of genes related to reactive astrocyte phenotype. Inhibition of this pathway has shown to prevent reactivity in astrocytes in diseases like AD (Ben Haim, Ceyzériat, et al., 2015a; Burda & Sofroniew, 2014; Ceyzériat et al., 2016; Reichenbach et al., 2019a).

On the contrary, a decrease in the CNS inflammation or injury/trauma is linked to the downregulation of the NF- κ B pathway in astrocytes in experimental autoimmune encephalomyelitis, an animal model of neuroinflammation (Brambilla et al., 2014) and optic nerve injury (Endo et al., 2022; H. G. Lee et al., 2022). Also, NF- κ B activation in preclinical models of Alzheimer's disease and in Huntington's disease (HD) (Ben Haim, Ceyzériat, et al., 2015b) trigger NF- κ B activation in astrocytes and causes increased pro-inflammatory cytokine release during neurodegeneration indicating this pathway can be a potential therapeutic target in CNS neurodegenerative diseases including AD, and HD (Ben Haim et al., 2015; Reichenbach et al., 2019 Lu et al., 2020). For

example, astrocytes in white matter adjacent to active MS lesions and in EAE mice (Harit et al., 2025) have elevated phosphorylated STAT3 (Lu et al., 2020), and it is also seen in preclinical models of AD and HD (Ben Haim, Ceyzériat, et al., 2015b). Also, conditional deletion of STAT3 from astrocytes results in fewer amyloid-beta plaques, and mild deficiencies in learning and memory in the APP/PS1 transgenic mouse model of Alzheimer's disease (Reichenbach et al., 2019b), which is consistent with studies conducted using STAT3 inhibitor (Mehla et al., 2021; Wen & Hu, 2024). Another pathway shown to be associated with reactive astrocytes involves calcineurin, which is a serine/threonine protein phosphatase found abundantly in the brain. Increased intracellular calcium, activates calcineurin leading to dephosphorylation of nuclear factor of activated T cells (NFAT) causing nuclear translocation and transcriptional activation (Furman et al., 2016; Furman & Norris, 2014). During CNS injury and neurological diseases, calcineurin-NFAT signalling hyperactivation is observed in the reactive astrocytes (Furman et al., 2016; Furman & Norris, 2014). For example, in patients with AD, calcineurin is expressed in reactive astrocytes and blocking of this pathway with a calcineurin inhibitor VIVIT ameliorates AD symptomology in 5xFAD (Sompol et al., 2017) and APP/PS1 mice models (Sompol & Norris, 2018) with a reduction in astrocytes and microglia activation in these models (Furman et al., 2012, 2016; Furman & Norris, 2014). Furthermore, in middle cerebral artery occlusion (MCAO) ischemic stroke model, the crosstalk between microglial C3aR and astrocytic C3 induces astrocytic reactivity, involving functional and morphological changes in astrocytes, by the activation of NF- κ B (Edison,

2024; Huang et al., 2025; Mederos et al., 2018). These diverse signalling pathways, ultimately drives the reactive astrocyte phenotypes by upregulating the expression of molecular signatures. Understanding these markers is necessary for distinguishing the detrimental and beneficial responses in astrocytes and to develop therapeutic interventions to modulate astrocytic reactivity.

1.5.5 Inducing Astrocytic Reactivity

There are various mechanisms to induce astrocyte reactivity. A commonly used and well-established way is using a cocktail of molecules such as cytokines TNF, IL-1 α , and C1q (Amlerova et al., 2024; Hasel & Liddelow, 2021; Liddelow, 2019b; Liddelow et al., 2024; Liddelow & Barres, 2017). This specific combination (Liddelow & Barres, 2017) was derived from the transcriptomic data (Kim et al., 2023; Liddelow et al., 2024; Patani et al., 2023) obtained by comparing the reactive astrocytes from brain tissues of patients with controls and the phenotype of these reactive astrocytes resembled those suffering from Alzheimer's disease (Sadick et al., 2022), Parkinson's disease (Wang et al., 2023), amyotrophic lateral sclerosis (Guttenplan et al., 2020). Reactive astrocytes can also be induced, using interferons (Fan et al., 2022; Hasel et al., 2021; Rothhammer et al., 2016; M. Sun et al., 2017), viruses and other pathogens with pathogen-associated molecular patterns (PAMPs) (Gildea & Liddelow, 2025b; Liddelow & Barres, 2017; Yates, 2017) or by releasing damage-associated molecular patterns (DAMPs), can also induce reactive phenotypes in astrocytes, which is generally observed in any physical trauma

(Rubartelli & Lotze, 2007; Vu et al., 2024). Amongst the other drivers of astrocytic reactivity include pathological proteins such as amyloid beta (Neniskyte et al., 2011; Neus Bosch et al., 2015; Velezmoro Jauregui et al., 2024), alpha synuclein (Yang et al., 2022; Zhang et al., 2023). However, it is unclear if each of these individual drivers produce a heterogeneous population of reactive state or a unique uniform one. The profound implications of this uncertainty, to understand whether pathological stimuli gives rise to a common reactive state or generate stimulus-specific astrocytic phenotypes, necessitates therapeutic targets for designing effective interventions and to provide a clear understanding of mechanisms of astrocytic reactivity. As emerging *in vitro* model iPSC-derived astrocytes provide opportunity to understand these mechanisms driving reactive responses from a human context.

1.5.6 iPSC-derived astrocytes for studying astrocyte reactivity

Although animal models have served as useful tools to study and understand neuron-glia interactions, however, rodent models do not sufficiently mimic the neurodegenerative state observed in certain neurodegenerative diseases (Gomes et al., 2024a). Studies have suggested functional, transcriptional and phenotypic differences between neuron and glia in rodents and primates (Hodge et al., 2019) but recent research suggest that, iPSCs may serve as physiologically relevant *in vitro* cellular models to overcome differences between rodent and human cells (Gomes et al., 2024a; Hodge et al., 2019; Lendemeijer et al., 2024). The establishment of experimental models such as

iPSC-derived astrocytes, may serve as an innovative approach to study and understand the pathophysiological mechanisms underlying neuroinflammatory diseases (Lendemeijer et al., 2024). Unlike the immortalised cell lines, and primary rodent astrocytes, iPSC-derived astrocytes provide advantages which include patient-specific genetic background, and scalability (Fossati, 2023). Importantly, as discussed in Section 1.4, the generation of fully differentiated and mature astrocytes is essential for accurately recapitulating human astrocyte biology and disease-relevant responses. Once mature, iPSC-derived astrocytes can be exposed to the inflammatory cytokine cocktail (TIC) (Gildea & Liddelow, 2025b; Liddelow & Barres, 2017) which induces a reactive phenotype characterised by molecular and functional changes similar to those observed during neuroinflammation and neurodegeneration in patients (Santos et al., 2017a, 2022; Szabo et al., 2021). Across multiple iPSC-derived astrocytic lines it has been demonstrated that they exhibit consistency in mimicking these phenotypic and molecular responses further validating their usage for disease modelling (Gomes et al., 2024a; Kamińska & Kochański, 2024; Matiukhova et al., 2025). Building on these advantages it is important to understand how experimental induction of reactivity in iPSC-derived astrocytes can serve as a model to study neuroinflammation.

1.5.6.1 *Experimental induction of astrocytic reactivity in iPSC-derived models.*

As discussed in an earlier section (1.5.5) reactivity can be induced in iPSC - derived astrocytes by exposing them to a cocktail of pro-inflammatory

cytokines, TIC (Liddelow, 2019b; Liddelow & Barres, 2017) for a period of 24h. This exposure has shown NF- κ B translocate from the cytoplasm to the nucleus within 60 minutes, of cytokine exposure, indicating the initial trigger of reactive response. Moreover, more than 50% astrocytes have shown activated NF- κ B translocation (Fereidoni et al., 2013; Fossati, 2023; Tarassishin et al., 2014) causing robust activation of astrocytes. As NF- κ B is an important transcriptional regulator of inflammatory gene expression, its activation causes functional and morphological changes and affects the downstream modulation of reactivity in physiological and disease conditions. To gain further knowledge on the consequences of experimentally induced reactive astrocytes it is important to characterise molecular features and phenotypic changes in reactive iPSC-derived astrocytes.

1.5.6.2 *Molecular characterisations of reactive iPSC-derived astrocytes.*

Upon exposure to proinflammatory cytokines, iPSC – derived astrocytes show profound changes in transcriptomics, with upregulation of genes that promote neuroinflammation and downregulation of genes involved in neuroprotection (Lee et al., 2024; Ren et al., 2024). Analysis of transcriptomics via bulk- RNA sequencing and using qRT-PCR have shown significant increase in reactive markers, including C3 and Gbp2 genes (Hyvärinen et al., 2019; McComish et al., 2024; Tarassishin et al., 2014), now recognised as markers to identify neurotoxic reactive astrocytes, as their expression strongly correlates to

neurodegeneration (Clayton et al., 2024; Clayton & Liddelow, 2025; Kim et al., 2022; McComish et al., 2024).

Apart from C3 and Gbp2, other upregulated genes include chemokines (CCL2, CXCL10), proinflammatory cytokines (IL-1 β , IL-6, TNF- α), and interferons (Clayton & Liddelow, 2025; Gildea & Liddelow, 2025b; Liddelow, 2019a; Liddelow & Barres, 2017). However, the canonical marker GFAP shows variable expression suggesting it cannot be a lone marker to identify reactive astrocytes (Gildea & Liddelow, 2025a; McComish et al., 2024; Patani et al., 2023). Comprehensive transcriptomic analysis, involving pathway enrichment studies, have suggested not only an upregulation of genes involved in cytokine signalling, but also in autophagy-associated signalling such as SQSTM1, optineurin/OPTN, PINK1 (Gomes et al., 2024b; Hou et al., 2025; S. Kim et al., 2023; McComish et al., 2024). However, the genes that are associated with programmed cell death and apoptotic processes are downregulated, consistent with studies showing these changes in reactive astrocytes (Gomes et al., 2024b; J. Huang et al., 2022). These studies also highlight altered expression of disease-associated genes, for example in mutations in SOD1, in ALS patients (Filipi et al., 2024; McComish et al., 2024; Shibata et al., 2009; Tarassishin et al., 2014), with the induction of reactivity inducing elevated SOD1 protein aggregation, mimicking the ALS disease state.

Moreover in reactive astrocytes, studies have shown reduced intracellular calcium levels (Neaverson et al., 2023; Santos et al., 2022; Shigetomi et al.,

2016; W. Sun et al., 2013) leading to altered calcium homeostasis. This affects the calcium dependent processes such as neurotransmitter regulation and gliotransmitter release (Baraibar et al., 2024; Shigetomi et al., 2019). Additionally reactive astrocytes have also show alterations in phagocytic activity, which is highly context and disease-dependent (Hyvärinen et al., 2019; McComish et al., 2024). For example, in AD astrocytes show impaired phagocytic activity resulting in reduced synaptic debris clearance (Galloway et al., 2019), but in MS reactive astrocytes have shown to engulf myelin debris, playing a role in lesion progression (T. Xu et al., 2023). These studies reflect the importance of modelling disease-specific astrocytes *in vitro* to investigate underlying mechanisms and to develop therapeutic targets.

1.5.6.3 *Disease-modelling applications using iPSC-derived astrocytes.*

iPSC-derived astrocytes can be a powerful tool to model neurodegenerative diseases, as it involves cell lines derived from patients, thereby generating authentic genetic models for the disease, to help capture patient-specific phenotype, and when they are subjected to reactive phenotype induction they exhibit disease-specific characteristics, compared to the health control iPSCs. For example, in patient derived iPSC astrocytes, with PSEN1 mutations reactivity induction leads to impaired phagocytic capacity for amyloid-beta peptides ($A\beta_{1-42}$), in *in vitro*, thereby increasing the pathological condition (Gomes et al., 2024b; Wasilewski et al., 2022). Similarly, in PD, patient derived astrocytes with LRRK2 mutations, reactivity induction activates STAT3 in dopaminergic neurons via the IL-6 mediated signalling leading to

the neuronal death when examined in co-cultures (Pons-Espinal et al., 2024a; Weir et al., 2019). Therefore, patient derived iPSC astrocytes provide a platform to investigate, and study disease mechanisms and identifying therapeutic targets (Lee et al., 2022; Liddelow & Barres, 2017; Wang et al., 2021; Wang et al., 2023).

1.5.6.4 *iPSC-Derived Astrocytes - Advantages And Limitations In Reactivity Studies.*

iPSC-derived astrocytes have both advantages and disadvantages for studying astrocytic reactivity mechanisms. Firstly, the origin is human therefore, the issues involving species specific difference is eliminated which often is compromised in studies involving rodents. Secondly, patient-derived cell lines provide mechanistic insights into disease modelling, making them genetically authentic. Thirdly, scalability, reproducibility across various patient lines, along with culture conditions provide superior experimental control and close related microenvironment when compared to immortalised cell lines or primary cultures. Fourth, is the easy storage conditions namely - the provision of long-term cryopreservation and standardised expansion (Chao et al., 2022; Gomes et al., 2024b; Guerra-Gomes et al., 2018; O'Toole et al., 2024).

However, they also have their limitations. Firstly, matured iPSC-derived astrocytes cannot recapitulate many complex functional aspects such as polarised structures, region specific specialization, or certain advanced uptake mechanisms which are generally observed in adult astrocytes *in vivo*.

Secondly, majority of the protocols involve 2D monoculture or co-culture systems, maintained in simple culture conditions and cannot mimic the complex, multicellular and three-dimensional microenvironment found *in vivo* (Benincasa et al., 2024; Mulica et al., 2023). Finally it is important to understand that astrocyte reactivity is heterogenous and complex in nature (Clayton & Liddelow, 2025; Matias et al., 2019; Matyash & Kettenmann, 2010; Stifani, 2021) and that *in vitro* stimulation cannot capture all aspects of disease modelling to get a mechanistic understanding of reactivity (Apostolou & Stadtfeld, 2018; Llorente et al., 2021; McComish et al., 2024).

However, having said that, iPSC-derived astrocyte is a powerful tool to investigate mechanisms neuroinflammation-associated neurodegeneration and for translational drug discovery (Feng et al., 2020; Pons-Espinal et al., 2024b). The use of 2D culture system provides limited ability to investigate and understand astrocyte interactions with neurons, microglia and the extracellular matrix but the advancements in 3D cultures and organoids have proven to be relevant models to understand cell-cell and cell-matrix interactions. 3D tri-culture and organoid systems do provide platforms to model neuroinflammation and neurodegeneration (Park et al., 2018; Tujula et al., 2025; Whitehouse et al., 2023). Despite various challenges iPSC-derived astrocytes used in 2D, 3D or organoids remain valuable platform to study, investigate and understand mechanisms in neuroinflammation and neurodegeneration (Lee et al., 2023a; Zhao et al., 2024).

1.5.7 Therapeutic Targets For Astrocyte Reactivity

Reactive astrocytes exhibit diverse roles in disease initiation, progression, maintenance, and recovery across various phases of diseases. Identifying these specific reactive astrocyte substates and their context-dependent roles is critical for developing targeted interventions that help enhance beneficial substates. G-protein coupled receptors (GPCRs) are the most promising therapeutic targets for such selective modulations (Lee et al., 2022; Mantas et al., 2022; Sun et al., 2017). Their ability to activate diverse downstream signalling and biased modulation of signalling pathways make them a suitable target for drug development (Borah et al., 2025; Gao & Jacobson, 2013; Lorente et al., 2025). They are the largest family of cell surface receptors and are highly expressed on astrocytes (Meron Asher & Goshen, 2025; Qin et al., 2023; Zinsmaier et al., 2025). They mediate responses in astrocytes via various extracellular signals including neurotransmitters, neuromodulators, cytokines and chemokines making them ideal for studying astrocyte reactivity in a context dependent manner (Ballabh et al., 2004; Hasel et al., 2021; Sadick et al., 2022; Verkhratsky & Nedergaard, 2018). With their pharmacological tractability one-third of the Food and Drug Administration (FDA) approved drugs targets are GPCRs (Sriram & Insel, 2018; Wheeler et al., 2019; Zhang et al., 2024), positioning them to be as a therapeutic target because of their functional diversity, and cell-type expression pattern (Cheng et al., 2017; Hasel et al., 2021). Therefore, studying astrocytic GPCRs could be promising to

understand and develop therapeutic targets to modulate neuroinflammation, neurodegeneration, without suppressing homeostatic roles.

1.5.8 GPCRs expressed in astrocytes

In addition to their neuronal supportive roles, a significant amount of evidence has suggested astrocytic roles in supporting CNS physiology via signalling exchange with neuronal synapses, which is achieved by various receptors and neurotransmitters that the astrocytes express (Kofuji & Araque, 2021). Astrocytes are known to express a wide variety of neurotransmitter receptors including N-methyl-D-aspartate (NMDA) and non-NMDA glutamate receptors such as AMPA receptors and kainate receptors (Chipman et al., 2021; Skowrońska et al., 2019), purinoceptors (Burnstock & Verkhratsky, 2012; von Kügelgen, 2021; Zhao et al., 2022), serotonergic (Miyazaki & Asanuma, 2016; Verkhratsky et al., 2021) receptors, as well as α and β adrenergic receptors (Iqbal et al., 2023; Wahis & Holt, 2021). Prominent among these receptors are the GPCRs, as their activation can lead to the intracellular mobilization of calcium if they are Gq-coupled (Kofuji & Araque, 2021). GPCRs present on the plasma membrane play a key role in recognizing and transmitting information resulting in activating various complex downstream signalling pathways (Mantas et al., 2022). Astrocytes are electrically unexcitable, and astrocytic GPCRs function as a sensory component to neural activity, where they are significantly responsive to neurotransmitters, responses to which are manifested in both their soma and their processes (Shigetomi et al., 2016; Guerra-Gomes et al., 2018). The calcium responses evoked by the GPCR activation are linked to the astrocytic cell body and are

linked to the Gq-coupled phospholipase C/inositol 1,4,5-triphosphate pathway, which initiates the release of calcium from the endoplasmic reticulum (Perea & Araque, 2005; Kofuji & Araque, 2021). Various neurotransmitters such as glutamate, GABA, and ATP activate GPCRs present in the astrocytes. For example, purinergic transmission is mediated by two subsets of GPCRs namely the P1 and the P2 of which P2 receptors exhibit both ionotropic P2X and metabotropic P2Y receptors on astrocytes. The activation of subsets of P2Y receptors, namely (P2Y_{1,2,4,6}, and 11) (von Kügelgen, 2021) which are Gq-coupled stimulates phospholipase C /inositol 1,4,5-triphosphate pathway and diacylglycerol production (Kofuji & Araque, 2021; Y. F. Zhao et al., 2022). Further, GABA is the major inhibitory neurotransmitter in the CNS and can act on both metabotropic (GABA_B) and ionotropic (GABA_A) receptors both of which are expressed in astrocytes (Perea & Araque, 2005; Kofuji & Araque, 2021). Although GABA_B-mediated intracellular calcium release from the internal store has been reported. However, it is known that GABA_B couples via Gi proteins (Meier et al., 2008). This reflects on the cell-specific mechanism, while in neurons they mediate via G_{i/o} pathways, but in astrocytes they are coupled through G_q signalling involving PLC/IP3 cascade leading to IP3-dependent calcium release. Inhibiting PLC/IP3 pathway leads to reduction in GABA_B-mediated intracellular calcium (Bostel et al., 2024; Chalifoux & Carter, 2011; Padgett & Slesinger, 2010). In addition, glutamate is the major excitatory neurotransmitter and has been divided into group I, II and III receptors of which group I receptors (mGlu1 and 5) are expressed on astrocytes. Major evidence points towards the existence of mGlu5 receptors in astrocytic cultures, where

calcium waves triggered by afferent hippocampal neurons can be inhibited by mGlu5 antagonists (Banasr et al., 2010; Khoodoruth et al., 2022). However, another GPCR family, protease-activated receptors (PARs), are broadly distributed in the rodent and human brain, including astrocytes.

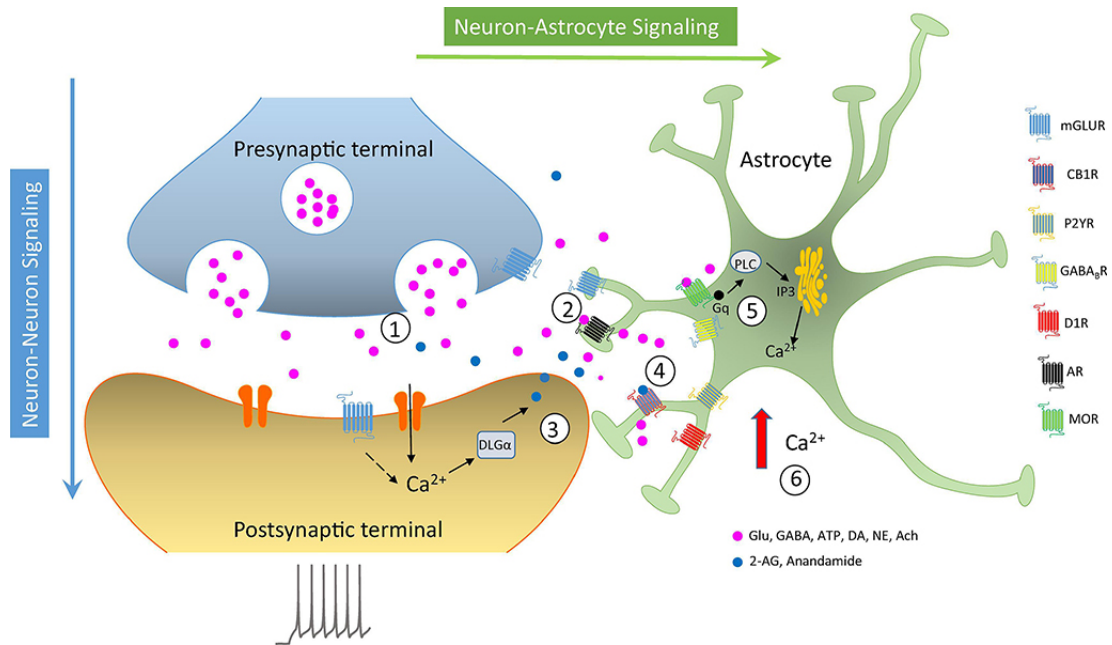


Figure 3: Schematic representation of GPCRs expressed on Astrocytes. In synaptic signalling, presynaptic neurons release neurotransmitters (1) that bind to G protein-coupled receptors (GPCRs) on postsynaptic neurons and astrocytes (2). In astrocytes, GPCR activation triggers the phospholipase C (PLC) signalling cascade (5), leading to an increase in intracellular calcium levels (6). Increased postsynaptic Ca^{2+} activates diacylglycerol lipase- α (DAGL α), leading to 2-arachidonoylglycerol (2-AG) production. Released 2-AG acts on astrocytic cannabinoid type 1 receptors (CB1Rs), inducing intracellular Ca^{2+} elevations in astrocytes. (4). GPCRs include metabotropic glutamate receptors (mGluRs), purinergic type 2 receptors (P2YRs), GABA type B receptors (GABABRs), dopamine type 1 receptors (D1Rs), adenosine receptors (ARs). Adapted from (Kofuji & Araque, 2021).

1.5.9 Protease-activated receptors

Protease-activated receptors (PARs) belong to the superfamily of G-protein-coupled receptors but differ from other GPCRs due to an unusual mechanism of activation. The PAR family includes four members PARs 1, 2, 3 and 4 and they belong to the class of group A rhodopsin-like subfamily of GPCRs (Chandrabalan & Ramachandran, 2021; J. Guo et al., 2024; Price et al., 2021). The functional roles of various sub-types of PARs have been established in homeostasis, coagulation and inflammation. However, their roles in physiological conditions of the brain remain unappreciated but they are widely expressed in the CNS including oligodendrocytes, microglia, astrocytes and neurons (Mavridis et al., 2025; Price et al., 2021; Spampinato et al., 2018; M. Zhang et al., 2024) and are known to be involved in several inflammatory and neurodegenerative diseases (Fredriksson et al., 2003; Crilly et al., 2012; Price et al., 2021, Habibi et al., 2025). PAR1, 3, and 4 are preferentially activated by thrombin while PAR2 is preferentially activated by serine protease trypsin and tryptases.

PARs consist of seven transmembrane helices bound by three intracellular and three extracellular loops. In comparison to other GPCRs, PARs activation in vivo is not through the binding of a soluble ligand but is actually triggered through the extracellular cleavage by protease at the extracellular amino terminus, exposing a six amino acid sequence termed the tethered ligand

(Fredriksson et al., 2003; Han & Nieman, 2020; Price et al., 2021; Sokolova et al., 2012; Uhl et al., 1998; Vu et al., 1991), which can then bind intramolecularly to the second extracellular loop causing a conformational change in the receptor evoking intracellular signal transduction through the recruitment of G-proteins. This activation of PARs is a general paradigm for all the PARs and is irreversible. It has been shown that PARs signal via recruitment of multiple G-proteins, including coupling via G α_q , G α_i and also G $\alpha_{12/13}$ which is activator dependent (Bushnell et al., 2016; Bushnell et al., 2006; Cheng et al., 2017; Gan et al., 2011; Guo et al., 2024b; Price et al., 2021b) (Fig 4). Given the unveiled tethered ligand following N-terminal cleavage, short peptide sequences also called the activating peptides (APs), matching these tethered ligands can also fully activate PARs without any proteolytic cleavage (Heuberger & Schuepbach, 2019a).

Signal transduction in PARs occurs through the recruitment of G-proteins, which is initiated through the coupling of the receptor to the membrane-related heterotrimeric complex wherein G α and dimeric subunits G $\beta\gamma$ are hydrolysed by GTP. Upon the receptor activation, the GDP bound to the G-protein is exchanged to GTP triggering the G α and G $\beta\gamma$ subunits to disassociation and causing various effector molecules to bind to specific G α subunits (Heuberger & Schuepbach, 2019; Price et al., 2021). The signalling pathway depends on the type of G-protein that is activated, for example, if the G-protein is stimulatory, i.e. G α_s then ATP is converted to cAMP through the activation of adenylyl cyclase, which directly activates protein kinase A (PKA), whereas the

activation of inhibitory G-proteins (Gai) inhibits adenylyl cyclase. Gαq/11 subunit activation stimulates phospholipase C (PLC) to hydrolyse phosphatidylinositol 4,5-bisphosphate (PIP₂) to diacylglycerol (DAG) and inositol triphosphate (IP₃). DAG activates – protein kinase C while IP₃ causes the release of calcium from the endoplasmic reticulum. Guanine nucleotide exchange factors (GEFs) are activated by Gα_{12/13} which is a G-protein for RhoA (Mavridis et al., 2025; Ossovskaia & Bunnett, 2004; Price et al., 2021a, 2021b; H. C. Wu, 2012). PARs 1 and 2 have been shown to couple with Gα_{12/13}, Gαq, and Gai (Macfarlane, 2001; Soh et al., 2010; Heuberger & Schuepbach, 2019) whilst PAR4 couples to Gαq and Gai/o (Hirano, 2007; Price et al., 2021,). In addition to G-protein-dependent signalling pathways, proteolytic cleavage of PARs can also activate G-protein-independent pathways (Adams et al., 2011; Chandrabalan & Ramachandran, 2021; Guo et al., 2024b; Heuberger & Schuepbach, 2019a; Ramachandran et al., 2012). GPCR activation involves downstream signal transduction via various Gα subunits but these signals are terminated through the recruitment of β-arrestins which bind to already phosphorylated C-terminal serine/threonine residues by GPCR kinases causing the receptor desensitization and internalization through the clathrin-dependent pathway (Dorn, 2009; Hamilton & Trejo, 2017; Thompson et al., 2024; Thomsen, 2023; Wolfe & Trejo, 2007; Zhai et al., 2023). Not all PARs desensitize and internalise identically. For example, PAR1 and PAR2 undergo clathrin-mediated endocytosis, whereas PAR3 and PAR4 lack β-arrestin involvement, a diversity in trafficking that prompted our investigation into PAR expression in rat primary hippocampal cultures and human astrocytes.

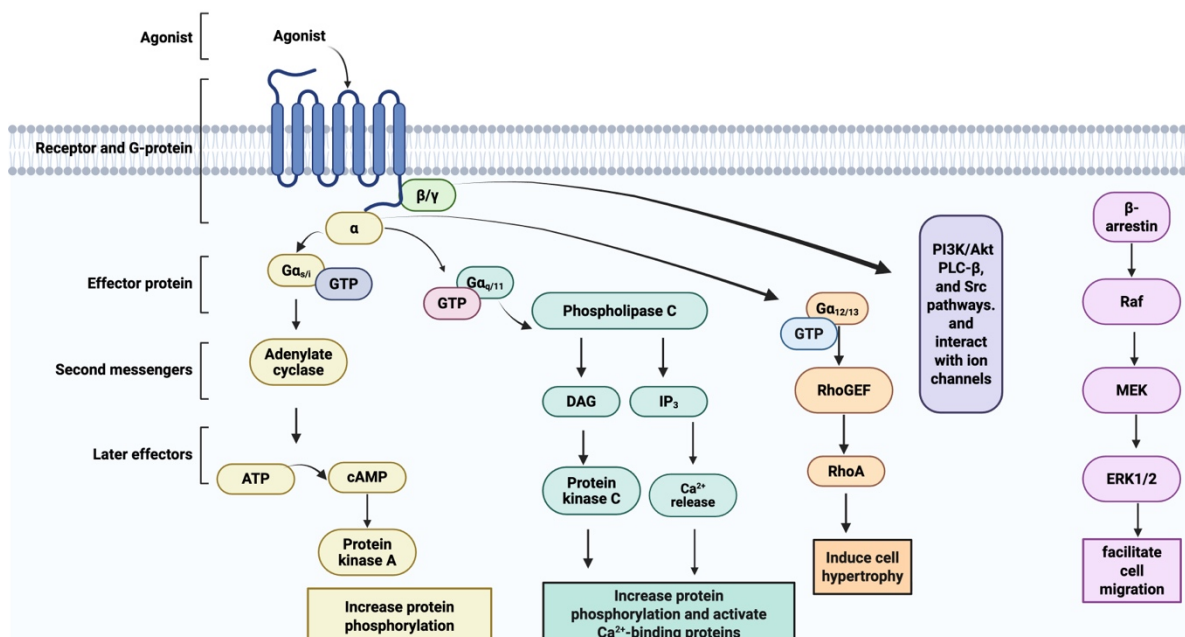


Figure 4: Schematic representation of PAR2 signalling. Upon receptor activation GDP bound to the heterotrimeric complex of G α and G $\beta\gamma$ is exchanged for GTP causing disassociation of the G $\beta\gamma$ from G α . G α is associated with different effector molecules and initiates various signalling pathways as outlined. furthermore Created in <https://BioRender.com>.

1.5.10 Protease activated receptor 2 (PAR2)

Given our increasing knowledge related to the multifactorial functionality of PARs, and their signalling pathways one constant link that has always been intriguing is the one between PAR2 and inflammation. PAR 2 is known to be present in both peripheral immune cells as well as in the cells within CNS and with its active role in conditions commonly related to inflammation (Bushell et al., 2016; Heuberger & Schuepbach, 2019a; K. Xu et al., 2024). Recent

evidence has highlighted PAR2's dual and context-dependent roles, where it can promote inflammation through immune cell activation while also facilitating tissue healing in damaged organs, with the specific outcome depending on the nature and location of the initial injury (Khoon & Piran, 2025; Reches & Piran, 2024a).

1.6 Innate immune system

1.6.1 Neutrophils

Within the innate immune system, PAR2 is known to be expressed in neutrophils (Bryzek et al., 2025). Activation of PAR2 promotes neutrophil recruitment and upregulates IL-17 receptor signaling, along with promoting chemokines and cytokines such as IL-23 and CXCL2 (Xu et al., 2024). The activation of PAR2 in neutrophils causes an increase in intracellular calcium and a shape change, with increased expression of macrophage-1 antigen and integrin VLA-4 upon exposure to PAR2-AP (Howells et al., 1997; Johansson et al., 2005). This leads to an increased release of cytokines (Cho et al., 2025; Thompson et al., 2024; Zeng et al., 2013a), including IL-6, IL-1 β , IL-8, and lactoferrin, which is not observed through activation of any other PARs (Shpacovitch et al., 2004; Wang & He, 2006). Recent investigations have revealed that PAR2 activation by endogenous protease triggers neutrophil extracellular trap (NET) formation through a non-classical mechanism, with these protease-induced NETs lacking bactericidal activity but exhibiting proteomic signatures indicative of roles in inflammatory disorders, including autoimmune diseases and carcinogenesis (Bryzek et al., 2025). Furthermore,

studies have demonstrated cooperation between platelet-activating factor receptor (PAFR) and PAR2 in mediating neutrophil recruitment and lung inflammation through NF- κ B transactivation (Silva et al., 2023).

1.6.2 Monocytes and Macrophages

PAR2 has been found to be expressed in human blood monocytes wherein its activation causes an increase in intracellular calcium and also the release of IL-6, IL-1 β , and IL-8 (Johansson et al., 2005; López et al., 2014). PAR 2 expression is also present in monocyte-derived macrophages, activation of which can cause a robust increase in intracellular calcium. Recent studies have demonstrated that macrophage-expressed PAR2 plays a crucial role in adverse cardiac remodelling and that PAR2 deficiency in hematopoietic lineages protects against myocardial infarction through attenuated inflammatory response and fibrosis (Reches & Piran, 2024a). In the context of inflammatory bowel disease, dysregulated PAR2 signaling induced by microbial protease from pathogenic bacteria like *Hungatella hathewayi* activates intracellular signalling cascades involving the p38 MAPK and NF- κ B pathways, triggering increased expression of pro-inflammatory cytokines including IL-1 β , IL-6, and TNF- α , thus contributing to chronic inflammatory responses (Khoon & Piran, 2025; Reches & Piran, 2024a).

1.6.3 Mast Cells

Finally, PAR 2 is known to be expressed in mast cells, causing degranulation of mast cells upon PAR 2 activation. Mast cells express PAR2 on both plasma membrane and intracellular granule membranes, and upon activation induce histamine or IL-8 release, thereby exacerbating inflammation and immune responses, with recent research identifying the tryptase/PAR2 axis as a critical component of crosstalk between mast cells and keratinocytes in skin inflammation (Xu et al., 2024). Mast cells release the serine protease tryptase which is a PAR 2 activator and has been implicated in many inflammatory diseases including (Crilly et al., 2012; Kalogera et al., 2021), synovial hyperemia, and many more (Chatterjea & Martinov, 2015; Oliveira et al., 2013; Bushell et al., 2016).

1.6.4 Adaptive immune system

In the adaptive immune system PAR2 is seen to be expressed in natural killer cells, and CD4⁺ T cells but is not seen to be expressed on CD8⁺ and $\gamma\delta$ T cells with both mouse and human T-cells expressing PAR2 but not the B cells (Jiang & Lu, 2024; López et al., 2014). Also, peptide agonists of PAR2 SLIGRL-NH₂ have shown to induce an increase in intracellular calcium in T cells (Jiang & Lu, 2024; Mari et al., 1996; Pfanzagl & Jensen-Jarolim, 2025). Also, studies have reported the secretion of IL-6 by T cells upon stimulation with trypsin and tryptase indicating their role in the immune response of the body (Jiang & Lu, 2024; Pfanzagl & Jensen-Jarolim, 2025). Further, these serine protease were also able to stimulate concentration-dependent

production of IL-6 (Li & He, 2006; Soh et al., 2023). This production of IL-6 benefits the adaptive immune system by driving differentiation of naïve CD4⁺ T cells into Th17 effector cells which are essential for pathogen clearance at mucosal sites) and T follicular helper cells, which enhance B cell maturation, antibody production, and germinal centre formation.

1.6.5 PAR2 in peripheral inflammation

Given the plethora of evidence of PAR 2-linked inflammatory disease, in the following, we discuss the pathophysiological roles of PARs in the musculoskeletal, gastrointestinal, and respiratory systems.

1.6.5.1 *Arthritis and musculoskeletal inflammation*

Evidence suggests the role of PAR2 in inflammatory diseases, which comes from studies that suggested that PAR2 AP's induced acute inflammatory response in the rat paw that was characterised by persistent oedema and also granulocyte infiltration (Vergnolle et al., 1999; Villano & Pontisso, 2024).

Intra-articular carrageenan/kaolin injection induces joint swelling and upregulates synovial PAR2, while inhibiting PAR2 upregulation reduces joint inflammation (Ferrell et al., 2003; Heuberger & Schuepbach, 2019b; Kelso et al., 2006; Kume et al., 2023). PAR2 activation in rodent models promotes prolonged chronic joint inflammation and synovial hyperemia, with PAR2-deficient models showing inhibited inflammation and heterozygous models displaying an intermediate phenotype, highlighting PAR2's role in mediating

potent proinflammatory effects in joint inflammation (Heuberger & Schuepbach, 2019b; Kume et al., 2023; Villano & Pontisso, 2024).

1.6.5.2 *Respiratory inflammation*

PAR2 is expressed in lung epithelial cells and fibroblasts and plays a vital role in the recruitment of inflammatory cells and has been proven to cause an increase in the inflammatory response in various respiratory diseases (Akers et al., 2000; Heuberger & Schuepbach, 2019a; Pfanzagl & Jensen-Jarolim, 2025; Silva et al., 2023). Also, in the number of allergic airway disease models. PAR2 activation causes an increased inflammatory response producing increased proinflammatory cytokines from the epithelial cells, which are supported by the data showing PAR2 activation causes the development of airway inflammation that is significantly reduced in PAR2 KO mice (Ebeling et al., 2005, 2007; Heuberger & Schuepbach, 2019a; Silva et al., 2023).

1.6.5.3 *Gastrointestinal inflammation*

PAR 2 is widely distributed and also expressed in the gastrointestinal tract and is known to play a crucial role in inflammatory bowel disease wherein activation of PAR2 on the epithelial cells causes diarrhoea-like symptoms due to increased chloride secretion via the prostaglandin E2-mediated process (Cenac et al., 2002, 2007; Lakemeyer et al., 2025). Also, colon administration of PAR2 agonists induced inflammatory reactions via granulocyte infiltration, tissue damage, and increased T-helper cell type 1 cytokine which were seen in wild-type mice but not in PAR2 deficient mice (Cattaruzza et al., 2011;

Cenac et al., 2002; Lakemeyer et al., 2025; Rondeau et al., 2024). Finally, studies have also suggested that activation of PAR1 and 2 causes increased intestinal permeability of macromolecules in vivo via the disruption of the epithelial tight junctions (Cattaruzza et al., 2011; Rondeau et al., 2024; Santiago et al., 2023). However, having discussed various inflammatory roles of PAR 2 in various peripheral systems we will further discuss and highlight their potential physiological and pathophysiological roles in CNS cells namely astrocytes and microglia.

1.7 PAR2 in the central nervous system

1.7.1 Expression on Microglia

PAR2 is reported to be present in microglia which are considered the innate immune system of CNS (Balcaitis et al., 2003; G. H. Park et al., 2010). Studies have reported that microglial PAR2 activation can cause the release of nitric oxide, reactive oxygen species (ROS) and also inflammatory cytokines which cause neuronal death (Afkhami-Goli et al., 2007a; Noorbakhsh et al., 2006a; Ramachandran et al., 2012), however, rodent studies conducted have reported altered cytokine profiles upon PAR 2 activation which was independent of microglial modulation (Moudio et al., 2022). In addition, microglial PAR2 activation causes the time-dependent release of brain-derived neurotrophic factor (BDNF) via the increased expression of the P2X4 receptor implicating the role of microglia in neuropathic pain (Afkhami-Goli et al., 2007a; Chen et al., 2012; Fan et al., 2014). However, in contrast, a recent study indicated that cytokine release was absent following application of PAR2

activators to primary mouse microglia (Moudio et al., 2022). With the evidence indicating the role of PAR 2 in peripheral inflammation, there is also a plethora of evidence linking PAR 2 activation and neuroinflammation to several inflammation-related disorders.

1.7.2 Function and expression of PAR2 in astrocytes

PAR2 demonstrates robust expression in both human and rodent astrocytes, as documented in numerous investigations spanning the past two decades and extending to recent studies. However, the initial characterization of astrocytic PAR2 expression originated from pioneering work by the Reiser group (Ubl et al., 1998b; Wang, Ubl, & Reiser, 2002; Wang, Ubl, Stricker, et al., 2002), where PAR2 activation in mouse astrocytes triggers rapid elevation of intracellular calcium levels. The discovery established the foundation for the subsequent need to investigate the role of PAR2 in astrocytic physiology and pathophysiology.

1.7.3 PAR2-mediated gliotransmitter release

Research has shown that PAR2 activation in astrocytes releases diverse gliotransmitters including cytokines (Cho et al., 2025; Thompson et al., 2024), chemokines (Greenwood & Bushell, 2010; Lakatos & Rosta, 2025; Sasaki et al., 2022; Wang et al., 2007b; Zeng et al., 2013), nitric oxide (Park et al., 2010), and glutamate (Gan et al., 2011; Greenwood & Bushell, 2010; Price et al., 2021a). Recent research has shown that activation of PAR2 on primary

cultured astrocytes induces the release of IL-6 and LIF, which is calcium-dependent (Zhang et al., 2025). Further, PAR2 mediated neuroinflammatory response was also associated with MAPK activation (Ocak et al., 2020a; Zeng et al., 2013; Zhang et al., 2012). In addition, PAR2 activation, induces the release of chemokines, such as CXCL1, CCL2 and CXCL10 and are known to play critical roles in mediating neuroinflammation (Boakye et al., 2021; Ni et al., 2019; Zhang et al., 2013).

1.7.4 PAR2 mediated neuroprotection

Interestingly, PAR2 activation is also associated with neuroprotection. Studies have reported that the release of chemokines upon PAR2 activation is linked to its neuroprotective nature. This has been shown in models of C2 ceramide-induced cell death and neurotoxicity induced by kainate (Greenwood & Bushell, 2010) in the organotypic hippocampal slice cultures (Moudio et al., 2025; Wang et al., 2007a, 2007c,) In these studies PAR2-mediated neuroprotection was via the CXCR2 chemokine receptor. Research has also shown that PAR2 activation reduced neurotoxicity in organotypic hippocampal slice cultures when co-applied with kainate in excitotoxicity assays (OSC's) which was mediated through the inhibition of MAPK signalling (Greenwood & Bushell, 2010; Moudio et al., 2025). Both of these studies proving PAR2 mediated neuroprotection is independent calcium-dependent mechanisms. However, available pharmacological tools for PAR2 often lack CNS penetration, selectivity, or oral bioavailability, significantly impeding in vivo elucidation of PAR2 function in the CNS. Consequently, genetic deletion of

PAR2 which reduces LPS-induced sickness-like behaviour in mice has been a critical alternative approach (Abulkassim et al., 2016).

1.7.5 PAR2 in the CNS inflammation

1.7.5.1 *PAR 2 and Multiple Sclerosis*

Studies have shown that PAR2 expression is significantly upregulated in the brain in inflammation-related diseases including MS. An elevated expression of PAR2 in astrocytes in MS has been observed (Noorbakhsh et al., 2003, 2006a), and is proposed to mediate oligodendrocyte toxicity in, experimental autoimmune encephalomyelitis (EAE) mouse models. In addition PAR2 KO mice have shown reduced neuroinflammation and myelin preservation in EAE models by increased macrophagic production of the anti-inflammatory cytokine IL-10, and diminished leukocyte infiltration compared to wild type (WT) mice. Furthermore, a recent study revealed reduced disease severity, reduced demyelination, and reduced infiltration of T-cells and macrophages upon the application of PAR2 antagonist P2pal-18S (Eftekhari et al., 2024a). Therefore, PAR2 represents a promising therapeutic target for mitigating neuroinflammation, demyelination, and immune cell infiltration in MS and related disorders.

1.7.5.2 *PAR2 and Alzheimer's disease*

The neurodegenerative role of PAR2 is reported in Alzheimer's disease, a disease which is characterised by inflammation, deposition of misfolded proteins and neuronal death. A study has shown reduced levels of IL-4 and an

inflammatory cytokine with increased expression of PAR2 in the proximal glial cells (Kiyota et al., 2010) and increased levels of pro-inflammatory cytokines. Suggesting increased glial PAR2 expression causes a shift from IL-4 mediated anti-inflammation towards pro-inflammatory state (Afkhami-Goli et al., 2007a). Studies utilizing conditioned media from PAR2-activated astrocytes and macrophages provide further support for a role of PAR2 in AD pathogenesis. Specifically, media from PAR2-activated macrophages exacerbated β -amyloid-induced neurotoxicity in human foetal neurons, whereas control media had no effect (Afkhami-Goli et al., 2007b). This is corroborated by findings that PAR2 knockout reduces amyloid plaque burden and cognitive deficits in AD mouse models (Luo et al., 2016).

Finally, neurotoxicity induced by β -amyloid in PAR2 deficient mice reported decreased glial activation while severe neuro-behavioural phenotypes were observed in wild-type mice, suggesting the role of glial PAR2 in assisting neurotoxicity induced by β -amyloid and disease progression through the activation of inflammatory signalling pathways (Afkhami-Goli et al., 2007a).

1.7.5.3 *PAR 2 and Parkinson's disease*

PD is characterised by degeneration of projecting dopaminergic neurons in the substantia nigra and reduced dopamine levels in the striatum.

Animal model studies have also indicated a role for PAR2 in Parkinson's disease (PD), wherein rats injected with MPTP (a neurotoxin widely used in to induce Parkinson's disease models by selectively destroying dopaminergic neurons in the substantia nigra) caused an increased expression of PAR2 in

the substantia nigra with increased production of α -synuclein. PAR2 inhibition reduced the production of α -synuclein and reduced motor dysfunction in rats when induced by MPTP. Also, in rats injected with MPTP, phosphorylation of the p65 subunit of NF- κ B was seen while this was abolished with PAR2 blockade (Liu et al., 2014). Also, post-mortem studies from patients with PD indicated reduced neuronal PAR2 expression in locus coeruleus and primary motor cortex and increased microglial PAR2 expression in substantia nigra and primary motor cortex highlighting a potential pathogenic role of glial PAR2 activation in PD (M. J. Hurley et al., 2015a). Finally, recent study has also reported that 1-Piperidin Propionic Acid (1-PPA), a novel inhibitor of PAR2 has shown reduced PAR2 expression in all patients observed at both transcriptional and a protein level. 1-PPA treatment downregulated the inflammatory profile involving IL-1 β , IL-6, and TNF- α , and reduced PAR2 expression in LPS-induced mice microglia pointing towards the inhibition of PAR2 as a therapeutic step to reduce neuroinflammation (M. J. Hurley et al., 2015b; Quarta et al., 2024; Reches & Piran, 2024b).

1.7.5.4 *PAR 2 and sickness-like behaviour*

With PAR2 linked to peripheral inflammation and also CNS inflammation, studies were conducted to investigate its role in inflammation-related behavioural changes. Rodent studies were conducted to understand whether behavioural changes linked to systemic inflammation were due to the activation of PAR 2 and the study reported the contribution of PAR 2 in the

onset and maintenance of sickness behaviour induced by LPS in PAR 2^{+/+} mice while in PAR 2^{-/-} mice showed reduced onset and increased recovery from LPS (Abulkassim et al., 2016).

Finally, these studies have indicated that inhibition of PAR2 may be a therapeutic target in peripheral and CNS inflammation (Eftekhari et al., 2024b; Göbel et al., 2019; Noorbakhsh et al., 2006b; Ocak et al., 2020b; Reches & Piran, 2024b). Collectively, these findings provide compelling evidence that PAR2 inhibition constitutes a high-priority therapeutic target for mitigating both peripheral and central neuroinflammation across diverse disease contexts (Eftekhari et al., 2024b; Göbel et al., 2019; Noorbakhsh et al., 2006b; Ocak et al., 2020b; Reches & Piran, 2024b).

1.8 PAR2 antagonists

Given that PAR2 has been implicated in several diseases including CNS disorders, having a potent and selective antagonist that would allow further investigation would be beneficial. Due to the unique activation mechanism of PARs, several strategies were outlined to develop antagonists that do not follow the classic pharmacological sense of receptor blocking but may involve using a small molecule to block the docking by tethered ligand, obstructing the cleavage site for the tethered ligand to stop the initiation of signal transduction through peptides. However, the desire for potent and selective PAR2 antagonists have been a challenge, which has led to the development of several different types including peptidomimetics, inhibitory peptides, cell-

penetrating pepducins and small molecules and antibodies (Muley et al., 2017; McIntosh et al., 2020).

1.8.1 Peptidomimetics

One of the first attempts to develop PAR2 antagonists was through the use of peptidomimetics that involves the use of small peptides that resemble the tethered ligand. This led to the discovery of peptide antagonists K-14585 and K-12940, which proved to be competitive antagonists for PAR2-ligand [3H]-2-furoyl-LIGRL-NH₂ and reduced PAR2-mediated intracellular calcium signalling in primary human keratinocytes (Goh et al., 2009; Kanke et al., 2009). However, K-14585 was able to inhibit signal transductions generated via synthetic peptides but not by trypsin and thereby wasn't considered a suitable therapeutic agent (Kanke et al., 2009).

Pepducins are short-lipidated peptides demonstrating high stability derived from various intracellular loop domains of GPCRs (Covic et al., 2002; Seigny et al., 2011). P2pal-18s is a pepducin that blocks the PAR2 activity by reducing the inflammatory response in vivo through the blockade of trypsin signalling and mast cell tryptase in mice models of paw oedema. However, it fails to block the trypsin-mediated interactions with arrestins (Seigny et al., 2011; M. S. Lee & Lerner, 2019). Therefore, P2pal-18S exemplifies a biased PAR2 antagonist with therapeutic potential for selectively targeting G protein-coupled inflammatory pathways while preserving β -arrestin-mediated signalling.

1.8.2 Non-peptide small molecule

Peptide antagonists have proven to have poor bioavailability and stability. Therefore, researchers examined new approaches to develop PAR2 antagonists. A study identified a small molecule ENMD-1068, which did inhibit joint inflammation via blockade of trypsin-mediated signalling but its IC_{50} of 5 mM made it unfit as an antagonist *in vivo* (Kelso et al., 2006). Following this, the GB series was developed which had improved bioavailability derived from GB110 agonists. GB83 and GB88 were developed of which GB88 has been shown to be selective for PAR2 and had good oral availability (Lohman et al., 2012; Suen et al., 2012). However, later studies using GB88 suggested it be a partial agonist as it selectively inhibits the $G_{\alpha q}/11$ pathway of PAR2 signalling and internalises the receptor (Moudio et al., 2025). More recently, the AZ series of PAR 2 antagonists has been developed including AZ8838 and AZ3451. It was initially proposed that AZ8838 is a negative allosteric modulator (NAM) of PAR 2 with slow binding kinetics (Cheng et al., 2017; McIntosh et al., 2020; Moudio et al., 2025). However, another study suggested that AZ8838 binds to the orthosteric site of PAR2 (Kennedy et al., 2018) indicating that AZ8838 is a competitive agonist for PAR2. To conclude, structure-based drug design could pose a promising approach in developing PAR2 inhibitors and combining various approaches could help develop ligands that can counteract PAR2 binding that will best suggest receptor status in a pathological condition, thus providing novel breakthroughs from various directions.

1.9 Aims and Hypothesis

Previous studies have shown PAR2-mediated neuroprotection is via astrocytes, further highlighting the role of astrocytes in PAR2-dependent signalling and neuroprotection (T. J. Bushell et al., 2006; Greenwood & Bushell, 2010; Moudio et al., 2025). Furthermore, unpublished data from our lab (Trevor Bushell's lab) has indicated a reduction in reactivity in astrocytes after injecting mouse models of amyloid pathology with AC264613, a PAR2 agonist. In summary this close linkage of astrocytic PAR2-mediated neuroprotection and reduction of reactivity has been shown in rodent models, but whether this astrocytic PAR2 neuroprotection and modulation of reactivity can be achieved in human astrocytes is unknown. Therefore, this study aims to understand and investigate astrocytic PAR2 expression, signalling and its function in human astrocytes.

1.9.1 Hypothesis

The central hypothesis of this study is that activation of protease-activated receptor 2 (PAR2) impairs the reactive phenotype and functions of human astrocytes.

1.9.2 Aims

1. To determine the presence and expression patterns of protease-activated receptors (PARs), including PAR2, in human astrocytes.
2. To elucidate the signalling pathways activated by PARs in human astrocytes, with emphasis on downstream molecular and cellular responses.

3. To investigate whether PAR2 activation alters astrocytic reactivity and functional properties under both basal (resting) conditions and in response to inflammatory stimuli.

1.9.3 Rationale

The data generated from these aims will establish a foundational framework for comparative studies using iPSC-derived astrocytes, in which reactive and phagocytic responses have previously been characterized. This approach will enable direct and systematic comparisons between cultured human astrocytes and their iPSC-derived counterparts, enriching our understanding of PAR2-mediated mechanisms within diverse astrocyte populations.

CHAPTER 2

Materials and Methods

2 Materials

Table 1: Materials and chemicals

Material	Supplier (UK unless stated otherwise)	Catalogue number
13mm coverslips	VWR International Ltd, UK	6311578
Mr Frosty™ freezing container	Thermo Scientific, UK	51000001
Cryovials	Star lab, UK	21121
1.5ml centrifuge tubes	Star lab, UK	K195636H
50ml falcon tube	Corning, UK	30622006
15ml falcon tube	Corning, UK	30022001
2ml centrifuge tubes	Eppendorf, UK	0030120094
T75 flask	Corning, UK	14423038
T25 flask	Techno Plastic Products AG, Switzerland	20210196
35mm cell culture treated surface petri dish	VWR International Ltd, UK	130180
BioLite™ 60mm cell Culture treated surface petri dish	Thermo Scientific, UK	130181
Phosphate buffered saline (PBS) tablets	Gibco™, UK	2662260
Bovine Serum Albumin (BSA)	Sigma-Aldrich, UK	A9647
Potassium Chloride KCl	Fisher, UK	10375810

Table 2: Materials

Solutions	Supplier (UK unless stated otherwise)	Catalogue number
HBSS (Hank's Balanced Salt Solution)	Gibco, UK	14185-045
Ethanol >99.8%	Fisher, UK	20847.307
Paraformaldehyde 4%	Alfa Aesar	J61899
Triton X-100 solution	Sigma-Aldrich, UK	93443
B-27 Supplement (50x), serum free	Gibco™, UK	17504-044
Fetal bovine Serum	Gibco™, UK	17974731
Neurobasal-A medium	Gibco™, UK	10888-022
Hibernate-A Medium	Gibco™, UK	12349-015
Astrocytic media (10% FBS, 1% P/S, 1% Astrocytic growth supplement)	ScienCell Research Laboratories, USA	1801
Penicillin-streptomycin (P/S)	Gibco™, UK	15140-122
Glutamax (0.5mM)	Gibco™, UK	35050061
1N NaOH	Sigma-Aldrich, UK	S2770
Gentamycin	Gibco™, UK	12664735
TrpIE	Gibco™, UK	10718463
DMSO Dimethyl Sulfoxide	Merck, Millipore, UK	D2650
Dulbeccos Modified Eagle Medium (DMEM)	Gibco™, UK	2662099
Ethanol	Supelco, UK	I1307083
Pierce™ BCA Protein Assay Kit	Thermo Fisher Scientific, UK	23227
Halt™ Protease and Phosphatase Inhibitor Cocktail (100X)	Thermo Fisher Scientific, UK	78440
PBS-Tween	Sigma, UK	P1379-1L

Table 3: Solutions

Solution	Composition
HEPES buffered saline (HBS)	(10mM HEPES, 140mM NaCl, 5mM KCl, 2mM CaCl ₂ , 2mM MgCl ₂ , 10mM D-glucose, pH
Digestion media	hibernate-A media, 2mg/ml papain from papaya latex, 1N NaOH, 0.1 M EGTA
Hibernate-A Medium complete	hibernate-A media, 10mg/ml gentamycin
Neurobasal-A complete	media (neurobasal-A media, 2% B27, 0.5mM glutamax)

Table 4: Antibodies

Antibodies	Supplier (UK unless stated otherwise)	Catalogue number	Dilution
Anti-Glial Fibrillary Acidic Protein antibody, clone GA5	Merck, Millipore, UK	MAB360	1:500
MAP-2 Chicken monoclonal antibody	Merck, Millipore, UK	AB15452	1:500
Gbp2 – rabbit monoclonal antibody	Proteintech, Europe	11854	1:500
C3 – mouse monoclonal antibody	Hycult Biotech, Netherlands	HM1045	1:500
SOX-9 rabbit monoclonal antibody	Abcam, UK	AB185966	1:500
Donkey anti-mouse ALEXA fluor 555	Life Technologies, UK	A-31570	1:200

Antibodies	Supplier (UK unless stated otherwise)	Catalogue number	Dilution
Donkey anti-mouse ALEXA fluor 488	Life Technologies, UK	A-21202	1:200
goat anti-chicken ALEXA fluor 555	Life Technologies, UK	A21437	1:200
goat anti-chicken ALEXA fluor 488	Life Technologies, UK	A11039	1:200
goat anti-rabbit ALEXA fluor 555	Life Technologies, UK	A2142	1:200
goat anti-rabbit ALEXA fluor 488	Abcam, UK	Ab150077	1:200
Cell Mask	Invitrogen, UK	H32713-A	1:500
Phospho-p38 MAPK (Thr180/Tyr182)	Cell Signalling, UK	9211	1:1000
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	Cell Signalling, UK	9101	1:1000
Total P-38 p38 MAPK (D13E1)	Cell Signalling, UK	8690	1:1000
Total ERK p44/42 MAPK (Erk1/2)	Cell Signalling, UK	4695	1:1000
goat anti-rabbit ALEXA fluor 700	Life Technologies, UK	A21038	1:1000
goat anti-rabbit ALEXA fluor 800	Life Technologies, UK	A32735	1:1000

Table 5: Drugs for calcium Imaging

Drugs	Concentration	Supplier (UK unless stated otherwise)	Catalogue number
AC264613	1nM- 100µM	Biotechnne, UK	3370
SLIGRL-NH ₂	1nM- 100µM	PeptideSynthetics, UK	1468
SLIGKV-NH ₂	1nM- 100µM	PeptideSynthetics, , UK	3010
AYPGKF	1nM- 100µM	Biotechnne, UK	1487
Trypsin	1nM- 100µM	Tocris, UK	AZ3451
ADP	1nM- 100µM	Tocris, UK	A2754
DHPG	1nM- 100µM	Abcam, UK	Ab120007
TFLLR-NH ₂	1nM- 100µM	PeptideSynthetics, , UK	1464
U71322	1µM	Merck Life Sciences	662035

Table 6: Materials for iPSC culturing

Material	Supplier (UK unless stated otherwise)	Catalogue number
DMEM/F-12, GlutaMAX™ supplement	Thermo Fisher Scientific, UK	10565-018
N2 supplement	Gibco™ N-2 Supplement (100X),, UK	17502-048
Non-essential amino acids	Gibco™ Non-essential amino acids MEM NEAA (100X),, UK	11140-050
B- mercaptoethanol	Gibco™ 2-Mercaptoethanol (50 mM), UK	31350-010
Pen/strep	Gibco™ Penicillin- Streptomycin (5,000 U/mL), UK	15070-063
Insulin	Sigma-Aldrich, UK	19278

Table 7: Materials for iPSC culturing

Material	Supplier (UK unless stated otherwise)	Catalogue number
Neurobasal – A media	Thermo Fisher Scientific, UK	12348-017
B27	Gibco, UK	17504-044
L glutamine	Thermo Fisher Scientific, UK	25030-024
LIF	Sigma-Aldrich, UK	L5283

Table 8: Recombinant Proteins for iPSC culturing

Material	Supplier (UK unless stated otherwise)	Catalogue number
BMP4	Thermo Fisher Scientific, UK	PHC9534
Geltrex	Gibco, UK	A-14133-02
DPBS	Thermo Fisher Scientific, UK	14190-144
EDTA	Fisher scientific, UK	15825388
TNF -alpha	Gibco™Peprotech, UK	300-01A
IL1 alpha	Gibco™Peprotech, UK	200-01A
C1q	Sigma-Aldrich, UK	204876

Table 9: Buffers for Western Blots

Buffer/Solution	Composition	Preparation Method
Western blot transfer buffer (10× stock)	• 480 mM Tris base • 390 mM glycine • 0.06% (w/v) sodium dodecyl sulfate (SDS) • Distilled water (dH₂O)	Dissolve components in dH ₂ O. For 1× working buffer: dilute 100 ml of 10× stock in 900 ml of dH ₂ O.
Western blot wash buffer (PBST)	• 1× phosphate-buffered saline (PBS) • 0.1% (v/v) Tween 20	Supplement PBS with Tween 20.
Blocking buffer	• 1 g semi-skimmed milk powder (5% w/v) • 20 ml PBST	Dissolve milk powder in PBST.
Secondary antibody diluent	• 0.2 g semi-skimmed milk powder (1% w/v) • 20 ml PBST	Dissolve milk powder in PBST.
2× resolving gel buffer	• 750 mM Tris base • 4 mM EDTA • 0.2% (w/v) SDS • dH₂O, adjusted to pH 8.9	Prepare in dH ₂ O and adjust pH to 8.9.
2× stacking gel buffer	• 250 mM Tris base • 4 mM EDTA • 0.4% (w/v) SDS • dH₂O, adjusted to pH 6.8	Prepare in dH ₂ O and adjust pH to 6.8.

Table 10: Composition of gels for Western Blots

Buffer/Solution	Composition	Preparation Method
10× running buffer	• 250 mM Tris base • 1.92 M glycine • 1% (w/v) SDS • dH₂O	Dissolve components in dH ₂ O.
2× resolving gel solution	• 5 ml of 2× resolving gel buffer • 2.5 ml of 40% acrylamide (Carl Roth GmbH & Co.) • 2.5 ml of dH₂O • 10 µl TEMED • 100 µl of 10% ammonium persulfate (APS: 0.1 g in 1 ml PBS)	Mix components. Add TEMED and APS to initiate polymerization before casting.
2× stacking gel solution	• 4 ml of 2× stacking gel buffer • 0.9 ml of 40% acrylamide • 3.1 ml of dH₂O • TEMED and APS (added before casting)	Sufficient for four gels. Mix components and add TEMED and APS before casting.
RIPA buffer	• 50 mM Tris, • 150 mM NaCl, • 1 mM EDTA, • 1% Triton X-100, • 0.5% sodium Deoxycholate supplemented with protease and phosphatase inhibitor cocktail (10 µl/ml)	Add the protease and phosphatase inhibitor fresh every time.

2.2 Methods

2.2.1 (a) Animals

One to two day old Sprague Dawley rat pups were obtained from in-house colonies maintained in the Biological Procedures Unit at Strathclyde University. All animal care and experimental procedures were in accordance with the guidelines of the UK Home Office (UK Home Office Schedule 1 guidelines) under the agreement and authority of the UK Animals Act, 1986.

2.2.1 (b) iPSC – derived cell lines

Human induced pluripotent stem cell (iPSC)-derived glial progenitor cells were obtained from Dr. Charlie Arber at University College London. All iPSC lines were generated with informed donor consent under ethical approval from the relevant institutional review board, in accordance with national guidelines and regulations for the use of human biological materials in research. No new iPSC lines were generated specifically for this study and all work complied with the principles set forth by the UK guidelines and the International Society for Stem Cell Research (ISSCR).

2.2.2 Rat primary hippocampal culture

2.2.2.1 *Poly-D-lysine coating*

Poly-D-lysine (PDL, 0.01 mg/ml) was made fresh for each culture from a pre-prepared stock solution (0.1mg/ml). About 90 µl PDL was loaded directly onto freshly autoclaved 13mm glass coverslips. The coverslips were incubated at 37°C / 5% CO₂ for a period of 90 mins, followed by a wash with tissue-grade sterile water and then allowed to dry for 60 mins before introducing cells and media.

2.2.2.2 *Preparation of primary hippocampal culture*

Hippocampal cultures were prepared in a class II cell culture hood under aseptic conditions. The rat primary hippocampal culture preparation was similar to that described previously (Hridi et al., 2019; Megarity et al., 2022). Sprague Dawley rat pups (1-2 days old) were killed via cervical dislocation followed by decapitation with scissors. The skull was cut open using curved sharp 9cm scissors (FST, UK) in the transverse plane, followed by the rapid removal of the brain using a small spatula and placing it on a filter paper previously sterilised with 70% ethanol. Using a sterile blade No 11 (FST, UK), the brain was bisected, and the hippocampi were dissected from both hemispheres and placed in complete Hibernate-A media. The tissue was transferred to Ca²⁺- free Hibernate-A digestion media and incubated for 30 minutes in a water bath at 37°C. The tissue was then transferred into 3ml of complete Hibernate-A media to inactivate papain and centrifuged at 1000rpm for 4 min (Thermofisher Scientific CL2 centrifuge, UK). The pellet obtained was

resuspended into 5 ml complete Hibernate media-A and triturated using fine polished glass pasteur pipettes (Fisher, UK) with decreasing diameter, following which the supernatant was collected in a 15ml tube (Corning, UK) and centrifuged at 1100rpm for 4 min. The resulting pellet was resuspended in 1 ml of complete Neurobasal-A medium (see Table 2.1.1). The mechanically disassociated cells were plated on poly-D-lysine coated 13mm glass coverslips (3 coverslips per 35mm dish) and incubated for 1 hour at 37°C / 5% CO₂ for cell adhesion at a density of 3×10^5 cells/unit area (count obtained from a haemocytometer). After 1h incubation, the dishes were flooded with 2ml complete Neurobasal A medium with cultures used for experiments between 10-12 DIV (days *in vitro*), with cultures fed every 2-3 days by replacing half the media with fresh complete Neurobasal A media.

2.2.3 Human astrocyte culture

2.2.3.1 *Poly-D-lysine coating*

Poly-D-lysine (PDL, 0.01 mg/ml) was made fresh for each culture from a pre-prepared stock solution (0.1mg/ml) T25 flasks were loaded with 2mL PDL and gently rocked to ensure an even coating of the culture surface. Flasks were incubated at 37°C / 5% CO₂ for a period of 90 mins, followed by a wash with tissue-grade sterile water and then allowed to dry for 60 mins before introducing cells and media.

2.2.3.2 *Preparation of human astrocyte culture*

All cell culture work was performed in a class II cell culture hood under aseptic conditions. Human astrocytes were stored at -80°C until needed. Cells were

thawed and added to T25 flasks coated with poly-D-lysine. The flasks were flooded with 15 ml of astrocytic media and incubated for 24h at 37°C/ 5%CO₂. After 24h of incubation, the media was completely replaced with 5 ml of astrocytic media and further incubated for 48h. When cHAs reached 80% - 90% confluency, they were washed with 2ml HBSS, followed by trypsinisation with TrypLE (1.6ml/T25 flask, incubated at 37°C/5% CO₂ for 4-5mins). The cells were then transferred to a 50 ml tube containing 5 ml astrocytic media and centrifuged at 1000rpm for 5 min. The resulting pellet was resuspended in 1 ml of astrocytic media and triturated using a pipette, and the cells then plated on PDL coated coverslips at a desired dilution and incubated at 37°C/5% CO₂ for 90 min, following which the dishes were flooded with 2ml astrocytic media. The remaining cells were plated in PDL coated T25 flasks at a dilution of 1:2 with approximately 1.5 mL media added to the flasks and were incubated at 37°C/5% CO₂ until 80-90% of confluency was achieved. cHAs plated at 1:10 dilution were used for Ca²⁺ imaging between 3-5 days post-plating (DPP). Media in the petri dishes was completely replaced every 3 days.

2.2.4 Optimizing human astrocyte culture: culturing in serum and serum-free medium.

cHAs were cultured following the supplier's recommendations. For serum-based conditions, cells were maintained in astrocyte medium supplemented with 2% FBS, 1% AGS, and 1% P/S and maintained in an incubator at 37°C / 5% CO₂. For serum-free culture conditions, astrocytes were initially thawed and cultured in serum-containing medium for one passage to ensure viability. Cells were seeded at a 1:30 ratio onto PDL coated 13 mm coverslips in 12-

well plates and incubated at 37°C / 5% CO₂ for either 24 hours or 7 days. cHAs were sub-cultured every 3-4 days (up to a maximum of seven passages, to maintain cellular integrity), and with each passage cells were transferred into both serum-containing and serum-free media and maintained for either 24 hours or 7 days, with fixation (see section 2.2.6) performed immediately after 24 hours of treatment or on the seventh day to assess cellular responses under these conditions.

2.2.5 Optimizing human astrocyte culture: to study the effects of different media types with par activators and inflammatory cytokines.

cHAs were cultured under serum-based and serum-free conditions, as previously described, and subjected to two experimental timelines: 24-hour and 7-day cultures. The cells were maintained in their respective media for 24 hours, followed by treatment with protease-activated receptor (PAR) activators AC264613 (10 µM), SLIGKV-NH₂ (5 µM), and TFLLR-NH₂ (3 µM), and with a cocktail of inflammatory cytokines tumour necrosis factor (TNF-α, 30 ng/mL), interleukin-1β (IL-1β, 3 ng/mL), and complement component 1q (C1q, 400 ng/mL). Cells were fixed (see section 2.2.6) 24 hours after the initiation of treatment.

For the 7-day cultures, cHAs were maintained in their respective media, with media changes performed every three days. During each media change, fresh PAR activators (AC264613 at 10 µM, SLIGKV-NH₂ at 5 µM, TFLLR-NH₂ at 3 µM) and inflammatory cytokines (TNF-α, 30 ng/mL, IL-1β, 3 ng/mL and C1q, 400 ng/mL) were added to maintain consistent stimulation. On the seventh day, cells treated were fixed (see section 2.2.6). To investigate the effects of

PAR activators and inflammatory cytokines on astrocyte reactivity and their morphology, cells from both serum-based and serum-free conditions were stained post-treatment with antibodies against GFAP, C3, Gbp2.

To measure these morphological differences between cells maintained in different media containing serum and no serum, they were stained with CellMask™, a fluorescent dye used to visualise the plasma membrane of cells in microscopy and help study cell structure (Tavares et al., 2017; Yakovlev et al., 2024).

2.2.6 Immunocytochemistry

cHAs (3–5, DPP), primary hippocampal cultures (10–12, DIV), glial precursor cells and iPSC-derived matured astrocytes were washed 2–3 times with phosphate-buffered saline (PBS) to remove residual media. Cells were fixed using ice-cold 4% paraformaldehyde for 10 minutes, followed by three washes with PBS. Subsequently, cells were treated with ice-cold 100% methanol for 10 minutes. Following washing 3 times with PBS permeabilization was performed for 10 minutes using Triton X-100 (0.01% for primary hippocampal cultures, 0.1% for cHAs) diluted in a blocking solution composed of 5% (v/v) FBS and 1% (w/v) BSA in PBS. After permeabilization of cells, in order to minimize non-specific antibody binding, cells were incubated in the blocking solution for 1 hour at room temperature. Primary antibodies, diluted in the blocking solution (see Table 2.4 for specific dilutions), were applied directly to the coverslips and incubated overnight at 4°C in a wet box. The following day, unbound primary antibodies were removed by washing the coverslips 2–3 times with PBS. Fluorescently conjugated secondary antibodies (Alexa Fluor

488 or Alexa Fluor 555, as specified in Table 4) were then applied and incubated for 60 minutes in the dark at room temperature. After secondary antibody incubation, cells were washed 2–3 times with PBS to remove unbound antibodies. Imaging was performed using an upright epifluorescence microscope (Olympus BX51W, Japan) equipped with a 20×/0.50 water-immersion objective lens (Olympus UMPlanFLN, Tokyo, Japan) with appropriate optical LEDs were used to excite the fluorophores. Fluorescent signals were captured using a cooled CCD digital camera (01-RET-EXI-F-M-12-C, QImaging, UK) and WinFluor software (version 4.1.9). The acquired images were analysed using Fiji ImageJ software (version 2.14.0/1.54f, National Institutes of Health, USA) to assess fluorescence intensity. Furthermore, to analyse cellular morphology, following secondary antibody application, CellMask™ plasma membrane stain was applied to visualize cell boundaries. The CellMask™ working solution was prepared by diluting the stock solution (10 mg/ml) to achieve a final working concentration of 2 µg/ml. This solution was applied directly onto the coverslips and incubated in darkness at room temperature for 90 minutes to ensure optimal membrane labelling prior to imaging.

The quantitative analysis of immunofluorescence images was performed using ImageJ. For each image, regions of interest (ROIs) were first defined by applying an intensity threshold to the primary staining channel to isolate individual cells from the background. Using the wand (tracing) tool, each cell was manually selected based on the thresholded ROI to ensure accurate segmentation. The same ROI boundaries were then consistently applied

across corresponding channels stained with different antibodies to enable direct comparison of fluorescence signals within the same cellular area. Following ROI selection, the “Measure” function was used to extract quantitative parameters, including cell area, perimeter, and mean fluorescence intensity for each marker. All measurements were performed under identical thresholding and acquisition settings to maintain consistency across samples.

2.2.7 Calcium Imaging

Ca²⁺ imaging was conducted on live primary hippocampal cultures at 10–12 DIV and cHAs at 3–5 DPP, following established protocols (Tinning et al., 2018). Prior to imaging, cells were washed twice with HBS. Cells were then loaded with the ratio metric calcium indicator Fura-2 AM (5 µM in HBS), or Fluo4 AM (5 µM in HBS, experiments involving studying the signalling pathway), for a minimum of 45-60 min at room temperature in the dark. Following loading, cells were washed 2–3 times with HBS, and the coverslip was transferred to a perfusion bath for imaging. Imaging was performed using a 20×/0.50 water-dipping objective lens (Olympus UMPlanFLN, Tokyo, Japan) mounted on an upright widefield BX51W epifluorescent microscope (Olympus, Japan). Ratiometric images were acquired at excitation wavelengths of 340 nm and 380 nm using an OptoLED system (Cairn Research, UK) at a frequency of 0.5 Hz with the emission wavelength of 555 nm. Data were collected from both primary hippocampal cultures and cHAs using WinFluor V.4.1.9 imaging software (Dempster, 2002). At the conclusion of each imaging experiment, neurons and astrocytes within the primary hippocampal cultures were identified based on their response to high extracellular potassium

chloride (KCl, 2M, Figure 5 B). All drugs were made to final bath concentrations in HBS including vehicle controls and were applied through the perfusate at a constant flow rate of 2 ml/min. However, during the course of the study, technical limitations with the calcium imaging system prevented reliable acquisition of ratio metric images at the dual excitation wavelengths required for Fura-2 analysis. Consequently, subsequent experiments were performed using the single-wavelength calcium indicator Fluo-4 AM, which enabled robust measurement of agonist-evoked intracellular Ca^{2+} responses. This methodological change has now been clarified in the thesis.

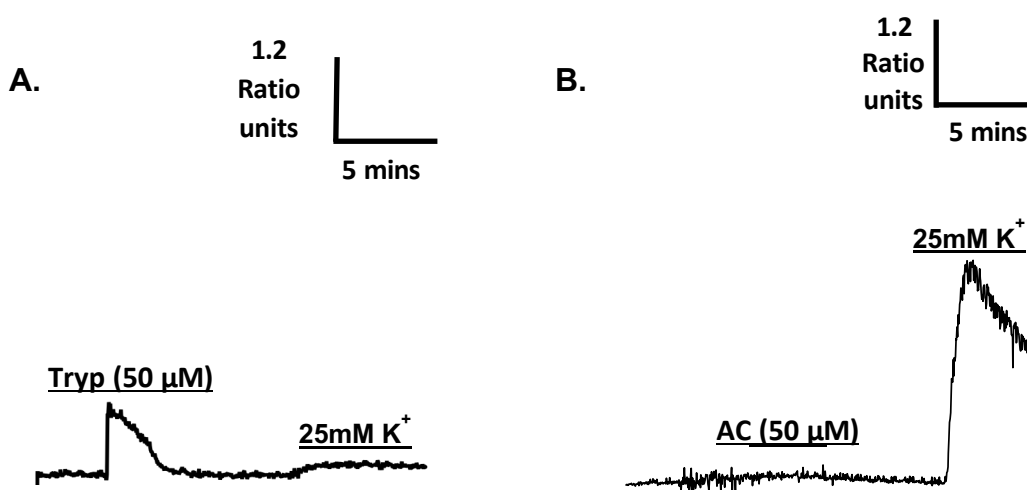


Figure 5: Identification of neurons and astrocytes in primary hippocampal cultures. Example traces showing responses to PAR2 agonist and KCl in (A) astrocytes and (B) neurons in the primary hippocampal cultures to PAR agonists.

2.2.8 Calcium imaging data analysis

Analysis of the images obtained was performed offline using Winfluor software v.4.1.9. with the ratio of fluorescence emission intensities at 340 nm and 380 nm (F_{340}/F_{380}) calculated for each ROI manually marked for individual cells,

including primary hippocampal cultures and cHAs. The acquired data was subsequently transferred to a Microsoft Excel spreadsheet, where the average baseline fluorescence of the HBS wash was calculated for each experimental condition. Each ROI was then normalized to its respective baseline value. These normalized emission ratios provided a quantitative measure of the average peak fluorescence change in response to the applied drugs, as described in previous studies (Bushell et al., 2006; Tinning et al., 2018).

2.2.9 Western blotting

2.2.9.1 *Sample Preparation*

Human astrocyte cultures were seeded in 6-well plates at a 1:5 dilution. At 2–3 DPP, cells were treated with PAR2 activators or vehicle control for 24 hours. Following treatment, cells were washed once with ice-cold phosphate-buffered saline (PBS). Cell lysis was performed by adding 80 µl of RIPA buffer to each well. Cells were scraped from the wells and transferred to Eppendorf tubes. The cell lysates were centrifuged at 16,000 rpm for 10 minutes at 4°C and the resulting supernatants were collected in fresh Eppendorf tubes and stored at -20°C until further analysis.

2.2.9.2 *Protein quantification*

The total protein concentration was analysed using a BCA assay using BSA standards. The standards were prepared as protein concentration references by diluting BSA in distilled water (dH₂O) to final concentrations of 0 - 2.0 mg/ml. cHA samples were diluted 1:10 with dH₂O. Both BSA standards and

cHA samples were vortexed and centrifuged prior to analysis with 10 μ L of each BSA standard and cHA samples were added in duplicate to a 96-well plate. A BCA working reagent, prepared by combining reagent A and reagent B at a 50:1 ratio, was added in a volume of 200 μ L to each well. The plate was incubated at 37°C / 5% CO₂ in the dark for 20 minutes.

Following incubation, absorbance at 560 nm was measured in a 96-well plate using a GloMax® Explorer Multimode Microplate Reader (Promega, UK). Protein concentrations (mg/ml) were calculated based on the absorbance readings from the BSA standards. As samples were analysed in duplicate, and the mean protein concentration was determined for each sample.

A standard curve was generated using bovine serum albumin (BSA) standards to determine the protein concentration in each sample. The protein content in 30 μ L of each sample was calculated, and 10 μ g of total cellular protein was used for each experiment to ensure consistency across all samples within individual experiments. Samples were prepared by mixing with 6 $\times$ SDS loading buffer and heated at 95°C for 5 minutes to denature the proteins. Following denaturation, samples were stored at -20°C until required for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The processed protein samples were stored at -80°C until required for further experiments.

2.2.9.3 *SDS-polyacrylamide gel electrophoresis*

Gels were cast using two different gel compositions: a resolving polyacrylamide gel (8-15% acrylamide) at the bottom and a polyacrylamide

stacking gel was then added on top spaced with a 1.0 mm comb. Samples were loaded into gel lanes and underwent SDS-PAGE in SDS running buffer alongside a molecular weight marker (BP3601-500, EZ-Run™ pre-stained protein marker, Fisher BioReagents™, UK). Gels were typically run at 80 V for 20 min followed by 150 V for 60 min (based on a 10 % resolving polyacrylamide gel). After SDS-PAGE, protein transfer to nitrocellulose membranes (6 x 9 cm) was conducted using a BioRad Trans-Blot® SD cell (Bio-Rad Laboratories, Inc, UK). For this, nitrocellulose sheets were positioned on top of gels and filter paper placed at either side (all components pre-soaked in transfer buffer) before being placed into the transfer cassette. Transfer was then performed at 120 mA overnight (~16 hours) in transfer buffer.

2.2.9.4 *Protein Transfer*

The transfer assembly was constructed by arranging the components in the following order within a transfer cassette, as illustrated in Figure 6: (1) sponge, (2) blotting paper, (3) nitrocellulose membrane, (4) gel, (5) blotting paper, and (6) sponge. All components were pre-soaked in transfer buffer. The assembled cassette was placed in the transfer tank filled with the same transfer buffer, and protein transfer was conducted at a constant current of 120 mA for approximately 16 hours overnight. **(Figure 6).**

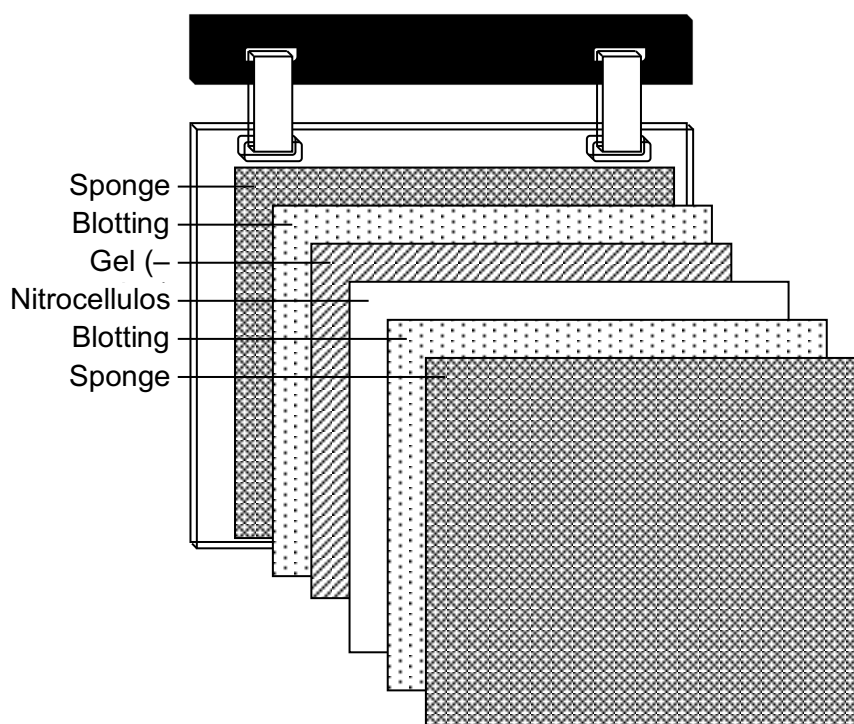


Figure 6: Cassette arrangement of western blotting. Above shown is the representative image of cassette arrangement in a typical western blotting in an order 1. sponge; 2. blotting paper; 3. nitrocellulose; 4. gel; 5. blotting paper; 6. Sponge.

2.2.9.5 *Western Blot immunodetection*

Following protein transfer to nitrocellulose membranes, non-specific binding sites were blocked by incubating the membranes in a blocking buffer consisting of 5% (w/v) non-fat dry milk in phosphate-buffered saline with 0.1% PBS-T for 45–60 min at room temperature. The incubation was performed on a shaker (IKA KS 250 Basic, Labortechnik) set to 100 RPM. Residual blocking buffer was removed by washing the membranes with distilled water (dH₂O) or PBS-T (three washes, 5 minutes each) on the shaker at 100 RPM. Membranes were incubated overnight at 4°C with specific primary antibodies (see Table 2.4 for specific dilutions) diluted in PBS-T. The following day, membranes were

washed four times with PBS-T (5 minutes per wash) on a shaker at 100 RPM to remove unbound primary antibodies. Membranes were then incubated with appropriate fluorophore-conjugated secondary antibodies (Alexa Fluor or infrared dye-conjugated, (see Table 2.4 for specific dilutions) in PBS-T or a diluent containing 1% non-fat dry milk in PBS-T for 50–60 minutes at room temperature. After incubation, membranes were washed three times with PBS-T (5 minutes per wash) to remove unbound secondary antibodies. Membranes were scanned using an Odyssey® Classic Imager or Odyssey 9120 IR Imager (LI-COR Biosciences UK Ltd, Cambridge, UK) with the appropriate infrared detection channels (700 nm for anti-rabbit or 800 nm for anti-rabbit, based on secondary antibody species). Protein band intensities were quantified using Image Studio™ Software (version 5.5, LI-COR Biosciences). Background signals were subtracted from the signal values to account for non-specific binding. Protein expression levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. Where applicable, phosphorylated protein expression was further normalized to total protein expression to assess phosphorylation states.

2.3.1. Culturing iPSC-derived astrocytes

All cell culture procedures were conducted under aseptic conditions within a Class II biological safety cabinet. GPCs, generously provided by Dr. Charlie Arber (University College London), were maintained in T25 flasks. GPCs were derived through continuous passaging of iPSC-derived cortical neurons at 80 DIV using EDTA, with the addition of FGF2 at 10 ng/mL to N2B27 medium to

promote gliogenesis. (Bose et al., 2025; O'Toole et al., 2024; Savchenko et al., 2019).

2.3.1.1. *Preparation of geltrex-coated coverslips*

Geltrex was thawed on ice for 1 hour in a cold room (4°C). The thawed Geltrex was diluted 1:100 in ice-cold DMEM/F12 medium to achieve a final protein concentration of 0.12–0.18 mg/mL, suitable for iPSC culture. For coating, 13 mm coverslips were treated with 0.2 mL of diluted Geltrex, 6-well plates with 1 mL, and T-75 flasks with 5 mL of diluted Geltrex.

2.2.9.6 *N2 / B27 medium preparation*

N2 / B27 medium was prepared as a 1:1 mixture of DMEM/F12 and Neurobasal medium. The medium was supplemented with the following components: N2 supplement, B27 supplement, L-glutamine, non-essential amino acids (NEAA), penicillin/streptomycin (25U), beta-mercaptoethanol, and insulin at 25 U. All components were combined under sterile conditions, filtered through a 0.22 µm filter, and stored at 4°C for up to two weeks.

2.2.9.7 *Passaging of glial precursor cells*

GPCs were passaged continuously using EDTA, with the addition of FGF2 (10 ng/mL) to N2B27 medium. This process induced a transition to gliogenesis during the FGF2-mediated propagation phase, facilitating the generation of glial lineage cells.

2.2.9.8 *Generation of iPSC-astrocytes*

Matured astrocytes were generated from GPCs that had reached a minimum of 150 DIV. These cells underwent a two-week maturation process in N2B27 medium supplemented with 10 ng/mL bone morphogenetic protein 4 (BMP4) and 10 ng/mL leukemia inhibitory factor (LIF) to promote terminal differentiation into mature astrocytes.

2.2.9.9 *Plating of glial precursor cells*

GPCs were passaged as described above and plated onto 13 mm Geltrex-coated coverslips at a 1:15 dilution. For terminal differentiation into matured astrocytes, the medium was replaced 24 hours post-plating with N2B27 medium supplemented with 10 ng/mL BMP4 and 10 ng/mL LIF. The medium changes were done every 2–3 days, monitoring for morphology and confluence to support consistent GPC expansion for downstream applications.

2.2.10 *Phagocytosis assay using latex beads*

CHAs were seeded at a 1:30 ratio onto poly-D-lysine-coated 13 mm coverslips, while iPSC-derived astrocytes were plated onto Geltrex-coated coverslips in 12-well plates and maintained at 37°C in a humidified incubator with 5% CO₂ for 24 hours prior to treatment. Cells were then treated with protease-activated receptor agonists AC264613 (10 µM), SLIGKV-NH₂ (5 µM), and TFLLR-NH₂ (3 µM), alongside an inflammatory cytokine cocktail consisting of tumour necrosis factor-α (TNF-α, 30 ng/mL), interleukin-1β (IL-1β, 3 ng/mL), and complement component 1q (C1q, 400 ng/mL). At the 23rd hour of treatment,

3 μ L of a suspension containing 1 μ m diameter FITC-labelled latex beads (10 mg/mL) was added to each well. Following a 1-hour incubation, excess extracellular beads were removed by washing. Cells were subsequently fixed at the 24-hour time point (see Section 2.2.6) and stained with CellMask according to the manufacturer's instruction.

2.2.11 Statistical Analysis

Data presented are as mean $\pm$ standard error of the mean (S.E.M) unless otherwise indicated. In calcium imaging experiments, the n value refers to the total number of cultures, not individual cells. To assess statistical significance, a normality test was conducted prior to selecting the appropriate statistical method. Wherever one-way analysis of variance (ANOVA) was used it was with Tukey's / Dunett's post-hoc comparison was conducted using GraphPad Prism v10.0.0 (Graphpad Software, Inc.) with $p < 0.05$ was taken to be statistically significant.

CHAPTER 3

Results

Characterisation of protease- activated receptor 2 in human astrocytes

3. Characterisation of PAR2 in human astrocytes and primary rat hippocampal cultures.

3.1 Introduction

Protease-activated receptors (PARs) constitute a unique family of GPCRs characterised by their activation through proteolytic cleavage, a mechanism that distinguishes them from other receptor types (Coughlin, 2000; Coughlin, 2005; Ishihara et al., 1997; Vu et al., 1991). Among the four identified PARs (PAR1–4), protease-activated receptor 2 (PAR2) has garnered significant attention due to its widespread expression and multifaceted roles in both the periphery and CNS physiology and pathology (Adams et al., 2011; Guo et al., 2024; Lyu et al., 2025; Ossovskaya & Bunnett, 2004; Rajala & Griffin, 2025; Ramachandran et al., 2012). PAR2 is activated by serine protease, such as trypsin and tryptase, which cleave the extracellular N-terminus to expose a tethered ligand that initiates intracellular signalling cascades, including the phospholipase C/inositol triphosphate pathway, leading to calcium mobilization and downstream effects on cellular function. In the CNS, PAR2 is expressed in various cell types, including neurons, astrocytes, and microglia, where it modulates processes ranging from synaptic plasticity to neuroinflammation and neurodegeneration (Adams et al., 2011; Afkhami-Goli et al., 2007a; Bushell, 2007; Eftekhari et al., 2024; Fan et al., 2024; Khoon & Piran, 2025;

Quarta et al., 2024; Ramachandran et al., 2012; Reches & Piran, 2024; Shrestha et al., 2014).

Astrocytes, the most abundant glial cells in the brain, play critical roles in maintaining CNS homeostasis, supporting neuronal function, and responding to injury or pathological conditions (Alhadidi et al., 2024; Lee et al., 2023; Li et al., 2025; Siracusa et al., 2019; Sofroniew & Vinters, 2010). These cells are integral to the regulation of synaptic activity, blood-brain barrier integrity, and the clearance of toxic protein aggregates, all of which are influenced by their dynamic responses to environmental cues (Verkhatsky & Nedergaard, 2018). Recent evidence suggests that PAR2 activation in astrocytes can trigger diverse responses, including morphological changes, cytokine release, and alterations in glutamate signalling, which may contribute to both neuroprotection and neurodegeneration depending on the context (Cocks & Moffatt, 2000; Noorbakhsh et al., 2006). For instance, PAR2 activation has been shown to reverse astrocyte stellation induced by serum deprivation, indicating its involvement in morphological plasticity, a hallmark of reactive astrogliosis (Cocks & Moffatt, 2000). Furthermore, PAR2 signalling in astrocytes has been implicated in the secretion of pro-inflammatory cytokines, such as interleukin-6 (IL-6), via pathways involving signal transducer and activator of transcription 3 (STAT3), which may exacerbate neuroinflammatory conditions (Alhadidi et al., 2024; Eftekhari et al., 2024; Khoon & Piran, 2025; Li et al., 2025; Pons-Espinal et al., 2024; Reches & Piran, 2024; Zhang et al., 2025). But, PAR2 activation can exert neuroprotective or neurotoxic

effects which is context dependent. For example, PAR2 activation in Alzheimer's disease exerts both neuroprotective and disease contributing effects depending on the cell type (Moudio et al., 2025; Widera et al., 2019a). In conditions of stroke PAR2 activation on astrocytes leads to inflammatory astrogliosis, classically hallmarked by an increase in the markers GFAP and vimentin along with increased tissue damage (Afkhami-Goli et al., 2007b; Burda & Sofroniew, 2014; Noorbakhsh et al., 2006). However, PAR2 is known to exert neuroprotective effects, in a mouse model of spinal cord injury, deletion of PAR2 reduces astrogliosis and leads to better recovery suggesting its neuroprotective effects of PAR2 (Noorbakhsh et al., 2006; Radulovic et al., 2015; Ramachandran et al., 2012) and in transient middle cerebral artery occlusion model of ischemia has shown larger infarct volume in mice with PAR2 KO when compared to WT (Jin et al., 2005).

Given the dual nature of PAR2 activation on astrocytes in neurotoxicity (Alhadidi et al., 2024; Liu et al., 2014; Lohman et al., 2012; Pons-Espinal et al., 2024; Reches & Piran, 2024; Zeng et al., 2013; Zhang et al., 2025) and promoting neuroprotection (Greenwood & Bushell, 2010; Moudio et al., 2025) it is essential for elucidating their context-dependent roles in maintaining brain homeostasis during health and diseases.

Studying this in human astrocytes is therefore crucial to enable more accurate translation to clinical therapies that modulate PAR2 for targeted neuroprotection without unintended inflammation.

Thereby, this study aims to investigate and seeks to elucidate the functional and the molecular characteristics of PAR2 in primary rodent hippocampal cultures and for the first time in human astrocyte cultures (cHAs). With specific aims to examine PAR2 expression profile, characterize its activation mechanisms and delineate downstream signalling cascades upon activation. These were investigated using calcium imaging, western blotting and immunostaining techniques (see sections 2.2.6 - 2.2.9).

3.2 RESULTS

3.2.1 Characterisation of primary mixed cultures by immunocytochemistry

To characterize the cellular composition of primary rodent hippocampal cultures, immunocytochemical staining was performed. Primary hippocampal cultures were stained with GFAP (glial fibrillary acidic protein) a marker to identify astrocytes, and MAP-2 (a somato-dendritic marker for the neurons) to differentiate neurons from astrocytes in rat primary hippocampal cultures. As expected, staining rat primary hippocampal (DIV 8-12) with MAP-2 (red) and GFAP (green) has resulted in positive staining of most neurons and astrocytes (Figure 7 A-C).

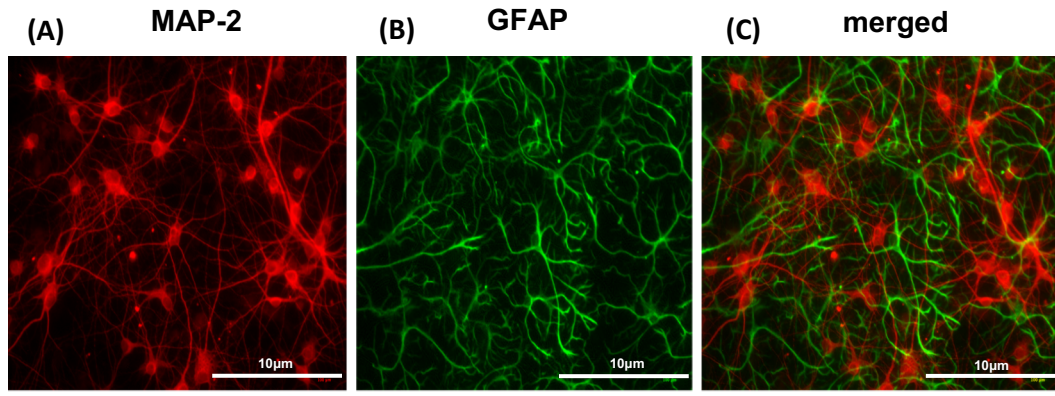


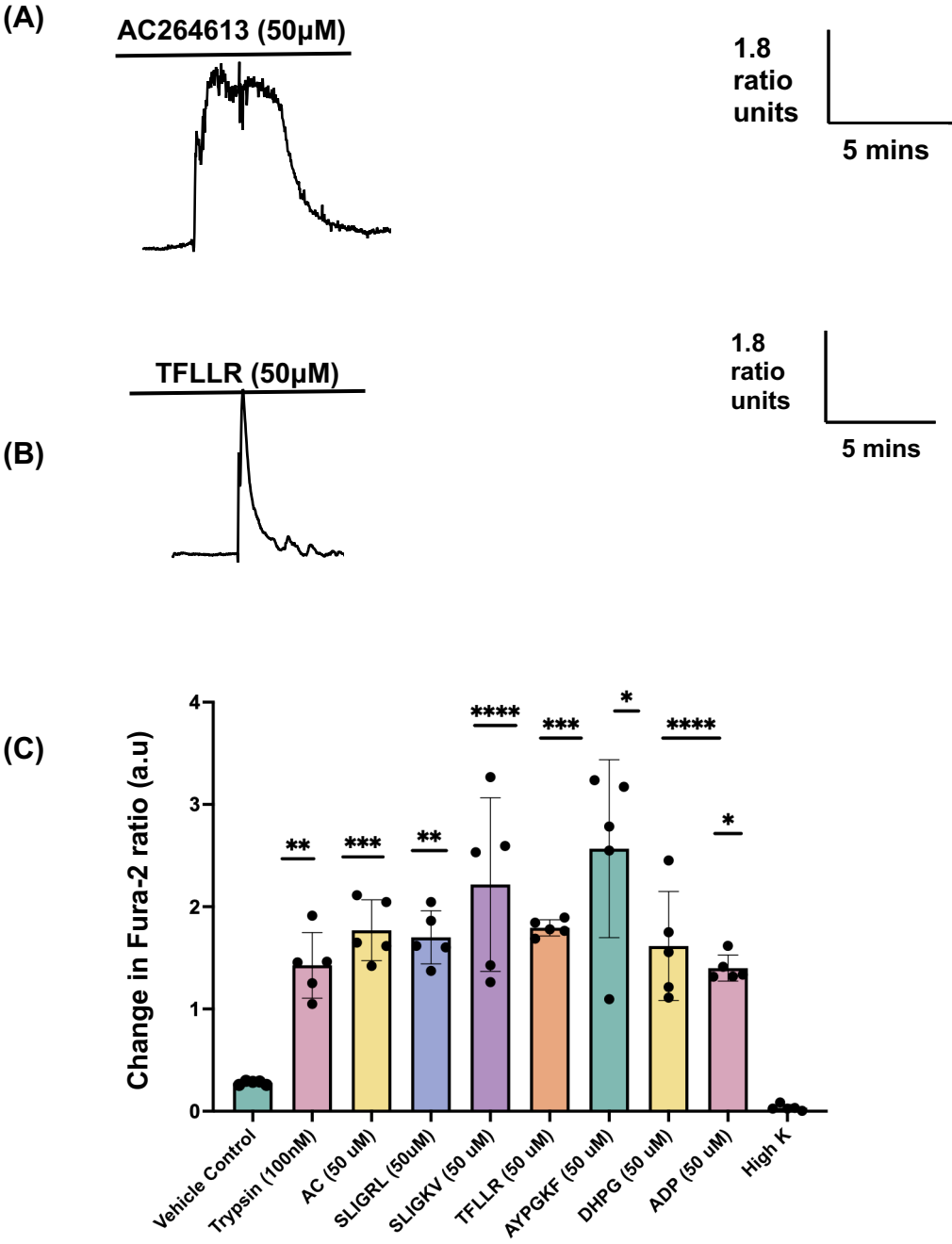
Figure 7: Characterizing primary hippocampal cultures. Representative images of immunostaining of rat primary hippocampal cultures **(A)** positive MAP-2 staining of the neurons in rat primary culture (DIV 10). **(B)** positive GFAP staining of the astrocytes in rat primary culture. **(C)** Overlay of primary hippocampal cultures stained with MAP-2(red) and GFAP (green). Scale bars 10 μm in (A-C), n=3.

3.2.2 PAR activators increase astrocytic but not neuronal $[Ca^{2+}]_i$ in rat primary hippocampal cultures

Application of the PAR2 activator, trypsin (100nM) to rat primary cultures (8-12 DIV) evoked a transitory rise in $[Ca^{2+}]_i$ of 1.42 ± 0.14 a.u., in astrocytes (**Figure 8C**, $n = 5$, $p < 0.0001$ vs vehicle control), but was without effect in neurons (0.02 ± 0.004 a.u., $n = 5$, $p > 0.9999$ vs vehicle control, **Figure 8D**). Similarly, PAR2 activating peptides (APs), SLIGRL-NH₂ (50μM) and SLIGKV-NH₂ (50μM) resulted in increases in astrocytic $[Ca^{2+}]_i$ of 1.7 ± 0.11 a.u. ($n = 5$, $p < 0.0001$ vs. vehicle control) and 2.2 ± 0.37 a.u. ($n = 5$, $p < 0.0001$ vs vehicle control **Figure 8C**) respectively. However, these PAR APs were without effect in neurons, resulting in changes in neuronal $[Ca^{2+}]_i$ of 0.03 ± 0.004 a.u. ($n = 5$, $p > 0.9999$ vs vehicle control **Figure 8D**) and 0.03 ± 0.005 a.u., ($n = 5$, $p >$

0.9999 vs vehicle control, **Figure 8D**). Similar effects of a rise in astrocytic $[Ca^{2+}]_i$ was seen with the application of PAR2 small molecule activator AC264613 (50 μ M) 1.7 ± 0.13 a.u., ($n = 5$, $p < 0.0001$ vs vehicle control, **Figure 8B & C**). Application of PAR1 activating peptide TFLLR-NH₂ (50 μ M) resulted in a robust rise in $[Ca^{2+}]_i$ in astrocytes of 1.79 ± 0.035 a.u., ($n = 5$, $p < 0.0001$ vs vehicle control, **Figure 8B & C**) but both PAR1 (TFLLR-NH₂) and 2 (AC264613) activators failed to elicit any rise in $[Ca^{2+}]_i$ in neurons with changes of 0.03 ± 0.004 a.u., ($n = 5$, $p > 0.9999$ vs vehicle control) and 0.04 ± 0.005 a.u., ($n = 5$, $p > 0.9999$ vs vehicle control, **Figure 8D**). The application of AYPGKF (50 μ M) a selective peptide agonist for PAR 4 elicited an increase in $[Ca^{2+}]_i$ in astrocytes (2.5 ± 0.3 a.u., $n = 5$, $p > 0.0001$ vs vehicle control) but not in neurons (0.04 ± 0.005 a.u., $n = 5$ cultures, $p > 0.9999$ vs vehicle control **Figure 8D**). To pharmacologically compare PAR activation induced effects, we compared other GPCRs that have shown to be present in hippocampal cultures ADP (50 μ M) a selective P2Y1 receptor agonist induced a transient rise of $[Ca^{2+}]_i$ in astrocytes 1.4 ± 0.05 a.u., ($n = 5$, $p < 0.0001$ vs vehicle control, **Figure 8C**) that was without effect in neurons 0.04 ± 0.03 a.u., ($n = 5$, $p > 0.9999$ vs vehicle control, **Figure 8D**). Similarly, (S)-3,5-dihydroxyphenylalanine (DHPG, 50 μ M) a selective group I mGlu receptors agonist induced increases in $[Ca^{2+}]_i$ in astrocytes of 1.6 ± 0.2 a.u., ($n = 5$, $p < 0.0001$ vs vehicle control, **Figure 8C**) and but did not increase $[Ca^{2+}]_i$ in neurons (0.03 ± 0.004 a.u., $n = 5$, $p > 0.9999$ vs vehicle control, **Figure 8D**). However, to assess the viability of primary neuronal cultures following drug washout, all cultures were exposed to a 25 mM K⁺ solution. Neuronal

responsiveness was confirmed by the consistent activation of neurons across all cultures in response to the 25 mM K⁺ treatment (**Figure 8D**). In contrast, astrocytes exhibited no response to the high-potassium wash, indicating a lack of sensitivity to this stimulus under the experimental conditions.



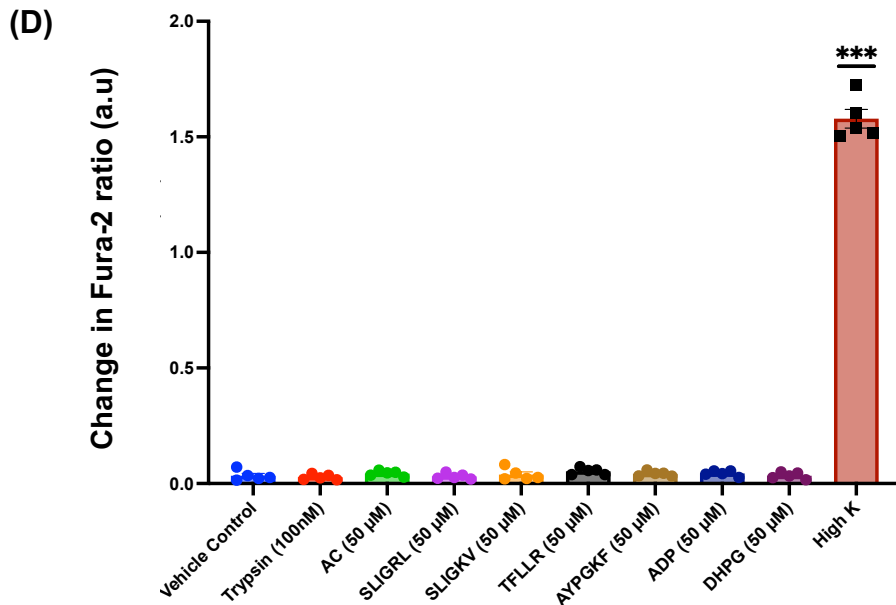


Figure 8: PAR activators increase astrocytic but not neuronal $[Ca^{2+}]_i$ in rat primary hippocampal cultures. (A, B) Representative traces of astrocytic (black) and neuronal (red) response to PAR activators and high potassium (C) Bar chart summarising the increases in astrocytic $[Ca^{2+}]_i$ observed in primary hippocampal cultures following the application of PAR activators. (D) Bar chart highlighting the lack of neuronal responses to PAR activators, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$ vs vehicle control, one-way ANOVA with Tukey's post hoc test, $n=5$.

3.2.3 Characterization of commercial human astrocytes (cHA).

To characterize the cellular composition of human astrocyte cultures, immunocytochemical staining was performed to examine and visualize astrocytes. Human astrocyte cultures were stained with GFAP (glial fibrillary acidic protein) a marker to identify astrocytes, SOX-9 (a nuclear marker for the astrocytes) and cell mask (a whole cell cytoplasmic stain) (**Figure 9 A-D**).

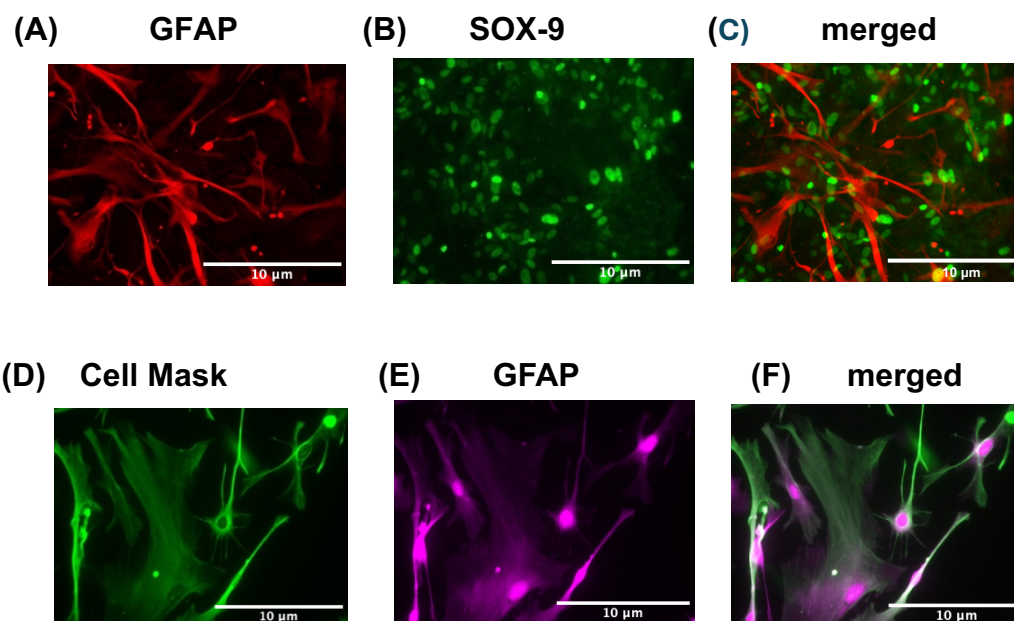
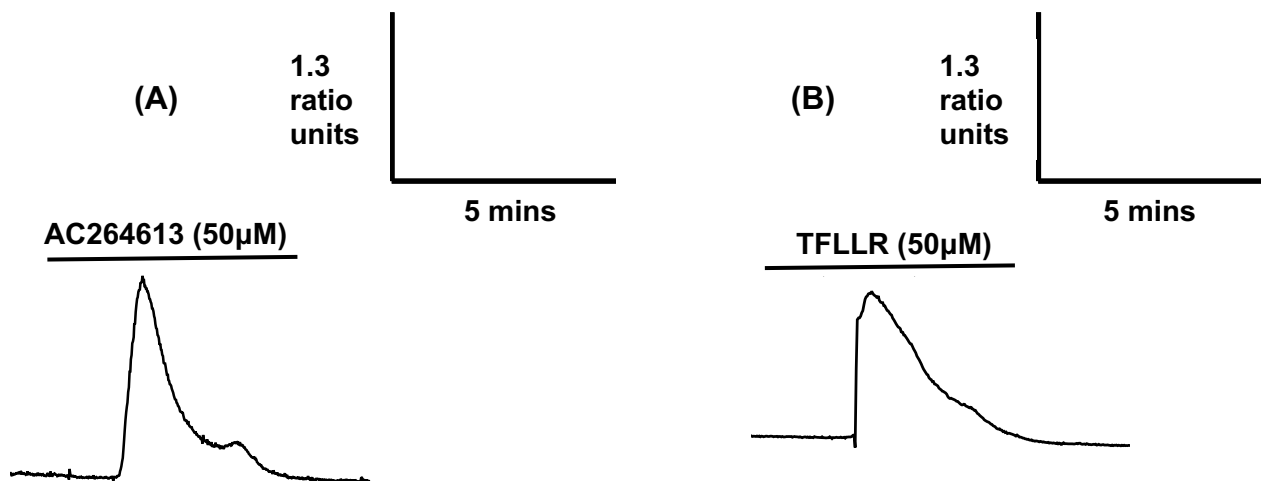


Figure 9: Representative images of immunostaining of commercial human astrocyte cultures. (A) human astrocyte cultures stained with GFAP (B) human astrocyte cultures stained with SOX-9 (C) overlay of human astrocyte culture showing positive SOX-9 (green) with comparatively little of GFAP staining (red) (D) whole cell staining of human astrocyte culture with Cell Mask, (E) human astrocyte cultures stained with GFAP, (F) overlay of human astrocyte culture showing positive cell mask (magenta) with GFAP staining (green). Scale bars 10µm in (A-F), n=3.

3.2.4 Human astrocytes express functional PARs.

Following the confirmation of protease-activated receptor (PAR) functional expression in primary rodent hippocampal cultures, subsequent experiments were conducted to determine whether these findings could be extended to human astrocyte cultures. This investigation aimed to assess the presence and functional relevance of PAR2 in human astrocytes. In the present study, we have investigated the presence of PARs in commercial human astrocytes (cHA) (3-5 DPP). Application of the PAR2 activators, trypsin (100nM) and

AC264613 (50 μ M) increased $[Ca^{2+}]_i$ in human astrocytes of 1.6 ± 0.16 a.u. ($n=6$, $p<0.0001$ vs vehicle control) and 1.5 ± 0.12 a.u., ($n=6$, $p<0.0001$ vs vehicle control, **Figure 10C**). Similarly, PAR2 activating peptides, SLIGRL-NH₂ (50 μ M) and SLIGKV-NH₂ (50 μ M) both induced an increase in $[Ca^{2+}]_i$ in human astrocytes of 2.06 ± 0.39 a.u. ($n=6$, $p<0.0001$ vs vehicle control) and 1.6 ± 0.2 a.u. ($n=6$, $p<0.0001$ vs vehicle control, **Figure 10C**). In addition, the PAR1 AP TFLLR- NH₂ (50 μ M) evoked an increase in $[Ca^{2+}]_i$ in human astrocytes of 2.0 ± 0.3 a.u., ($n=6$, $p<0.0001$ vs vehicle control, **Figure 10C**). In contrast to PAR1 & 2 activation, application of AYPGKF (50 μ M) a selective PAR4 AP did not alter $[Ca^{2+}]_i$ in human astrocytes 0.08 ± 0.03 a.u., ($n=6$, $p>0.6723$ vs vehicle control, **Figure 10C**). To pharmacologically characterize other GPCRs in the human astrocyte cultures, we applied the P2Y1 agonist ADP (50 μ M) the group 1 mGlu agonist (DHPG, 50 μ M). ADP (50 μ M) elicited a rise in $[Ca^{2+}]_i$ in human astrocytes of 1.6 ± 0.13 a.u., ($n=6$, $p<0.0001$ vs vehicle control **Figure 10C**) whereas DHPG was ineffective (0.047 ± 0.009 a.u., $n=6$, $p>0.9999$ vs vehicle control, **Figure 10C**).



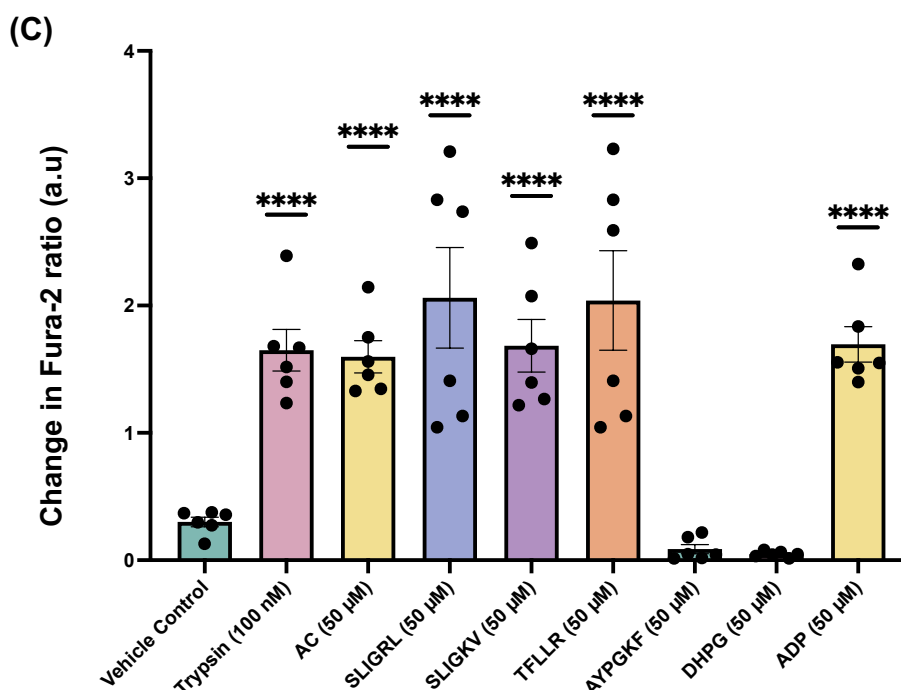
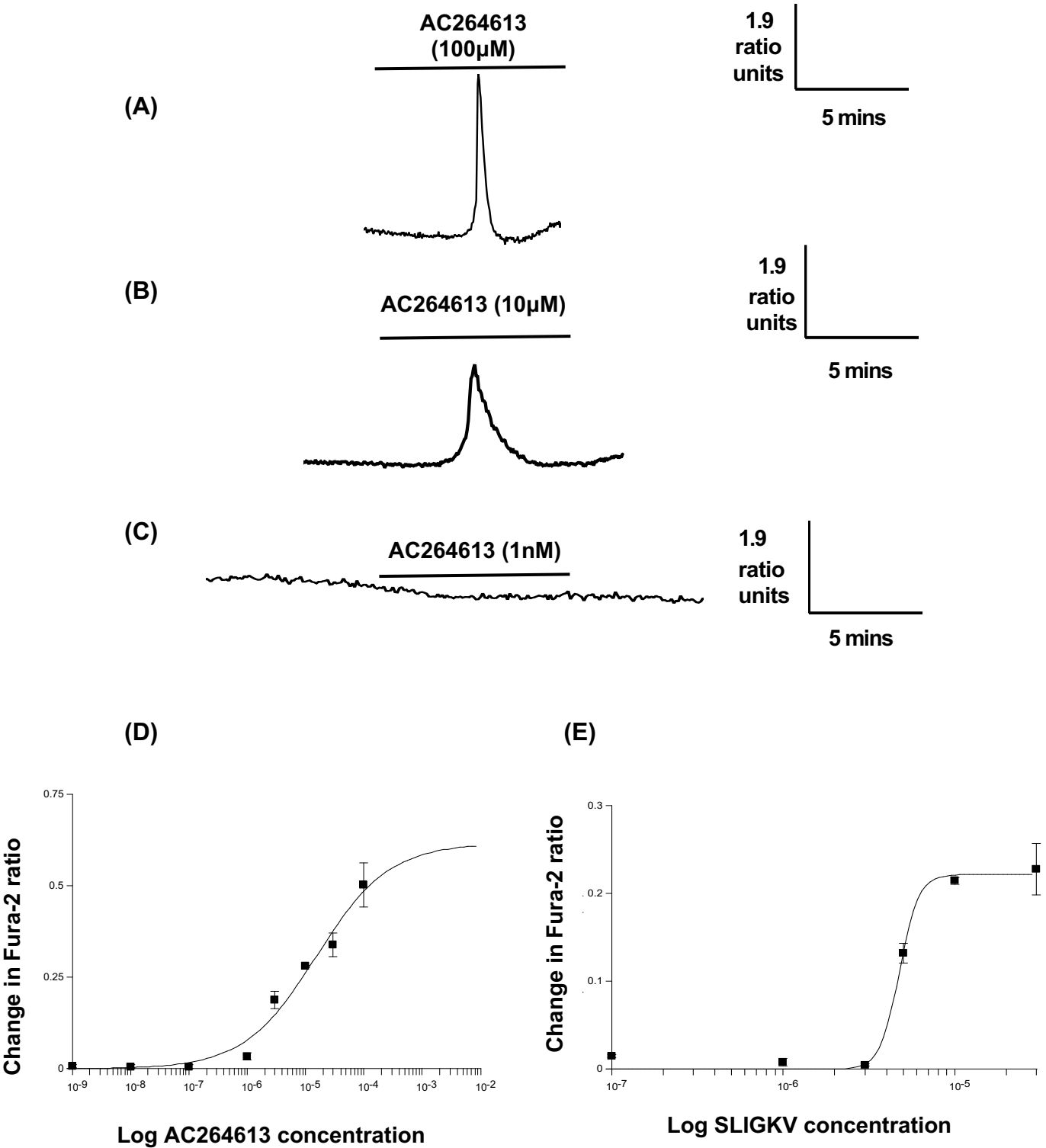


Figure 10: PAR activators increase $[Ca^{2+}]_i$ in commercial human astrocyte cultures. (A) and (B) Representative traces showing changes in astrocytic intracellular calcium concentration $[Ca^{2+}]_i$ induced by PAR2 activators AC264613 (50μM) and TFLLR-NH₂ (50μM). (C) Bar chart summarizing the increases in astrocytic $[Ca^{2+}]_i$ following application of PAR, P2Y1 and DHPG activators. ****p < 0.0001 compared to vehicle control, determined by one-way ANOVA with Tukey's post hoc test, n=6.

3.2.5 Concentration-dependent increase in astrocytic $[Ca^{2+}]_i$ induced by PAR activators.

Having established the functional expression of PAR1 and PAR2 in commercial human astrocytes (cHAs), concentration-response curves were generated using calcium imaging techniques. Application of PAR activators elicited concentration-dependent increases in intracellular calcium concentration $[Ca^{2+}]_i$ in cHAs with half-maximal effective concentration (EC₅₀) values of $15.5 \times 10^{-6} \mu\text{M}$ for AC264613 (**Figure 11D**), $4.8 \times 10^{-6} \mu\text{M}$ for

SLIGKV-NH2 (**Figure 11E**), and $2.7 \times 10^{-6} \mu\text{M}$ for TFLLR-NH2 (**Figure 11F**), with each agonist was applied over a range of concentrations, and each concentration was tested in replicate experiments to construct the dose–response curves.



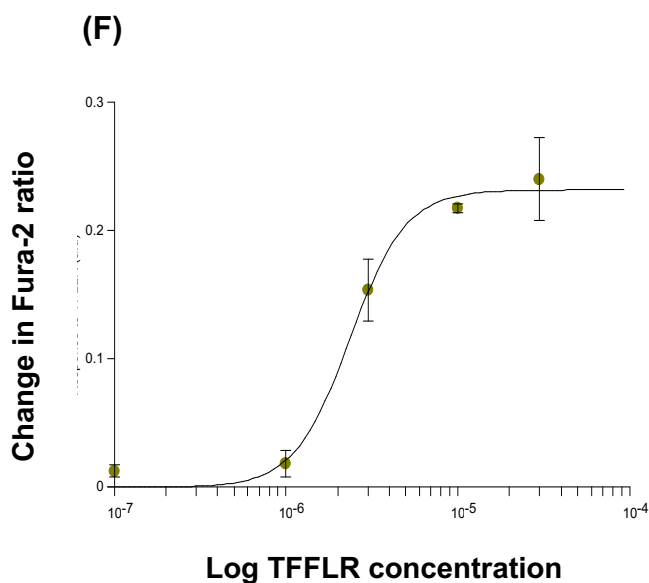
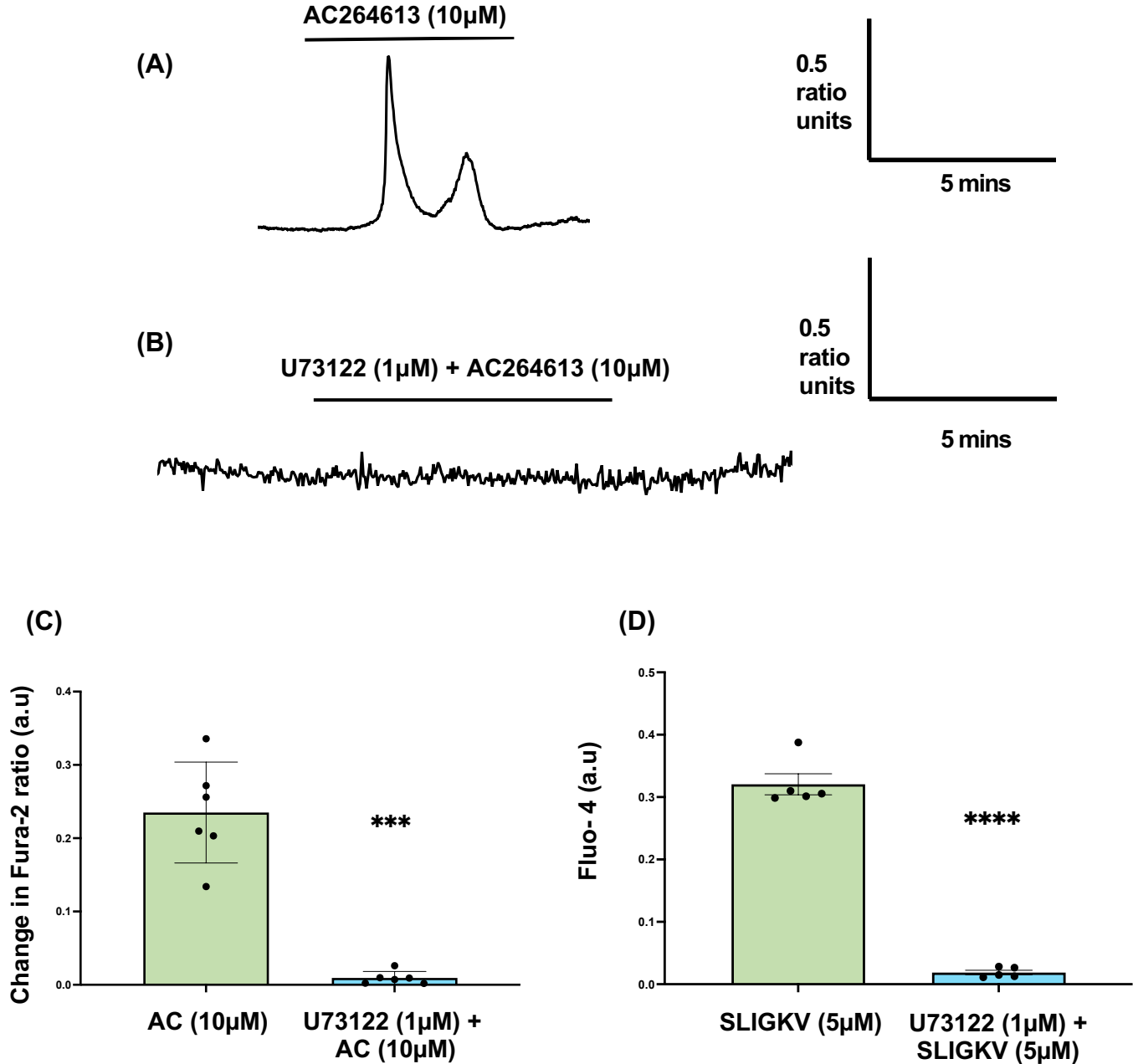


Figure 11: Concentration-response analysis of PAR activators in commercial human astrocyte. Representative traces of cultured human astrocytes response to (A) AC262413(100 μ M), (B) AC262413(10 μ M), (C) AC262413(1nM), (D) CR curve for AC264613 ($EC_{50} = 15.5 \times 10^{-6} \mu$ M) (E) CR curve for SLIGKV-NH2 ($EC_{50} = 4.8 \times 10^{-6} \mu$ M) and (F) CR curve for TFLLR-NH2 ($EC_{50} = 2.7 \times 10^{-6} \mu$ M).

3.2.6 PAR-mediated rise in $[Ca^{2+}]_i$ is via the PLC-IP₃ pathway

Given that activation of PAR1 and PAR2 elicits elevations in $[Ca^{2+}]_i$, we sought to elucidate the underlying signalling mechanisms in commercial human astrocyte cultures 3-5 DPP. To achieve this, we employed the selective phospholipase C (PLC) inhibitor U73122. Cultures were pre-incubated with U73122(1 μ M) for 10 minutes following which application of PAR2 activators AC264613 (10 μ M) and SLIGKV-NH₂ (5 μ M) in the continued presence of U73122 resulted in a 0.0094 ± 0.003 a.u., (n= 5, p< 0.05 vs AC264613 alone) (Figure 12C), 0.01873 ± 0.0035 a.u. (n= 5, p<0.05 vs SLIGKV-NH₂ alone) (Figure 12D). Similarly, TFLLR-NH₂ (3 μ M) in presence of U73122 induced a peak change of 0.02566 ± 0.0063 a.u. (n=5, p<0.05 vs TFLLR-NH₂ alone) in

human astrocytes (**Figure 12E**). Furthermore, For the TFLLR-NH₂ and SLIGKV-NH₂ experiments, astrocytes were loaded with Fluo-4 AM prior to Ca²⁺ imaging, as described in the methods.



(E)

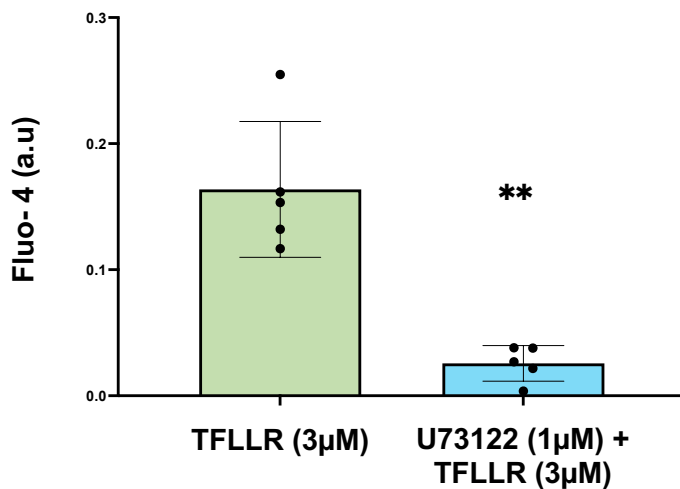


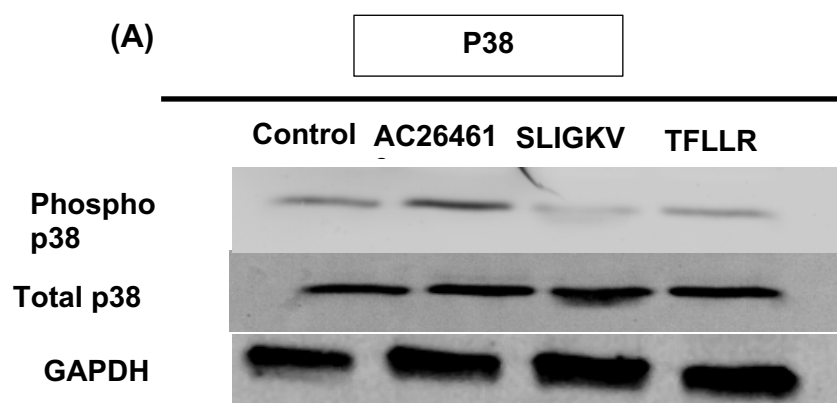
Figure 12: PAR1 and 2 induces [Ca²⁺]_i signalling via the phospholipase C pathway.

Representative trace showing changes in intracellular calcium concentration [Ca²⁺]_i in commercial human astrocytes (cHAs) induced by the (A) PAR2 agonist AC264613 (10 µM). (B) Response of cHAs to AC264613 (10µM) in the presence of U73122(1µM). Quantification showing significant inhibition of [Ca²⁺]_i increases by U73122 (1µM) (D) AC264613 (10µM) (E) SLIGKV (5µM). (F) TFLLR-NH₂ (3µM). Data represented mean ± SEM; ** p = 0.0004 *** p = 0.0001 **** p = 0.0035 vs. PAR1 and 2 activators, unpaired t-test, n=5 (SLIGKV and TFLLR), n=6 (AC264613).

3.2.7 PAR activation does not modulate the MAPK pathway

To further elucidate the downstream signalling mechanisms of protease-activated receptor activation in cHAs, we investigated the impact of PAR activators on mitogen-activated protein kinase (MAPK) signalling pathways, which prior studies have demonstrated to be modulated by PAR activation primarily through experimental assays like Western blotting for phospho-MAPK (Cheng et al., 2017; Gan et al., 2011; Greenwood & Bushell, 2010). cHAs were treated with the PAR activators AC264613 (10 µM), SLIGKV-NH₂ (5 µM), and TFLLR-NH₂ (3 µM) for 24

h. Whole-cell lysates were then subjected to western blotting using phospho-specific antibodies against p38 MAPK, normalised to total p38 and GAPDH loading controls; however, no significant effect on p38 MAPK phosphorylation was observed compared to vehicle controls. The phosphorylation ratios for MAPK signaling were determined as follows: AC264613 ($100.8 \pm 27.5\%$), SLIGKV-NH₂ ($119.1 \pm 29.6\%$), and TFLLR-NH₂ ($92.8 \pm 19.0\%$) (n=6), with none of these values showing significant differences compared to the vehicle control ($100 \pm 33.8\%$) (**Figure 13 A, B**). Similarly, the impact of PAR activation on extracellular signal-regulated kinase 1/2 (ERK1/2) activity was assessed using western blotting using phospho-specific antibodies against ERK1/2, normalised to total ERK1/2 and GAPDH loading controls; however, no significant effect on ERK1/2 phosphorylation was observed compared to vehicle controls. The phosphorylation ratios for ERK1/2 were recorded as AC264613 ($97.1 \pm 31\%$), SLIGKV-NH₂ ($115.5 \pm 39.7\%$), and TFLLR-NH₂ ($108.5 \pm 34.8\%$), with no significant differences observed relative to the vehicle control ($100 \pm 36.7\%$) (**Figure 13 C, D**). These findings suggest that PAR activation does not significantly alter MAPK or ERK1/2 phosphorylation under the tested conditions.



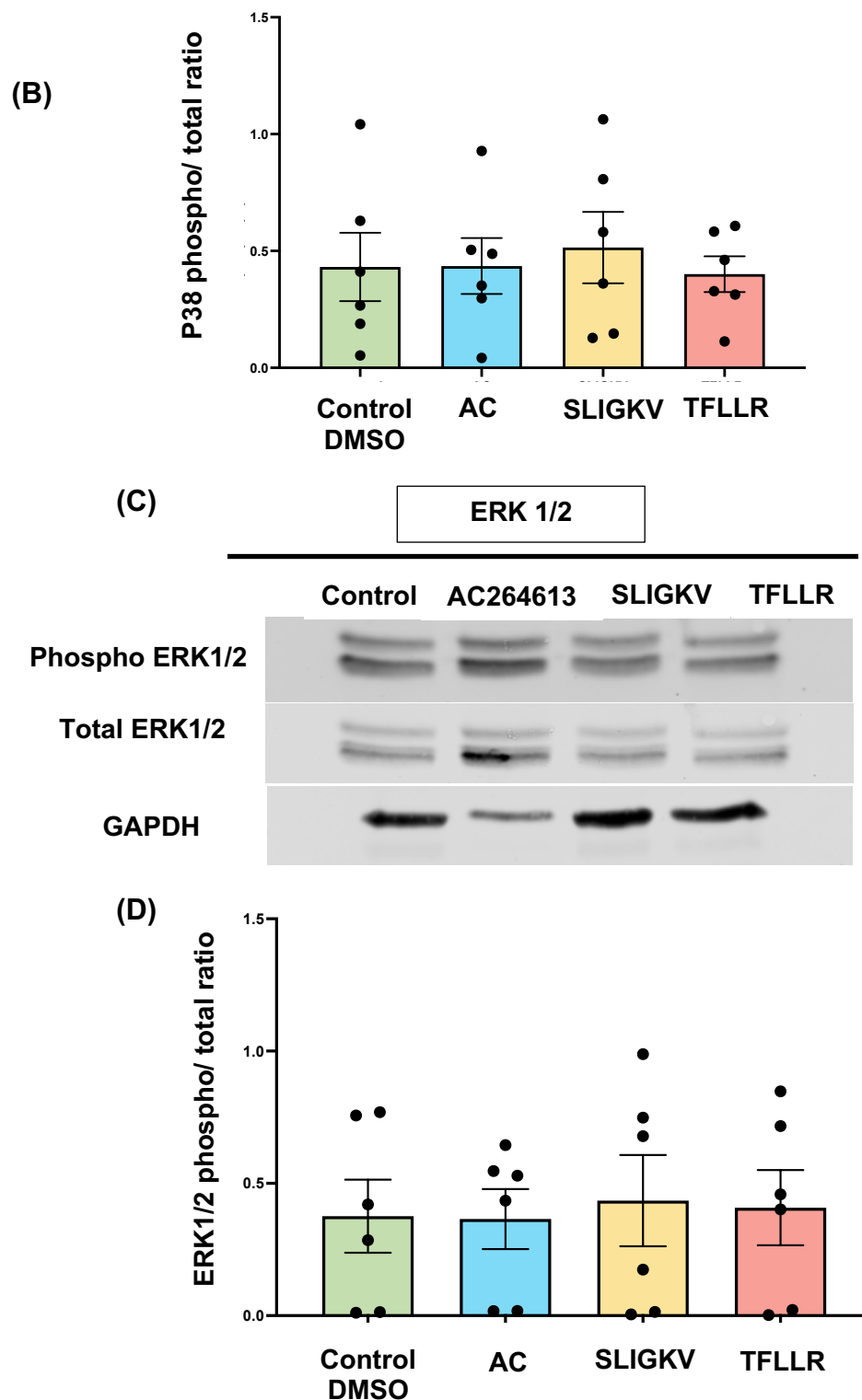


Figure 13: The effect of PAR activation on MAPK downstream signalling in human astrocytes. Representative blots of PAR activation on (A) p38 and (C) ERK 1/2 (B) Bar graphs summarizing the effects of PAR activation on MAPK activity (D)

ERK 1/2 activity. Data are mean $\pm$ SEM for n = 6 cultures, p > 0.05 versus vehicle control.

3.3 Discussion

3.3.1 Expression of PARs in primary hippocampal cultures.

PARs constitute a distinctive family of GPCRs activated by proteolytic cleavage to the N-terminal, playing pivotal roles in cellular signalling within the central nervous system, including modulation of calcium dynamics, synaptic plasticity, and neuroinflammatory responses. Primary hippocampal cultures provide an essential *in vitro* model for elucidating PAR functionality in neuronal and glial contexts. In the current investigation, selective activation of PAR2 using peptide agonists such as SLIGRL-NH₂ and SLIGKV-NH₂, the small molecule activator AC264613, and the endogenous protease trypsin elicited robust elevations in [Ca²⁺]_i exclusively in astrocytes, with no detectable responses in neurons. This data suggests that PAR2 may be absent in neurons or fails to couple effectively to Ca²⁺ signalling pathways in the cultures employed here. However, this observation contrasts with earlier reports demonstrating PAR2 functional expression in hippocampal neurons, where activation induced [Ca²⁺]_i increases (Bushell et al., 2006a; Moudio et al., 2025; Shavit-Stein et al., 2017). For instance, studies have shown that PAR2 agonists like trypsin or SLIGRL-NH₂ trigger calcium transients in both neuronal and astrocytic populations, contributing to synaptic modulation such as long-term depression at Schaffer collateral-CA1 synapses via transient receptor potential vanilloid 4 channels (Shavit-Stein et al., 2017) and

neuroprotection against kainite induced excitotoxicity assays (Moudio et al., 2025). This variance can be explained due to the culture condition and composition. Previous studies have indicated that primary hippocampal cultures can vary in neuron-astrocyte ratios which can alter pharmacological responses contributed by each cell-type (van Deijk et al., 2017). This suggests that culture composition can modulate PAR signalling dynamics. These observations suggest one possible explanation to astrocytic responses is that astrocytes were enriched compared to neurons, but this remains speculative as there was no direct cell-type quantification was conducted in our study (Stoppelkamp et al., 2010). Furthermore the media type also matters: Neurobasal-A suppresses glial proliferation and excitability, potentially reducing neuronal responses (Bardy C et al., 2015). In contrast, MEM supports higher responder rates in both, aligning with older studies using richer media where both cells responded similarly (Bushell et al., 2006b; Greenwood & Bushell, 2010; Moudio et al., 2025; Shavit-Stein et al., 2017; Stoppelkamp et al., 2010). The PAR1-selective peptide agonist TFLLR-NH₂ elevated [Ca²⁺]_i specifically in astrocytes but elicited no comparable response in neurons. Nevertheless, this observation is in contrast to the previous studies reported where PAR1 activation increases [Ca²⁺]_i in both neurons and astrocytes (Almonte et al., 2007, 2013; Bushell et al., 2006b). But, this finding aligns with prior investigations demonstrating that protease-activated receptor 1 (PAR1), a G-protein-coupled receptor (GPCR) responsive to thrombin and related protease, is predominantly functional in glial cells rather than neuronal populations in certain brain regions (Sweeney et al., 2017). Such selectivity is

likely attributable to differential PAR1 expression profiles, where immunohistochemical and functional assays have revealed robust PAR1 presence in astrocytes, particularly in hippocampal strata, but minimal or absent responsiveness in neighbouring neurons (Rakela et al., 2018). For instance, in acute hippocampal slices, PAR1 activation via TFLLR-NH₂ (typically at 30µM) triggers a pronounced [Ca²⁺]_i increase in astrocytic processes and somata, exhibiting a prolonged duration, no such calcium mobilization is observed in pyramidal neurons under similar conditions, highlighting a glial-centric role for PAR1 in calcium-dependent signalling (Rakela et al., 2018; Shigetomi et al., 2008; Sweeney et al., 2017).

In our study, the application of AYPGKF, a hexapeptide known to selectively activate PAR4 through G-protein-coupled mechanisms (Farndale, 2006; Hollenberg & Saifeddine, 2001), failed to elicit measurable calcium increases responses in neurons derived from primary hippocampal cultures, but did elicit responses in astrocytes. These results align with prior observations in the literature (Wang & Reiser, 2003), who reported calcium mobilization in astrocytes following application of PAR4-selective agonists and align with our previous lab work, wherein application of PAR4 activator did not elicit any noticeable increase in [Ca²⁺]_i in neurons (Bushell et al., 2006a). The absence of response to AYPGKF in neurons in our experimental paradigm can relate to the nature of PAR4 signalling in the CNS, that may influence receptor activation and downstream effector pathways.

However, PAR3 wasn't studied within our experimental paradigm due to several key factors rooted in the current understanding of PAR3 biology, its pharmacological tractability, and its expression and functional role in astrocyte or neuronal systems. PAR3 lacks a reliable, selective agonist. Studies indicate that these peptides fail to elicit robust PAR3-mediated responses in isolation, often due to cross-reactivity with other PARs or insufficient activation of downstream pathways (Vergnolle, 2005). This pharmacological limitation is compounded by PAR3's atypical signalling profile. PAR3 does not independently couple to G proteins or initiate canonical signalling cascades in the same manner as other PARs; instead, it primarily functions as a co-receptor, heterodimerizing with PAR1 or PAR4 to modulate their thrombin-induced responses (Houle & Hollenberg, 2007; McLaughlin et al., 2007).

Furthermore, we investigated other GPCRs known to be present in glia and neurons, such as the P2Y1 receptor and group I mGlu receptors (mGlu1/5). These were selected for their prominent roles in astrocyte-neuron communication: P2Y1 (Gq-coupled) drives ATP/ADP-induced Ca^{2+} oscillations and gliotransmitter release in astrocytes while regulating neuronal excitability bidirectionally, whereas group I mGluRs trigger PLC-IP₃-mediated Ca^{2+} release, synaptic modulation, and neuroinflammatory responses in both cell types serving as positive controls for Ca^{2+} imaging specificity against PAR agonists. This allowed assessment of cell-type selective GPCR signalling in mixed cultures. Specifically, the application of ADP (50 μM), a preferential agonist for P2Y1 receptors, elicited a significant increase in $[\text{Ca}^{2+}]_i$ in astrocytes but not in neurons. This observation aligns partially with prior

studies reporting P2Y1 receptor-mediated calcium responses in astrocytes (Jacob et al., 2014; Shigetomi et al., 2018; Skowrońska et al., 2019; Wellmann et al., 2018). The P2Y1 receptor, expressed in both glia and neurons and coupled to Gq/G11 signalling pathways (Rodrigues et al., 2022) appears to predominantly drive calcium mobilization in astrocytes under our experimental conditions. The lack of a detectable calcium response in neurons may reflect differences in receptor expression levels, coupling efficiency, or downstream signalling pathways, potentially influenced by the culture conditions or neuronal subtype specificity. Similarly, the application of DHPG, a selective agonist for group I metabotropic glutamate receptors (mGlu1 and mGlu5), induced a robust $[Ca^{2+}]_i$ increase in astrocytes within primary hippocampal cultures, consistent with previous findings (Bernstein et al., 1998; Casley et al., 2009; Spampinato et al., 2018). However, no significant response was observed in neurons, which contrasts with expectations given the established role of mGlu1 and mGlu5 receptors in neuronal signalling. This discrepancy may be attributed to the culture duration, as our experiments utilized cultures at 10–12 DIV. Previous studies suggest that mGluR expression and functional coupling in neurons may require extended culture periods or specific environmental cues not fully recapitulated in our system (Majumder et al., 2016). The absence of neuronal responses could also stem from variations in receptor localization, desensitization, or differences in intracellular signalling cascades between astrocytes and neurons in vitro (Mannaioni et al., 2001; Mao et al., 2022).

Finally to confirm the viability and functionality of neurons within these mixed hippocampal cultures, high potassium (25mM) was applied at the end of each experiment. This consistently induced neuronal depolarization, as evidenced by a robust increase in $[Ca^{2+}]_i$, thereby validating the health of the neuronal population. This approach also facilitated the identification of neurons from astrocytes in the mixed culture, as astrocytes typically do not exhibit calcium responses to high potassium depolarization. These findings underscore the utility of potassium-induced depolarization as a reliable method to assess neuronal functionality and distinguish cell types in mixed cultures, as extracellular high potassium diminishes the concentration gradient and causing the membrane to depolarise. Collectively, these results highlight the differential responsiveness of astrocytes and neurons to GPCR agonists in primary hippocampal cultures. The robust astrocytic responses to ADP and DHPG suggest a prominent role for P2Y1 and group I mGlu receptors in glial calcium signalling, potentially contributing to glial-neuronal communication. The lack of neuronal responses, particularly to DHPG, warrants further investigation into the factors governing mGluR expression and function in cultured neurons, such as culture duration, or neuronal subtype heterogeneity.

3.3.2 Presence of functional PARs on commercial human astrocytes.

The current study provides compelling evidence for the functional expression of PARs, specifically PAR1 and PAR2, in human astrocytic cultures, as demonstrated by increases in $[Ca^{2+}]_i$ following stimulation with SLIGRL-NH₂ and SLIGKV-NH₂, the small molecule activator AC264613 PAR2 activators, as well as

PAR1 agonists TFLLR-NH₂. These findings align with previous research indicating that PAR1 and PAR2 signal primarily through Gq proteins, activating the phospholipase C (PLC) pathway to mobilize intracellular calcium (Bushell, 2007; Cheng et al., 2017; Gan et al., 2011; Greenwood & Bushell, 2010; Price et al., 2021). The robust [Ca²⁺]_i responses observed in our experiments confirm for the first time the presence of functional PAR1 and PAR2 in commercial human astrocytes, underscoring their potential role in astrocytic signalling. Moreover, the concentration-response curves for PAR2 and PAR1 activators SLIGKV-NH₂, AC264613 and TFLLR-NH₂ revealed EC₅₀ values of 5µM, 10µM and 3µM respectively. These values for SLIGKV-NH₂ have been reported the same in various other cell lines including human colon carcinoma and human keratinocytes (S.-G. Wang et al., 2015; Widera et al., 2019b), while for AC264613 previous studies in HEK293 cell lines have reported the EC₅₀ as 30nM (Gardell et al., 2008) and finally for the PAR1 activator TFLLR-NH₂ studies have reported an EC₅₀ value between 1-1.9µM when tested in cultured hippocampal neurons (Garavilla et al., 2001), and in rat astrocytes (J. Lee et al., 2015). The comparison of EC₅₀ values across different cell types is essential to interpret ligand potency in a broader biological context, as receptor abundance, downstream signalling pathways, and cell-specific factors can substantially influence dose-response relationships. Any, agreement with previously reported EC₅₀ values in other cell lines supports the validity of the assay and allows direct assessment of whether the present model exhibits altered sensitivity to PAR agonists.

However, the lack of response to AYPGKF, a peptide agonist typically associated with PAR4 activation, suggests either the absence of functional PAR4 in these

cultures or that PAR4 signalling may couple to alternative effector pathways that do not elicit a detectable $[Ca^{2+}]_i$ rise. This observation warrants further investigation to elucidate the expression profile of PAR4 and its downstream signalling mechanisms in human astrocytes.

Additionally, our study explored the expression and functionality of purinergic P2Y1 receptors and group I metabotropic glutamate (mGlu) receptors, both of which are GPCRs implicated in astrocytic calcium signalling (Abbracchio & Ceruti, 2006; Skowrońska et al., 2019; Y. F. Zhao et al., 2022). Application of ADP, a preferential agonist for P2Y1 receptors, induced a significant increase in $[Ca^{2+}]_i$, confirming the presence of functional P2Y1 receptors that likely couple to Gq proteins, consistent with their established role in mobilizing intracellular calcium stores (Burnstock, 2004; Burnstock & Verkhratsky, 2012; Verkhratsky et al., 2012). In contrast, the lack of a $[Ca^{2+}]_i$ response to DHPG, an agonist for group I mGlu receptors (mGlu1 and mGlu5), suggests either the absence of these receptors in our commercial human astrocytic cultures or that their activation engages signalling pathways independent of Gq-mediated calcium mobilization. This finding is particularly intriguing given the reported expression of group I mGlu receptors in astrocytes (Bradley & Challiss, 2012; Mannaioni et al., 2001; Mao et al., 2022; Sun et al., 2013). The absence of a DHPG-induced response may reflect species-specific differences, culture conditions, or the preferential coupling of mGlu receptors to alternative pathways, such as Gs or Gi/o, which do not directly trigger $[Ca^{2+}]_i$ increases (Cimadevila et al., 2025; Kos et al., 2024). These results collectively highlight the complexity of GPCR-mediated signalling in commercial human astrocytes and suggest a selective expression or functional coupling of

receptors in these cultures. The presence of functional PAR1, PAR2, and P2Y1 receptors, coupled with the apparent lack of functional PAR4 and group I mGlu receptors, points to a tailored set of GPCRs that may regulate astrocytic responses to injury, inflammation, or synaptic activity. Future studies should focus on delineating the downstream consequences of PAR and P2Y1 activation, including their roles in cytokine release, gliotransmitter secretion, and astrocyte-neuron interactions, to better understand their contributions to neurological and neurodegenerative conditions.

3.3.3 PAR2 activation induces Ca^{2+} signalling via the PLC Pathway.

A key downstream effect of PAR2 activation is the induction of Ca^{2+} signalling through the PLC pathway. This pathway plays a critical role in modulating cellular responses, including calcium mobilization, which regulates processes such as inflammation, pain, and tissue repair. Understanding the mechanisms of PAR2-mediated Ca^{2+} signalling via PLC is essential for elucidating its physiological and pathological roles, offering insights into potential therapeutic targets. Our investigation into the downstream signalling pathways mediating the transient increase in $[\text{Ca}^{2+}]_i$ in commercial human astrocytes following activation of PAR1 and PAR2 aligned with the previous studies conducted in CNS preparations (Hermann et al., 2009; P. R. Lee et al., 2017; wang et al., 2010). The application of the selective phospholipase C (PLC) inhibitor U73122 completely abolished the PAR1- and PAR2-mediated rise in $[\text{Ca}^{2+}]_i$ induced by the small-molecule PAR2 agonist AC264613, as well as the peptide agonists (PAR1) TFLLR-NH2 and (PAR2) SLIGKV-NH2. These results

indicate that PAR1 and PAR2 activation in commercial human astrocytes triggers an elevation in $[Ca^{2+}]_i$ predominantly through the Gq/PLC/IP3 signalling pathway. This finding aligns with previous studies that have established the critical role of the Gq/PLC/IP3 axis in PAR-mediated calcium signalling across various cell types (Bushell et al., 2016; Bushell et al., 2006b; Gan et al., 2011; Greenwood & Bushell, 2010; Heuberger & Schuepbach, 2019) including astrocytes. The consistency of our observations with existing literature highlights the conserved nature of this signalling pathway in human astrocytes, emphasizing its importance in mediating calcium-dependent responses to PAR1 and PAR2 activation. Specifically, PAR2 activation leads to the production of inositol trisphosphate (IP3), which subsequently triggers the release of calcium from intracellular stores, such as the endoplasmic reticulum, thereby facilitating astrocytic calcium transients. This mechanism is consistent with established GPCR signalling paradigms for PARs, wherein receptor activation couples to Gq/11 subunits to stimulate PLC- β isoforms (Bushell et al., 2006b; Heuberger & Schuepbach, 2019). The pronounced inhibitory effect of U73122 underscores the dependency of the $[Ca^{2+}]_i$ response on PLC activity, effectively ruling out significant contributions from alternative pathways, such as store-operated calcium entry or extracellular calcium influx, during the initial phase of the response. These results confirm that the Gq/PLC/IP3 pathway is the primary mediator of PAR1- and PAR2-induced calcium signalling in cultured human astrocytes. The potential implications of this pathway for astrocyte physiology, including its roles in glio-

transmission and neuroinflammatory responses, warrant further investigation in future studies.

3.3.4 PAR activation does not modulate the MAPK pathway in human astrocytes.

The MAPK/ERK1/2 signalling pathway is a critical regulator of cellular processes, including proliferation, differentiation, and inflammatory responses in astrocytes (Johnson & Lapadat, 2002). Given that PARs, particularly PAR1 and PAR2, are G protein-coupled receptors known to mediate inflammatory and stress responses in glial cells (Afkhami-Goli et al., 2007c; Lee-Rivera et al., 2022; Noorbakhsh et al., 2003, 2006; Ramachandran et al., 2012), based on these previous studies we hypothesized that PAR activation would influence MAPK/ERK1/2 signalling in commercial human astrocytes. However, our findings indicate that (PAR) activation does not significantly modulate nor lead to significant phosphorylation or activation of the mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase 1/2 (ERK1/2) signalling pathways in human astrocytes. This observation is in contrast with previous studies that have reported PAR-mediated activation of MAPK pathways in astrocytes (Chen et al., 2022; Nicole et al., 2005; P. Zhao et al., 2014) and other cell types (Lin & Trejo, 2013; McCoy et al., 2010; P. Zhao et al., 2014). However, there have been contrasting studies of PAR activation on MAPK and ERK 1/2 signalling, for instance, (H. Wang et al., 2002), demonstrated that PAR1 activation by thrombin in rat astrocytes induces robust ERK1/2 phosphorylation, and studies by (Greenwood & Bushell, 2010),

indicated inhibition of MAPK and ERK1/2 upon PAR2 activation in organotypic hippocampal slices cultures (OHSC). The lack of such modulation in human astrocytes may indicate a unique regulatory mechanism in these cells, potentially involving alternative signalling pathways or desensitization of PAR-mediated responses. This could counteract any transient activation induced by PARs, resulting in no net change in MAPK and ERK1/2 phosphorylation. Alternatively, the downstream effects of PAR activation in astrocytes may be mediated through other signalling cascades (Luo et al., 2007; H. Wang & Reiser, 2003).

In conclusion, this chapter presents novel evidence demonstrating the presence of functional PAR1 and PAR2 receptors on commercial human astrocytes, with EC₅₀ values comparable to those observed in other cell types. The activation of PAR1 and PAR2 engages the PLC signalling pathway, while notably, it does not influence the MAPK or ERK1/2 signalling cascades.

CHAPTER 4

Results

Modulation of reactivity and phagocytosis in human astrocytes

4 PAR1 and PAR2-mediated modulation of astrocytic reactivity in commercial human astrocytes.

4.1 Introduction

Astrocytes are known for playing crucial roles in maintaining brain homeostasis, providing metabolic support to neurons, regulating synaptic transmission, and orchestrating neuroinflammatory responses (Andersen & Schousboe, 2023; Siracusa et al., 2019; Sofroniew & Vinters, 2010). Another key role played by them is undergoing phenotypic and functional changes termed as “reactive astrogliosis”. This transition is orchestrated via the bidirectional astrocyte-microglia crosstalk, where activated microglia releases inflammatory mediators, driving astrocytic reactivity and this reactivity amplifies neuroinflammation via secretion of extracellular signalling molecules and pro-inflammatory factors (Escartin et al., 2021; Liddelow & Barres, 2017). Finally, in acute injury and chronic neurodegeneration, the magnitude and the neuroinflammation persistence is dependent on this astrocyte-microglial crosstalk.

While acute reactivity is necessary for safeguarding healthy brain functioning it is proposed that chronic astrocyte reactivity contributes to neurodegeneration in certain CNS diseases (Andersen & Schousboe, 2023; Brandebura et al., 2023; Price et al., 2021). Astrocyte reactivity is defined and marked by a response to an extrinsic signal where astrocytes undergo alterations involving transcriptional, morphological and functional changes (Escartin et al., 2021; Hasel & Liddelow, 2021; Liddelow & Barres, 2017) and

understanding the molecular changes that cause this reactive transformation is a central focus in neuroscience research for identification of potential therapeutic targets for neurodegenerative diseases. (Ponath et al., 2017).

Recent studies have shown the role of PAR1 in mediating astrocytic reactivity in various neuropathological conditions. For example, studies have indicated the a role for PAR1 in induction of morphological changes and inflammatory responses in cultured astrocytes (Chen et al., 2022a; Nicole et al., 2005a) and also induces the release of pro-inflammatory mediators such as interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF- α), and various other chemokines (Chen et al., 2022b; Li et al., 2023; Yang et al., 2022) via activation of the MAPK/NF- κ B cascade. However, PAR2 has been less studied in the context of astrocyte reactivity but studies are indicative of its role in neuroprotection (Greenwood & Bushell, 2010; Moudio et al., 2025).

In previous studies, experiments to recapitulate reactive astrocyte phenotype *in vitro*, have been done wherein cHAs are subjected to a cocktail of inflammatory cytokines TIC (Hasel et al., 2021; Hyvärinen et al., 2019a; Liddelow, 2019a; Liddelow & Barres, 2017; Negro-Demontel et al., 2024). This model recapitulates neuroinflammatory conditions that are observed in various disease conditions, such as Alzheimer's disease (Han et al., 2025), Parkinson's disease (Chi et al., 2025; Yun et al., 2018) and multiple sclerosis (Mohamadi & Borhani-Haghighi, 2023; Ponath et al., 2018). Recent studies have also established models for investigating serum-induced reactivity of human astrocytes (White et al., 2024a). Furthermore, exposure of astrocytes

to inflammatory cytokines triggers characteristic morphological changes and induces inflammatory phenotypes (Prah et al., 2019; Sánchez-Juan et al., 2024; White et al., 2024a).

Our previous results have demonstrated for the first time the presence of functional PAR1 and PAR2 on commercial human astrocytes. Here we further build on these findings to evaluate the impact of serum and serum-free media conditions on the reactivity of commercial human astrocytes, and to understand whether PAR1 and PAR2 activation modulates their reactivity. Immunostaining was performed to assess the astrocyte reactivity through the expression of GFAP (de Reus et al., 2024; Phillips et al., 2024; Sánchez-Juan et al., 2024), C3 (van Erp et al., 2023; Zhang et al., 2023), and Gbp2 (Kim et al., 2025; Kumar et al., 2023; Peng et al., 2024). To investigate the impact of culture conditions on astrocytic phenotype, we characterized morphological differences between human astrocytes maintained in serum-containing versus serum-free media. This comparison is particularly relevant given that astrocytes undergoing reactive gliosis typically exhibit marked morphological remodelling, characterized primarily by cellular hypertrophy. Such architectural changes involve significant increases in both soma size and the complexity of major processes, serving as a hallmark indicator of the reactive state. By quantifying these morphological parameters, we aimed to determine whether the absence of serum significantly influences the baseline reactivity of our human astrocyte models (Chi et al., 2025; Filipi et al., 2024; Kim et al., 2025).

4.2 Results

4.2.1 Assessing the effect of media conditions on human astrocyte reactivity

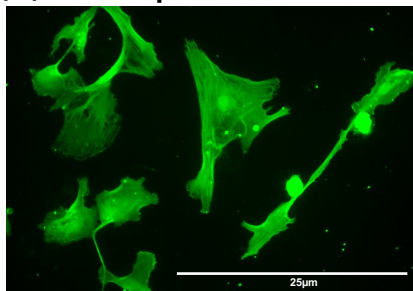
Initially, we investigated the effects of varying media conditions on both the reactivity and morphology of cHAs.

4.2.1.1 *Impact of 24-hours media conditioning on astrocyte reactivity.*

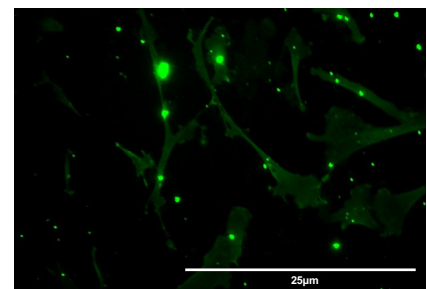
To assess the effects on cell reactivity in both serum (AM) and serum-free media (NB), the cells were stained for GFAP C3 and, markers regularly used to examine astrocytic reactivity (Bohlen et al., 2017, 2019; Guttenplan & Liddelow, 2019a; Liddelow et al., 2017a, 2017b). From our experiments, maintaining human astrocytes under two different media conditions (serum and serum-free) revealed no change in reactivity when assessed for the reactive markers GFAP and C3. GFAP and C3 expression was similar in both serum media (GFAP, 1723.0 ± 592.4 a.u; 95% CI 78.35 - 3368 n = 5; C3 624.0 ± 50.1 , a.u; 95% CI 484.7 – 763.3 n = 5) and serum-free media (GFAP 1498.0 ± 357.2 a.u, p = 0.9101 vs serum media; 95% CI 506.1- 2940 n = 5; C3, 1174.0 ± 797.4 a.u, p = 0.9757 vs serum media; 95% CI -440.1 – 3988, n = 5, **Figure 14 C&D**). However, Gbp2 expression was higher in serum media (8242.0 ± 4071 a.u; 95% CI -3060 – 19544, n = 5) compared to serum-free media (1362.0 ± 334.2 a.u, p = 0.0317, vs serum media; 95% CI 433.8 -2290, n = 5, **Figure 14 E**).

Assessing the morphological changes in serum and serum-free media, no changes were observed in astrocyte area or perimeter, with the area in serum media ($730.3 \pm 65.1 \mu\text{m}^2$; 95% CI 549.5 - 911 n = 5) being comparable to that in serum-free media ($788.9 \pm 42.91 \mu\text{m}^2$, $p = 0.0658$ vs serum media; 95% CI 669.7 – 908, n = 5). Similarly, the perimeter of cells cultured in serum containing media ($705.3 \pm 58.3 \mu\text{m}$; 95% CI 543.2 – 867.3 n = 5) was not altered when compared to the cells cultured in serum-free media ($522.2 \pm 54.9 \mu\text{m}$, $p = 0.0518$ vs serum media; 95% CI 369.7 – 674.6, n = 5, **Figure 15 C&D**).

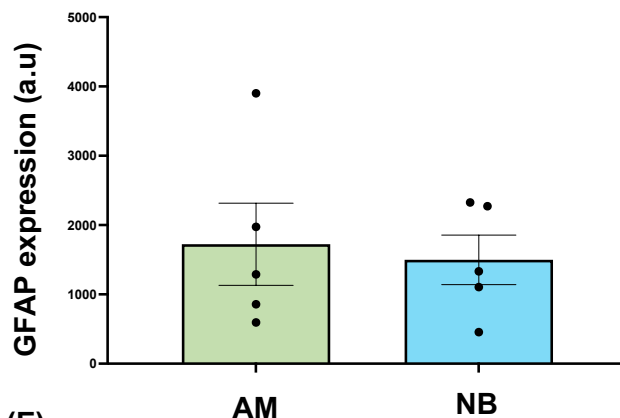
(A) Gbp2 AM



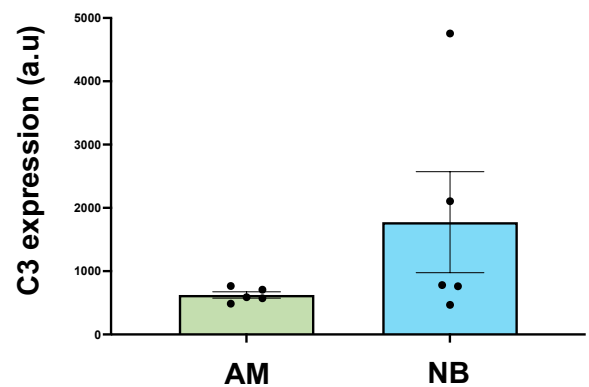
(B) Gbp2 NB



(C)



(D)



(E)

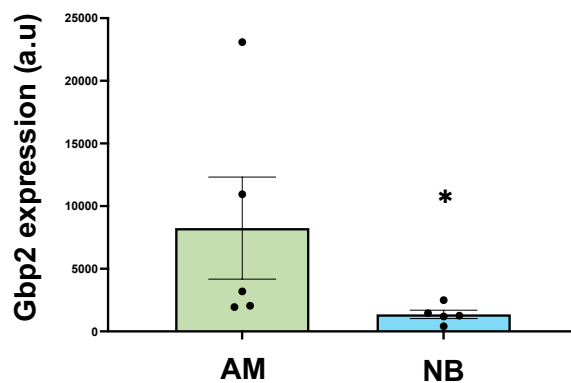


Figure 14: Downregulation of Gbp2 but not GFAP or C3 expression in serum-free conditions after 24 hours of culture. Representative images of human astrocyte Gbp2 expression in (A) serum (B) serum-free media. Bar graph summarising no change in (C) GFAP expression or (D) C3 expression but (E) Gbp2 expression is lower in serum-free media. * = $p < 0.05$ versus serum media. Data expressed is mean $\pm$ S.E.M. for $n = 5$, statistical significance was determined using unpaired t-test. Scale bar 20 μ m.

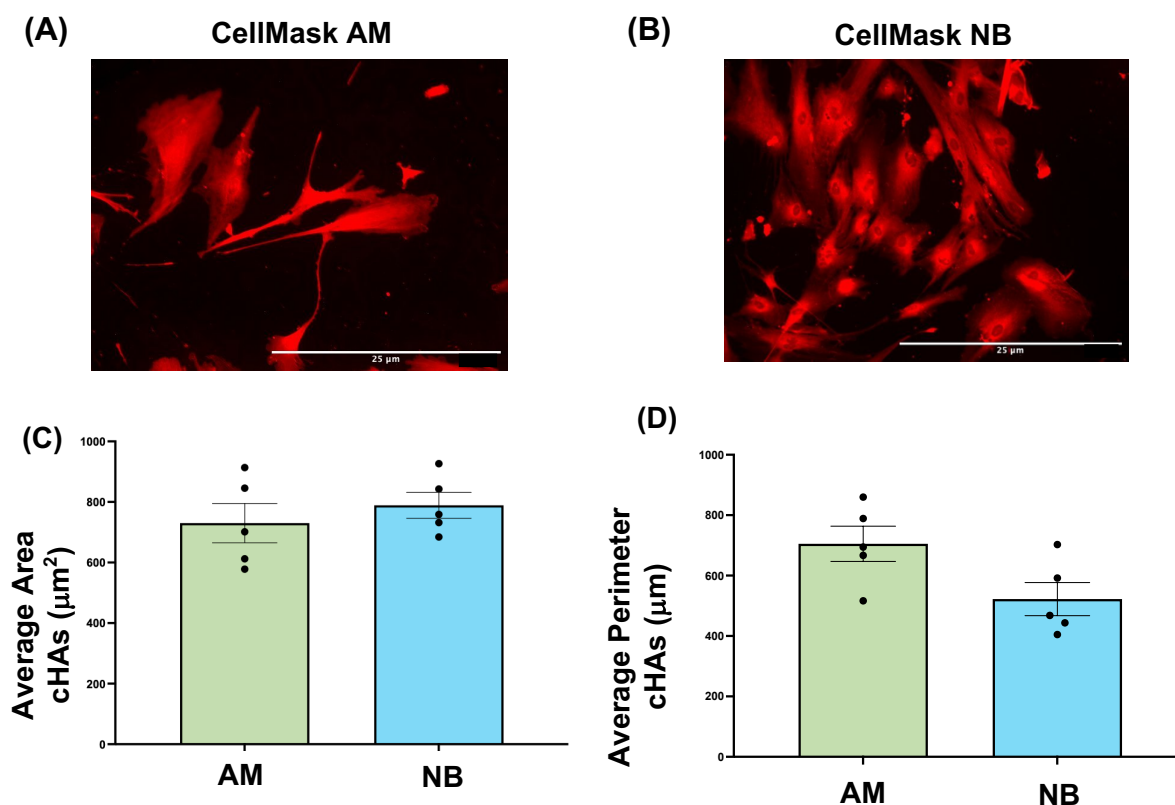


Figure 15: Media conditions have no effect on astrocytic morphology. Representative images of commercial human astrocyte stained with CellMaskTM in (A) serum and (B) serum-free media. Bar graphs summarising (C) average area and (D) average perimeter of human astrocytes. Data is expressed as mean $\pm$ S.E.M. for $n = 5$, statistical significance was determined using unpaired t-test. Scale bar 25 μ m.

4.2.1.2 *Impact of seven - day media conditioning on astrocyte reactivity.*

As limited effects were seen with 24h changes in culture conditions, cells were cultured for seven days in both serum-containing and serum-free media and assessed for the same reactive markers.

Quantification of reactivity markers revealed GFAP and C3 expression was similar in both serum (GFAP, 16.03 ± 1.3 a.u; 95% CI 11.61 – 20.45, $n = 5$; C3, 14.51 ± 2.4 a.u; 95% CI 6.685 – 22.33 $n = 4$) and serum-free media (GFAP, 15.78 ± 1.5 a.u, $p = 0.9101$ vs serum media; 95% CI 10.71 – 20.85 $n = 4$; C3, 14.62 ± 2.6 a.u, $p = 0.9757$ vs serum media; 95% CI 6.315 – 22.93 $n = 4$, **Figure 16 C and D**). However, Gbp2 expression was higher in serum-free media (47.73 ± 2.4 a.u; 95% CI 19.97 – 41.80, $n = 5$) compared to serum-containing media (30.89 ± 3.4 a.u, $p = 0.0017$ vs serum media; 95% CI 39.99 – 55.44, $n = 4$), indicating a differential response to culture conditions for this marker (**Figure 16E**).

Morphological analysis revealed no differences in cell area or perimeter between the two culture conditions. The cell area in serum media was ($3745.0 \pm 1439.0 \mu\text{m}^2$; 95% CI -836.1 – 8326, $n = 5$) compared to serum-free media ($1465.0 \pm 339.8 \mu\text{m}^2$, $p = 0.1356$ vs serum media; 95% CI 384.2 – 2547, $n = 4$). Similarly, the cell perimeter in serum-containing media was ($542.4 \pm 101.2 \mu\text{m}$; 95% CI 220.2 – 864.6, $n = 4$) compared to in serum-free media ($284.2 \pm 38.6 \mu\text{m}$, $p = 0.0571$ vs serum media; 95% CI 161.5 – 407.1, $n = 4$). These findings suggest that the morphological characteristics of astrocytes showed

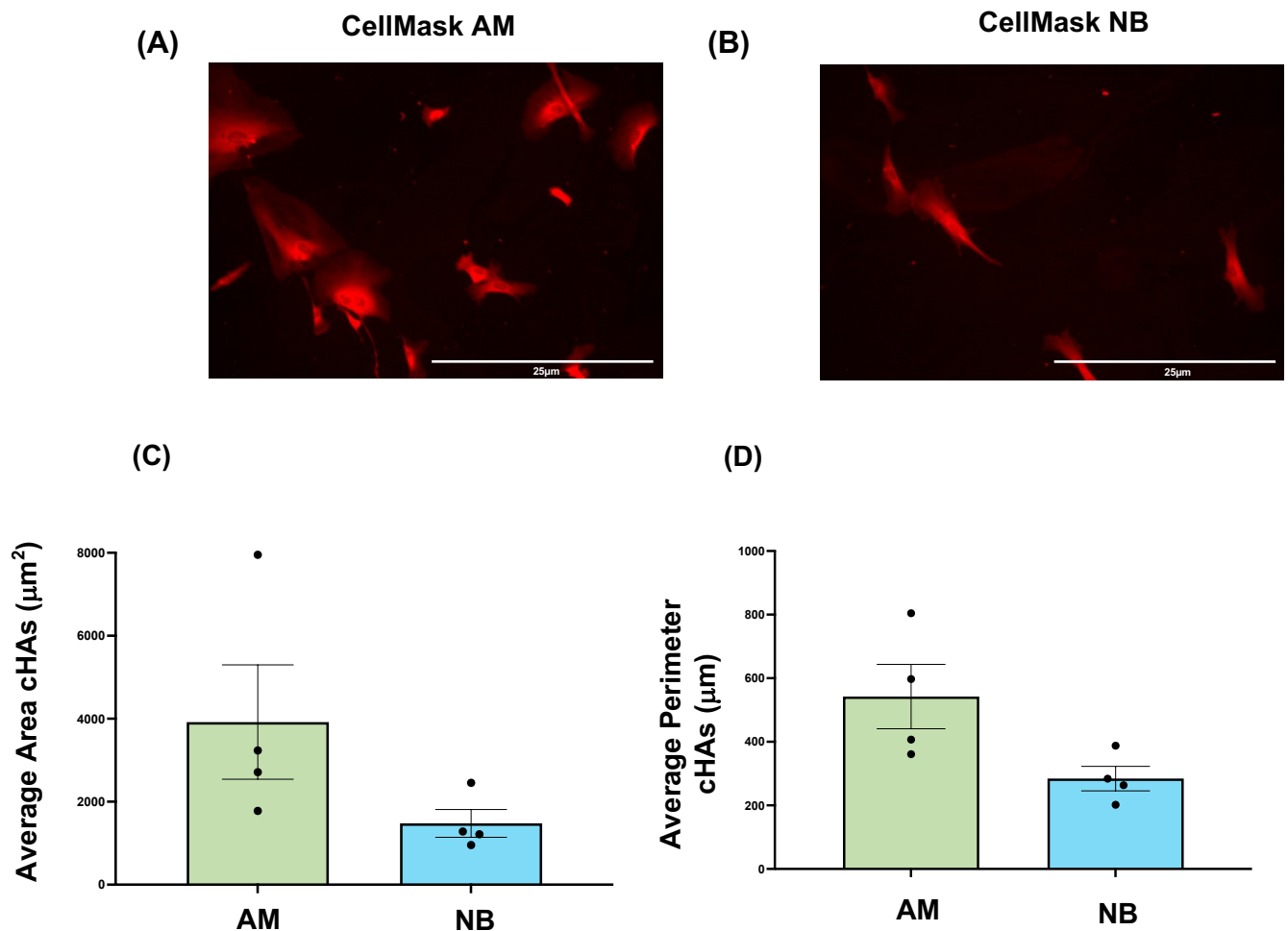


Figure 17: 7-day media conditions have no effect on astrocytic morphology. Representative images of human astrocyte stained with CellMask™ in (A) serum and (B) Serum-free media. Bar graphs summarising (C) average area and (D) average perimeter of human astrocytes following 7 days in two distinct culture conditions. Data expressed is mean $\pm$ S.E.M. for n = 4, unpaired t-test. Scale bar 25µm.

4.3 The effect of PAR activators on HA reactivity

Expanding on prior investigations, this study builds upon previous research examining human astrocyte reactivity in both serum-containing and serum-free media to investigate whether protease-activated receptor (PAR) activators modulate astrocyte reactivity. To evaluate the effects of PAR activators, astrocytes were analysed using immunocytochemical staining for established reactivity markers, including GFAP, C3, and Gbp2.

4.3.1 Evaluation of PAR activation on human astrocyte reactivity following 24-hour treatment.

Following 24 hours of treatment with PAR activators, immunostaining revealed that cells cultured in serum media showed higher GFAP expression when treated with PAR 1 and 2 activators, TFLLR- NH₂ (563.8 ± 112.9 a.u, $p = 0.0065$, 95% CI 250.5 – 877.1 $n = 5$) and SLIGKV-NH₂ (473.6 ± 117 a.u, $p = 0.0013$; 95% CI 148.9 – 798.4, $n = 5$) when compared to vehicle control (56.2 ± 2.2 a.u; 95% CI 49.98 – 62.61, $n = 5$). Similarly, cells cultured in serum-free media showed an increase in GFAP expression when treated with AC264613 (527.6 ± 90.6 a.u, $p = 0.0050$; 95% CI 275.8 – 779.4, $n = 5$), SLIGKV-NH₂ (423.6 ± 116.8 a.u, $p = 0.0300$; 95% CI 99.4 – 747.8, $n = 5$) and TFLLR-NH₂ (351.8 ± 81.4 a.u, $p = 0.00957$; 95% CI 125.8 – 577.8, $n = 5$) compared to vehicle control (83.17 ± 5.5 a.u, $n = 5$, **Figure 18 C**).

No change in C3 expression was seen in cells maintained in serum media and treated with PAR activators AC264613 (353.2 ± 54.9 a.u, $p = 0.8070$; 95% CI 200.8 – 505.7, $n = 5$), SLIGKV-NH₂ (477.3 ± 60.2 a.u, $p = 0.0545$; 95% CI

310.1 – 644.6 n = 5) and TFLLR-NH₂ (229.9 ± 19.7 a.u, p = 0.5858; 95% CI 175.1 – 284.6, n = 5) when compared to vehicle control (303.2 ± 48.0; 95% CI 169.9 – 436.6 n = 5) (**Figure 18 D**). While C3 expression was higher in cells in serum-free media treated with AC264613 (381.3 ± 61.1 a.u, p = 0.0468; 95% CI 211.7 – 551.0, n = 5) when compared to vehicle control (258.5 ± 12.5 a.u, ; 95% CI 223.8 – 293.3, n = 5), but not in cells maintained in serum-free media and treated with SLIGKV-NH₂ (293.6 ± 17.6 a.u, p = 0.7960; 95% CI 244.5 – 342.7, n = 5) and TFLLR-NH₂ (269.2 ± 12.9 a.u, p = 0.9916; 95% CI 233.2 – 305.2, n = 5) when compared to vehicle control (258.5 ± 12.5 a.u; 95% CI 223.8 – 293.3, n = 5, **Figure 18 D**) .

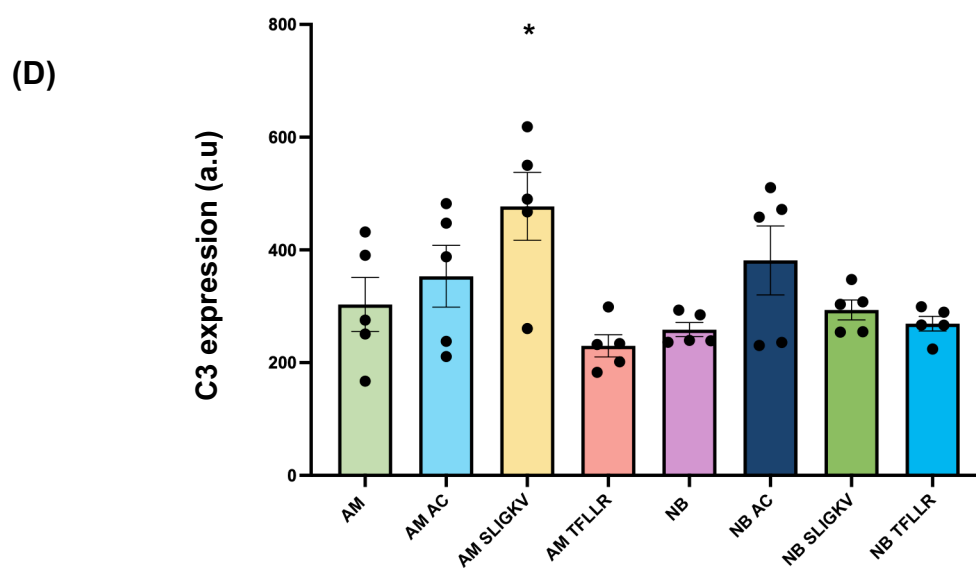
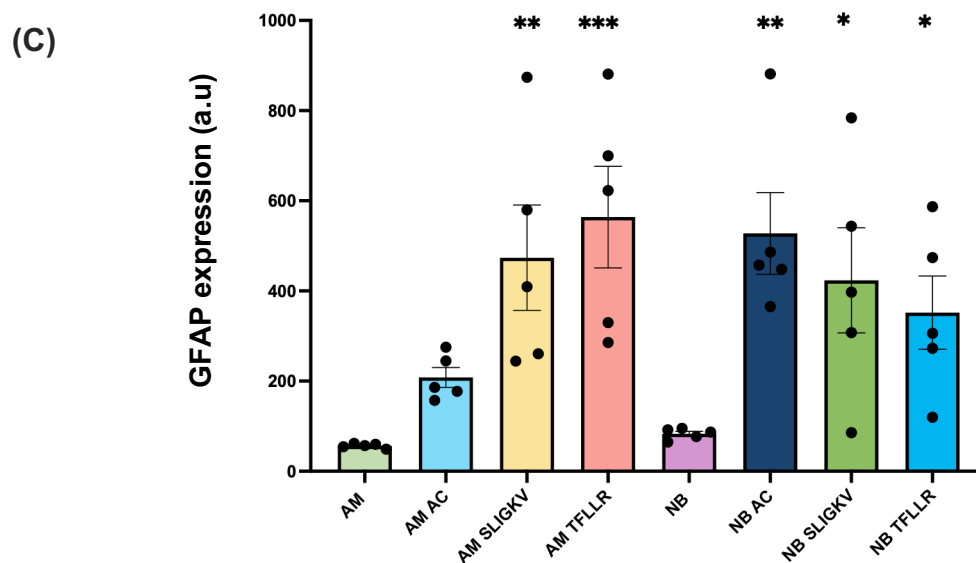
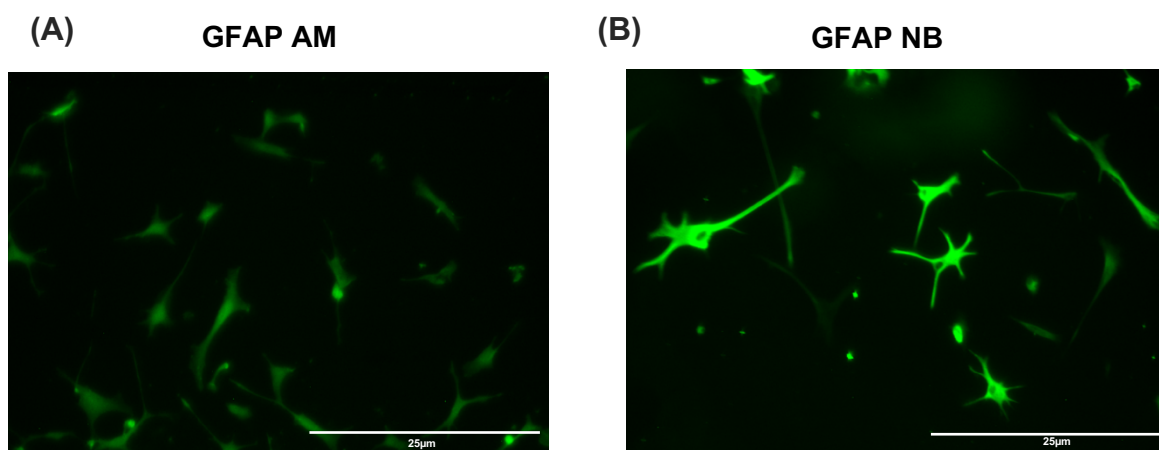
There was no change in expression of Gbp2 in cells maintained in serum and treated with AC264613 (498.2 ± 124.8 a.u, p = 0.9932; 95% CI 151.1 – 844.8, n = 5), SLIGKV-NH₂ (577.6 ± 145.9 a.u, p = 0.8106; 95% CI 172.6 – 982.5, n = 5) and TFLLR-NH₂ (479 ± 74.5 a.u, p = 0.9995; 95% CI 92.01 – 1666, n = 5), compared to vehicle control (465.6 ± 72.2 a.u,; 95% CI 264.9 – 666.3, n = 5, **Figure 18 E**). Similarly, no change in Gbp2 expression was seen serum-free media as well and treated with AC264613 (531.1 ± 112.7 a.u, p = 0.4565; 95% CI 218.1 – 844.1, n = 5), SLIGKV-NH₂ (588 ± 201.7 a.u, p = 0.6692; 95% CI 27.99 – 1148, n = 5) and TFLLR-NH₂ (575.2 ± 59.0 a.u, p = 0.6197; 95% CI 411.1 – 739.2, n = 5) compared to vehicle control (750.6 ± 44.8 a.u; 95% CI 626.1 – 875.2, n = 5, **Figure 18 E**).

Finally, morphological analysis of the cells cultured serum and serum-free media revealed no changes observed in area, when treated with PAR activators compared to vehicle control. Where the area of cells cultured in

serum and treated with AC264613 ($2579 \pm 700.9 \mu\text{m}^2$, $p = 0.4817$; 95% CI 2.472 – 19.59 $n = 5$), SLIGKV-NH₂ ($2038 \pm 676.7 \mu\text{m}^2$, $p = 0.8631$, 95% CI 0.6849 – 16.96, $n = 5$), and TFLLR-NH₂ ($1711 \pm 555.7 \mu\text{m}^2$, $p = 0.9668$, 95% CI 0.7291 – 14.09, $n = 5$) compared to the vehicle control ($1474 \pm 580.5 \mu\text{m}^2$, 95% CI -0.5961 – 13.36, $n = 5$) and the area of cells maintained in serum-free and treated with AC264613 ($1803 \pm 571.6 \mu\text{m}^2$, $p = 0.9646$, 95% CI 0.9336 – 14.67, $n = 5$), SLIGKV-NH₂ ($2243 \pm 728.6 \mu\text{m}^2$, $p = 0.7466$, 95% CI 0.9518 – 18.47, $n = 5$), and TFLLR-NH₂ ($2137 \pm 967 \mu\text{m}^2$, $p = 0.8135$, 95% CI -2.371 – 20.87 $n = 5$), when compared to the vehicle control ($1437 \pm 304.3 \mu\text{m}^2$, 95% CI 2.565 – 9.880 $n = 5$, **Figure 19 C**).

The perimeter of cells cultured in serum show no increase when treated with AC264613 ($531.5 \pm 18.0 \mu\text{m}$, $p = 0.0243$, 95% CI 481.3 – 581.7, $n = 5$) compared to the vehicle control ($414.1 \pm 31.2 \mu\text{m}$, 95% CI 327.4 – 500.7, $n = 5$), and treated with SLIGKV-NH₂ ($45.9 \pm 39.5 \mu\text{m}$, $p = 0.5835$, 95% CI 347.2 – 566.6 $n = 5$) or TFLLR-NH₂ ($469.1 \pm 17.2 \mu\text{m}$, $p = 0.3957$, 95% CI 421.2 – 517.1, $n = 5$) compared to the vehicle control (**Figure 19D**).

However, no changes in perimeter were seen in cells cultured in serum-free and treated with AC264613 ($361.8 \pm 26.7 \mu\text{m}$, $p = 0.9653$, 95% CI 287.5 – 436.2, $n = 5$) and, SLIGKV-NH₂ ($434.4 \pm 43.6 \mu\text{m}$, $p = 0.5835$, 95% CI 313.2 – 555.6 $n = 5$) and, TFLLR-NH₂ ($447.8 \pm 75.8 \mu\text{m}$, $p = 0.3957$, 95% CI 237.1 – 658.5, $n = 5$) compared to the vehicle control, ($387 \pm 25.97 \mu\text{m}$, $n = 5$, **Figure 19D**).



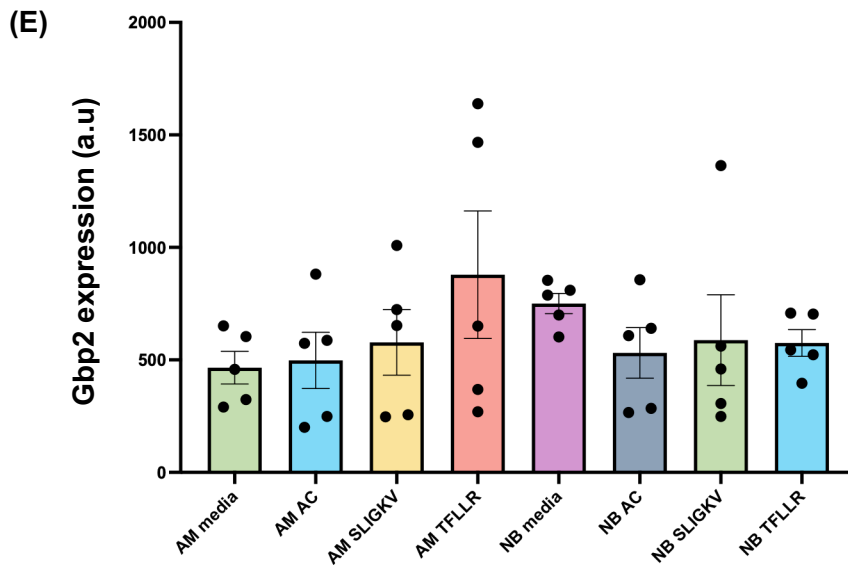
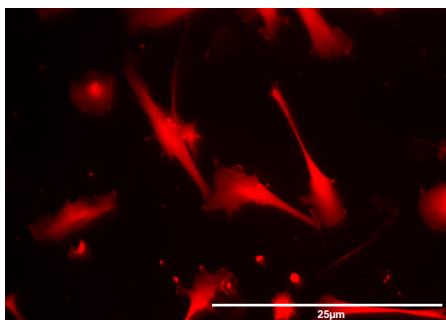
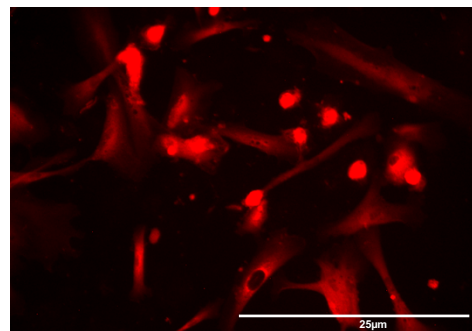


Figure 18 : Differential effects of PAR activators on human astrocytes under varied culture conditions. Representative images demonstrating GFAP expression in human astrocytes cultured in (A) serum (B) serum-free media, in response to the PAR2 activator AC264613. Bar graphs illustrating the differential effects of PAR1 & 2 activators on astrocyte reactivity marker expression, GFAP (C) serum & serum-free, C3 (D) serum & serum-free and Gbp2 (E) serum & serum-free, where (AM) serum and (NB) serum-free culture media. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data are mean $\pm$ S.E.M, one-way ANOVA, with Tukey's post-hoc test. Scale bar 25 μ m.

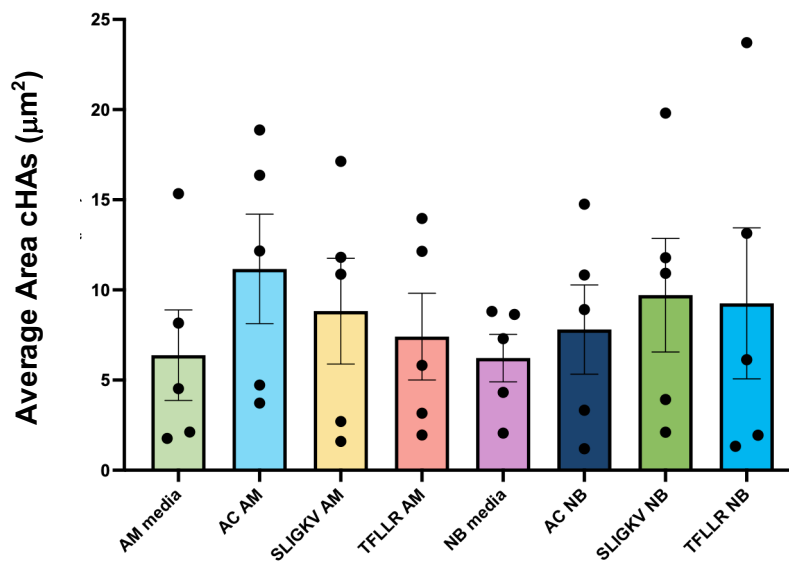
(A) CellMask AM



(B) CellMask NB



(C)



(D)

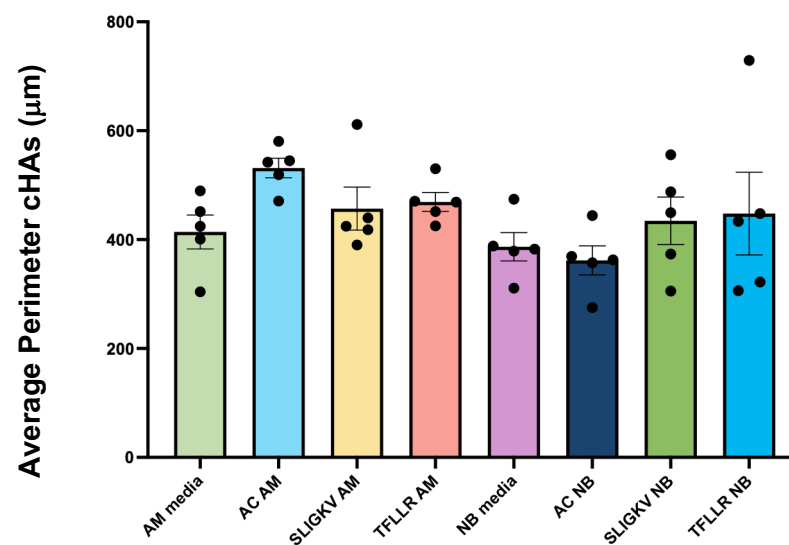


Figure 19: PAR activators alone exhibit no impact on astrocyte area and perimeter. Representative images of human astrocyte stained with CellMask™ in (A) serum and (B) serum-free media. Bar graphs showing area (C) in serum & serum-free, and perimeter (D) of human astrocytes maintained in serum & serum-free following 24-hour exposure to PAR1 and PAR2 activators. Data are mean $\pm$ S.E.M, one-way ANOVA, with Tukey's post-hoc test. Scale bar 25μm.

4.3.2 Evaluation of PAR activation on human astrocyte reactivity following 7-day treatment.

To investigate whether prolonged exposure to PAR activators modulates human astrocyte reactivity under extended culture conditions, astrocytes were maintained for 7 days in serum or serum-free media prior to a 24-hour treatment with PAR activators on day 7. Immunostaining for GFAP subsequently revealed no change in cells cultured under either serum media or serum-free media conditions following treatment with the PAR activators AC264613, SLIGKV-NH₂, or TFLLR-NH₂, relative to their vehicle control. Where the fluorescence values of GFAP expression of cells in serum and treated with AC264613 (119.0 ± 57.8 a.u, $p = 0.8029$, 95% CI -41.50 – 279.5, $n = 5$), SLIGKV-NH₂ (46.4 ± 11.08 a.u, $p = 0.8029$, 95% CI -8.283 – 141.1, $n = 5$), TFLLR-NH₂ (90.97 ± 48.0 a.u, $p = 0.8029$, 95% CI -42.40 – 224.3, $n = 5$) compared to vehicle control ($125. \pm 51.2$ a.u, 95% CI -16.48 – 268.0, $n = 5$). While the fluorescence values of GFAP expression of cells in serum-free and treated with AC264613 (91.5 ± 31.1 a.u, $p = 0.7313$, 95% CI 5.0 – 178.0 $n = 5$), SLIGKV-NH₂ (112.2 ± 44.7 a.u, $p = 0.8958$, 95% CI -11.8 – 236.5, $n = 5$), TFLLR-NH₂ (174.2 ± 71.8 a.u, $p = 0.9820$, 95% CI -25.15 – 373.5, $n = 5$) compared to vehicle control (152.9 ± 47.2 a.u, 95% CI 21.70 – 284.1, $n = 5$, **Figure 20 C**).

No change in C3 expression was observed in response to PAR activation, irrespective of whether cells were cultured in serum or serum-free media (**Figure 20 D**). This consistency in C3 expression indicates that PAR activators did not significantly alter this expression under either culture condition. The

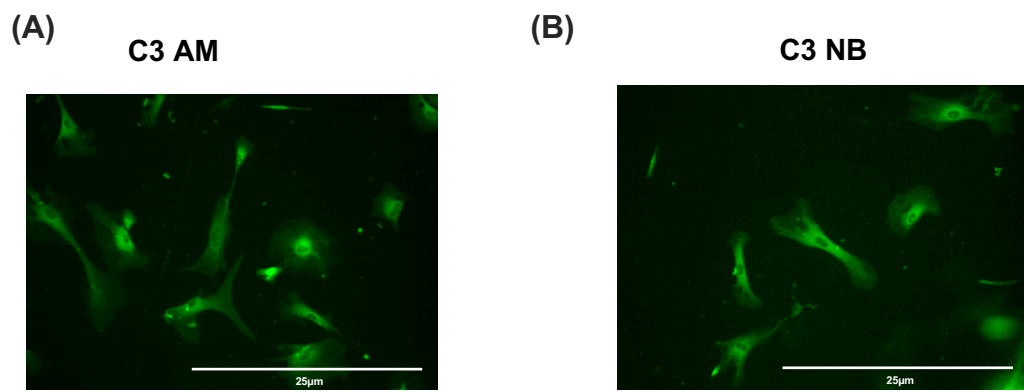
fluorescence values of C3 expression of cells in serum media and treated with AC264613 (42.52 ± 3.9 a.u, $p = 0.3495$; 95% CI 31.46 – 53.58, $n = 5$), SLIGKV-NH₂ (42.24 ± 4.0 a.u, $p = 0.3495$; 95% CI 31.06 – 53.43, $n = 5$), TFLLR-NH₂ (33.55 ± 2.7 a.u, $p = 0.3495$; 95% CI 26.03 – 41.06, $n = 5$) compared to vehicle control (41.51 ± 4.8 a.u; 95% CI 28.13 – 54.89, $n = 5$). The fluorescence values of C3 expression of cells in serum-free media AC264613 (43.77 ± 3.9 a.u, $p = 0.6657$; 95% CI 32.89 – 54.66, $n = 5$), SLIGKV-NH₂ (39.41 ± 4.2 a.u, $p = 0.8512$; 95% CI 27.55 – 51.27, $n = 5$), TFLLR-NH₂ (37.76 ± 3.2 a.u, $p = 0.8512$; 95% CI 28.86 – 46.67, $n = 5$) compared to vehicle control (38.98 ± 2.5 a.u; 95% CI 31.78 – 46.17, $n = 5$).

Moreover, evaluation of Gbp2 expression in human astrocytes cultured in either serum or serum-free media revealed no changes following treatment with PAR activators on the reactivity, compared to their vehicle-treated control. With AC264613 (160.1 ± 65.0 a.u, $p = 0.9822$; 95% CI -20.60 – 340.8, $n = 5$), SLIGKV-NH₂ (145.5 ± 56.9 a.u, $p = 0.9822$; 95% CI -12.5 – 303.6, $n = 5$), TFLLR-NH₂ (175.2 ± 83.8 a.u, $p = 0.9822$; 95% CI -57.47 – 408, $n = 5$) compared to vehicle control (178 ± 42.1 a.u; 95% CI 60.96 – 295.1, $n = 5$). While serum-free media for AC264613 (43.77 ± 3.9 a.u, $p = 0.9922$; 95% CI -66.48 – 492.9, $n = 5$), SLIGKV-NH₂ (39.41 ± 4.2 a.u, $p = 0.9922$; 95% CI -119.3 – 554, $n = 5$), TFLLR-NH₂ (37.76 ± 3.2 a.u, $p = 0.9922$; 95% CI -95.23 – 491.7, $n = 5$) compared to vehicle control (38.98 ± 2.5 a.u, $p = 0.9922$; 95% CI -48.41 – 436.7, $n = 5$, **Figure 20 E**).

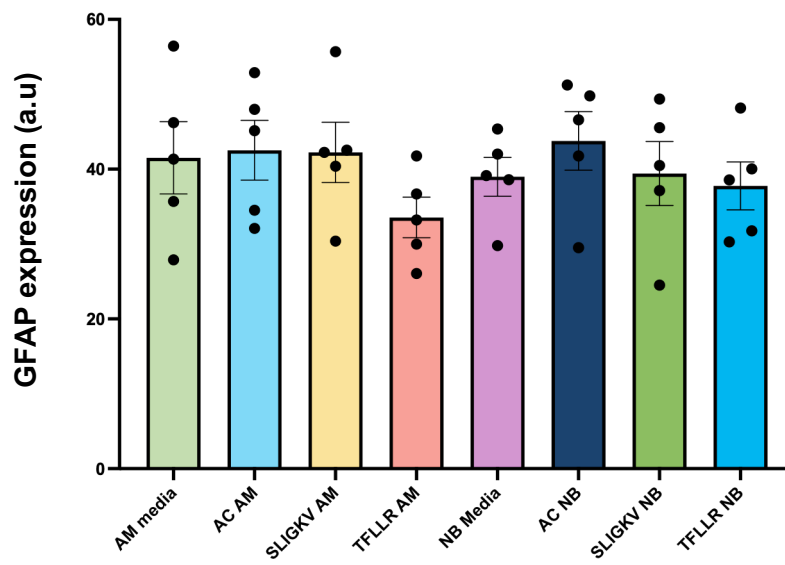
Finally, morphological analysis of the cells cultured serum and serum-free media revealed no changes observed in area, when treated with PAR

activators and vehicle control. Where the area of cells cultured in serum and treated with AC264613 ($7124 \pm 986.1 \mu\text{m}^2$, $p = 0.4432$; 95% CI 4386 – 9861, $n = 5$), SLIGKV-NH₂ ($5595 \pm 1020 \mu\text{m}^2$, $p = 0.8228$; 95% CI 2763 – 8426, $n = 5$), and TFLLR-NH₂ ($5153 \pm 275.6 \mu\text{m}^2$, $p = 0.5784$; 95% CI 4388 – 5918, $n = 5$), compared to vehicle control ($7002 \pm 813.3 \mu\text{m}^2$; 95% CI 4744 – 9260, $n = 5$), while the area of cells maintained in serum-free and treated AC264613 ($5170 \pm 917.3 \mu\text{m}^2$, $p = 0.8187$; 95% CI 2623 – 7717, $n = 5$), SLIGKV-NH₂ and, ($5364 \pm 751 \mu\text{m}^2$, $p = 0.9999$; 95% CI 3279 – 7449, $n = 5$) and TFLLR-NH₂ ($3887 \pm 464.9 \mu\text{m}^2$, $p = 0.8713$; 95% CI 2597 – 5178, $n = 5$) compared to the vehicle control ($3965 \pm 747.3 \mu\text{m}^2$; 95% CI 1890 – 7449, $n = 5$, **Figure 21 C**).

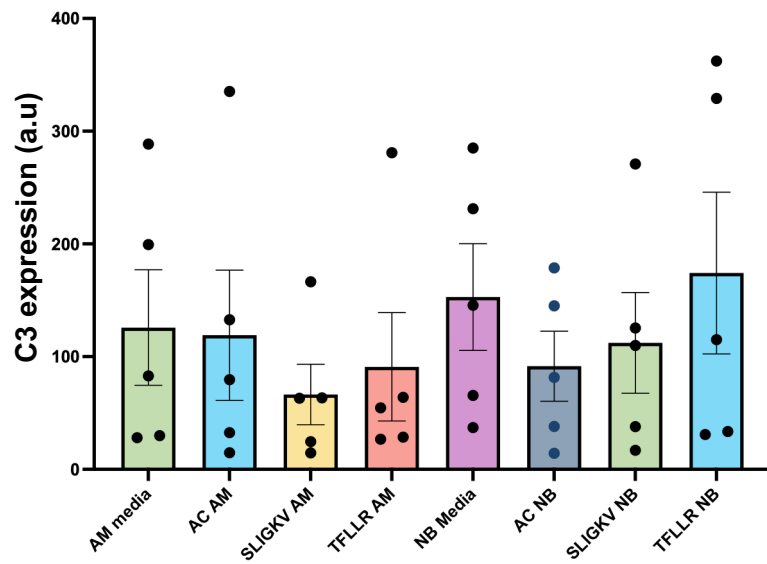
Similarly, PAR receptor activation did not significantly alter cell perimeter under either serum or serum-free culture conditions. In serum-containing media, cells treated with AC264613 ($785.4 \pm 52.58 \mu\text{m}$, $p = 0.4276$; 95% CI 639.4 – 931.4, $n = 5$), SLIGKV-NH₂ ($840 \pm 55.38 \mu\text{m}$, $p = 0.4276$; 95% CI 686.2 – 993.7, $n = 5$), and TFLLR-NH₂ ($656.8 \pm 29.83 \mu\text{m}$, $p = 0.4276$; 95% CI 574.0 – 739.7, $n = 5$), compared to the vehicle treated control ($863.9 \pm 77.79 \mu\text{m}$; 95% CI 647.9 – 1080, $n = 5$). Likewise, under serum-free conditions, no significant differences in cell perimeter were observed following treatment with AC264613 ($587.9 \pm 70.66 \mu\text{m}$, $p = 0.7107$; 95% CI 391.8 – 784.1, $n = 5$), SLIGKV-NH₂ ($590.4 \pm 54.03 \mu\text{m}$, $p = 0.7107$; 95% CI 440.4 – 740.4, $n = 5$) and TFLLR-NH₂ ($561.3 \pm 61.96 \mu\text{m}$, $p = 0.7107$; 95% CI 393.3 – 733.4, $n = 5$) when compared with vehicle-treated cells ($696.5 \pm 128.3 \mu\text{m}$; 95% CI 340.2 – 1053, $n = 5$, **Figure 21 D**).



(C)



(D)



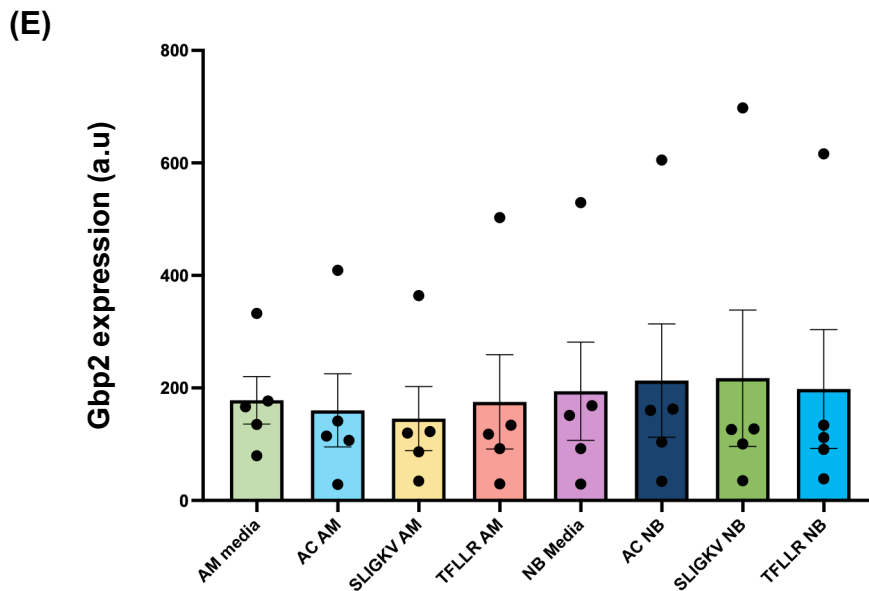
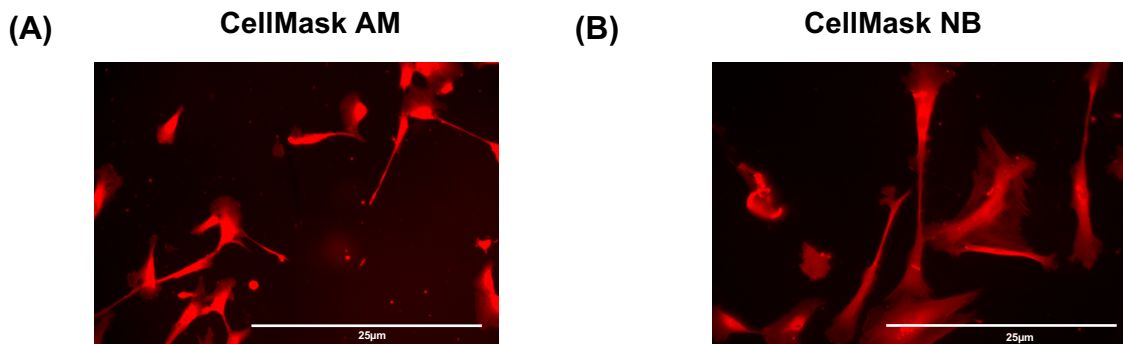
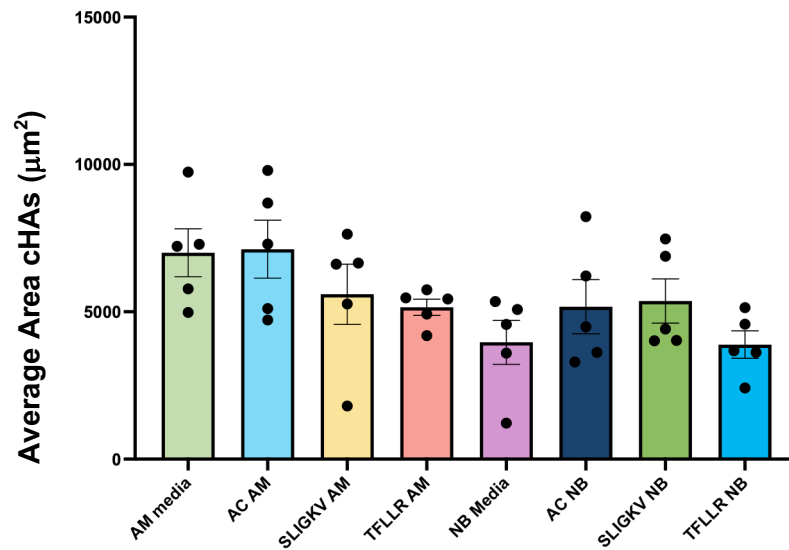


Figure 20: PAR activators show no effect on reactive markers in human astrocytes under prolonged culture conditions. Representative images illustrating C3 expression in human astrocytes cultured in (A) serum media and (B) serum-free media in response to the PAR2 activator AC264613. Bar graphs illustrating effects of PAR2 and PAR1 activators on GFAP (C) Serum (D) Serum-free, C3 (E) Serum (F) Serum-free, and Gbp2 (G) Serum (H) Serum-free, where (AM) serum and (NB) serum-free culture conditions. Data expressed is mean $\pm$ S.E.M. for $n = 5$, one-way ANOVA, with Tukey's post-hoc test. Scale bar 10 μ m.



(C)



(D)

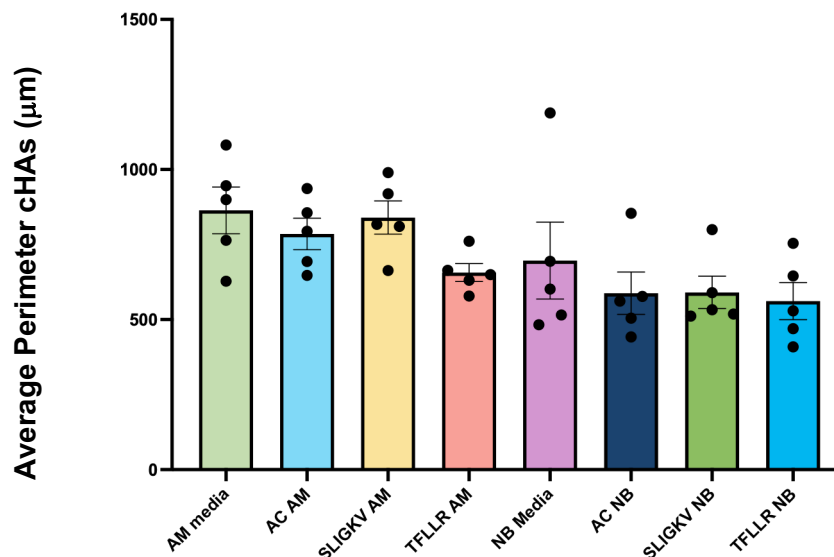


Figure 21: PAR activators show no effect on reactive markers in human astrocytes under prolonged culture conditions. Representative images of human astrocyte stained with CellMask™ in (A) serum and (B) Serum-free media when exposed to PAR1 & 2 activators. Bar graphs showing no changes in area (C) serum, and serum-free and perimeter (D) serum and serum-free following 7day culture period and exposure to PAR1 and PAR2 activators, where serum (AM) and serum-free (NB) culture conditions. Data expressed is mean $\pm$ S.E.M. for n = 5, one-way ANOVA with Tukey's post-hoc test. Scale bar 25μm.

4.3.3 Evaluation of Effects on Human Astrocyte Reactivity Following 24-hour inflammatory cytokine treatment.

Previous studies which have demonstrated elevated expression of GFAP, C3, and Gbp2 in astrocytes following exposure to inflammatory cytokines, specifically tumour necrosis factor (TNF), interleukin-1 β (IL-1 β), and complement component 1q (C1q), referred to as TIC (Clarke et al., 2018; Guttenplan et al., 2020; Guttenplan & Liddelow, 2019a; Hasel et al., 2021; Liddelow, 2019a; Liddelow & Barres, 2017; Zhao et al., 2024).

Building on these findings, the present study we treated human astrocytes with the inflammatory cytokines cocktail TIC and employed immunocytochemical staining to evaluate the expression levels of GFAP, C3, and Gbp2, thereby assessing the reactive state of astrocytes under these conditions. Immunostaining demonstrated that high expression of GFAP in cells maintained in serum media and treated with TIC (157.3 ± 58.57 a.u, $p = 0.0056$, 95% CI -5.43 -319.9, $n = 5$) compared to the vehicle control (57.14 ± 2.1 a.u, 95% CI 51.19 – 63.09, $n = 5$) however, cell maintained in serum-free media and treated with TIC showed no change in expression of GFAP (92.28 ± 3.31 a.u, $p = 0.5476$, 95% CI 83.07 – 101.5, $n = 5$) when compared to vehicle control (84.54 ± 5.9 a.u, 95% CI 68.13 – 100.9, $n = 5$, **Figure 22 C**).

The expression of C3 of cells in serum media treated with TIC showed no change (277.9 ± 13.0 a.u, $p = 0.8358$, 95% CI 241.7 – 314, $n = 5$) in comparison to vehicle control (300.2 ± 47.9 a.u, 95% CI 167 – 433.3, $n = 5$). While the expression of C3 shows no change in cells serum-free treated with TIC, (337.8

± 28.2 a.u, $p = 0.1883$, 95% CI 259.3 – 416.4, $n = 5$) when compared to vehicle control (265.9 ± 10.3 a.u, 95% CI 237.1 – 294.1, $n = 5$, **Figure 22 D**).

The expression of Gbp2 in the cells maintained in serum (786.1 ± 143.9 a.u, $p = 0.2524$, 95% CI 386.6 – 1186, $n = 5$) and compared to vehicle control (475.7 ± 67.3 a.u, 95% CI 288.9 – 662.6, $n = 5$) showed no change. Similarly, Gbp2 expression in serum-free media (724.4 ± 224.5 a.u, $p = 0.9836$, 95% CI 101 – 1348, $n = 5$) showed no change when compared to vehicle control (756.7 ± 47.2 a.u, 95% CI 625.4 – 888, $n = 5$, **Figure 22 E**).

Morphological studies have revealed there was no changes observed in the area of astrocytes maintained in either serum or serum-free media and exposed to the TIC when compared to vehicle control. With the area of cells in serum ($25.29 \pm 6.7 \mu\text{m}^2$, $p = 0.1129$, 95% CI 6.66 – 43.92, $n = 5$) vs vehicle control ($13.45 \pm 2.68 \mu\text{m}^2$, 95% CI 6.15 -20.75, $n = 5$), while the serum-free reported was ($10.23 \pm 1.5 \mu\text{m}^2$, $p = 0.9779$, 95% CI 5.9 – 14.5, $n = 5$) when compared to the vehicle control ($11.33 \pm 3.6 \mu\text{m}^2$, 95% CI 1.3 – 21.36, $n = 5$, **Figure 23 C**).

Similarly, no changes were observed in the perimeter of the cells maintained in either serum, or serum-free medium when exposed to TIC. With the area of ($19.17 \pm 2.8 \mu\text{m}$, $p = 0.1016$, 95% CI 11.36 – 26.97, $n = 5$) vs vehicle control ($14.25 \pm 1.5 \mu\text{m}$, 95% CI 10.0 – 18.42, $n = 5$), while the serum-free reported was ($12.54 \pm 0.9489 \mu\text{m}$, $p = 0.8755$, 95% CI 9.9 – 15.18, $n = 5$) when compared to the vehicle control ($387 \pm 25.97 \mu\text{m}$, 95% CI 314.9 – 459.1, $n = 5$, **Figure 23 D**).

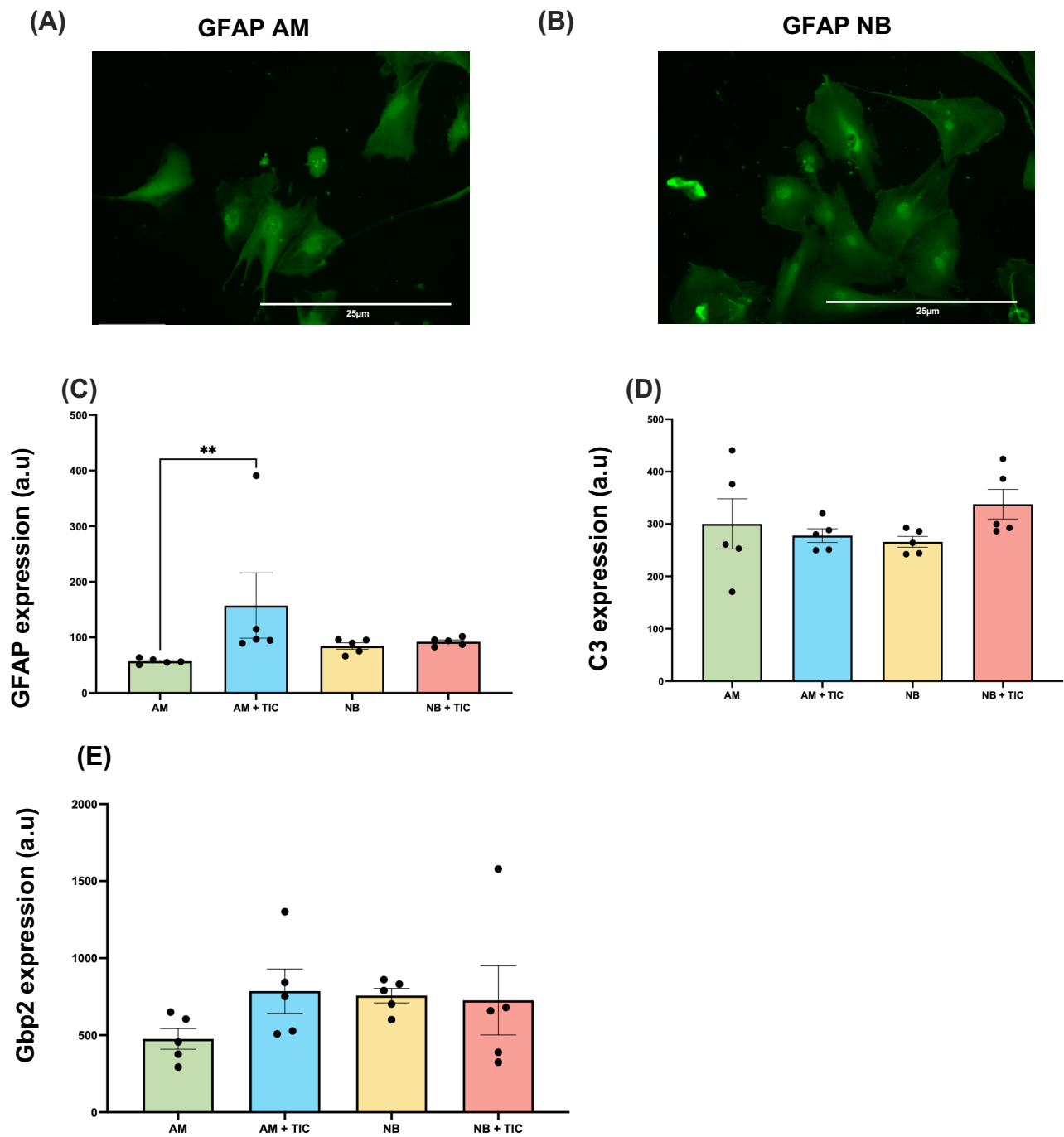


Figure 22: 24-hour TIC treatment upregulates GFAP but not C3 and Gbp2 expression in human astrocyte. Representative images illustrating higher GFAP expression in human astrocytes to TIC in **(A)** serum compared to **(B)** serum-free media. Bar charts summarising the effects of TIC on GFAP expression **(C)** serum, **(D)** Serum-free, C3 expression **(E)** serum, **(F)** Serum-free and Gbp2 expression **(G)** serum, **(H)** Serum-free. Serum (AM) and serum-free media (NB). Data expressed is mean $\pm$ S.E.M. for $n = 5$, $** = p < 0.05$ versus serum or serum-free media, statistical significance was determined using unpaired t-test. Scale bar 25 μ m.

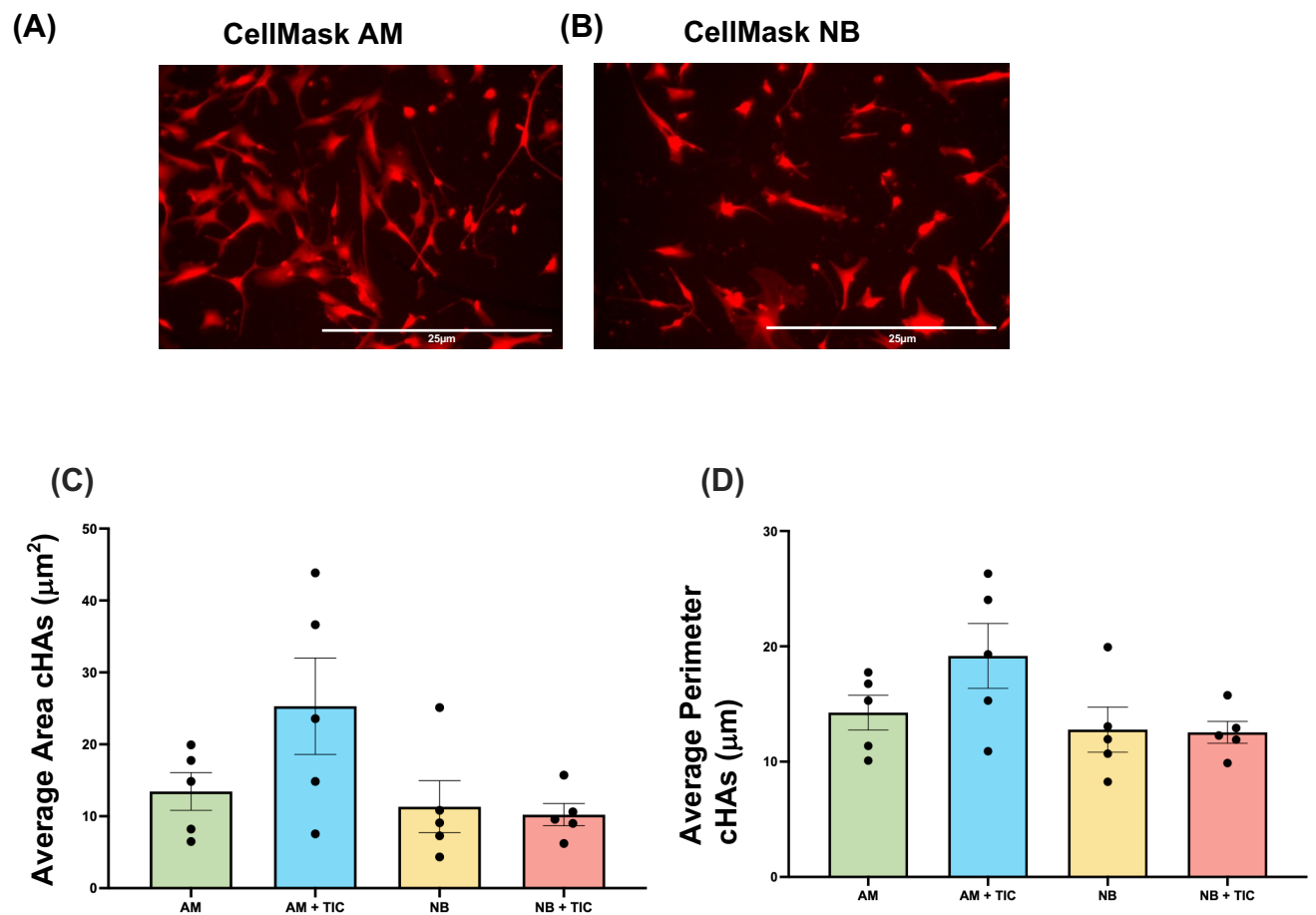


Figure 23: 24-hour TIC treatment exhibits no impact on human astrocyte morphology. Representative images of human astrocyte stained with CellMask™ in (A) serum and (B) Serum-free media in response to TIC treatment. Bar charts summarising the effects of TIC on area (C) serum and serum-free, and perimeter (D) serum and serum-free, where (AM) serum and (NB) serum-free media. Data expressed is mean ± S.E.M. for n = 5, statistical significance was determined using unpaired t-test. Scale bar 25μm.

4.3.4 Evaluation of effects on human astrocyte reactivity following inflammatory cytokine treatment over 7-day period.

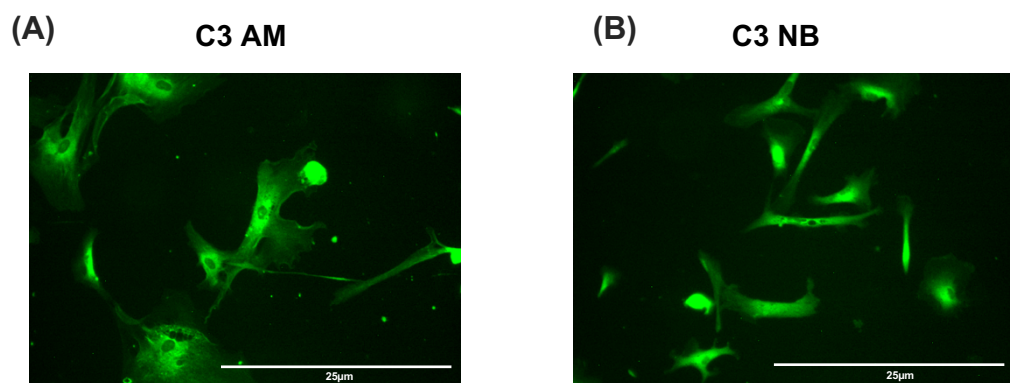
We treated human astrocytes with the inflammatory cytokines cocktail TIC to evaluate the expression levels of GFAP, C3, and Gbp2, over a prolonged period of exposure to TIC. expression of GFAP in cells maintained in serum media (87.77 ± 27.1 a.u, $p = 0.7506$, 95% CI 12.44 – 163.1, $n = 5$), compared to the vehicle control (122.4 ± 49.60 a.u, 95% CI -15.3 – 260.1 $n = 5$), while GFAP expression in serum-free media treated with TIC (82.74 ± 20.9 a.u, $p = 0.4194$, 95% CI 24.6 -140.8, $n = 5$) compared to the vehicle control (144.3 ± 37.5 a.u, 95% CI 39.98 – 248.6, $n = 5$, **Figure 24 C**).

No change in C3 expression in cells treated with TIC, maintained in serum media (38.02 ± 2.5 a.u, $p = 0.1961$, 95% CI 30.8 – 45.1, $n = 5$) when compared to vehicle control (46.02 ± 5.2 a.u, 95% CI 32.2- 61.1, $n = 5$), and in the serum-free media treated with TIC (38.7 ± 2.4 a.u, $p = 0.6168$, 95% CI 31.9 – 45.5, $n = 5$) in comparison to vehicle control (43.3 ± 3.2 a.u, 95% CI 34.2 – 52.39, $n = 5$, **Figure 24 D**). Finally, Gbp2 expression in cells maintained in serum (180.1 ± 49.7 a.u, $p = 0.9947$, 95% CI 42.1 – 318.3, $n = 5$) compare to vehicle control (179.7 ± 41.67 a.u, 95% CI 64 – 295.4, $n = 5$) and in serum-free media (175.1 ± 77.8 a.u, $p = 0.9779$, 95% CI -41 – 391, $n = 5$) compared to respective vehicle control (193.1 ± 86.7 a.u, 95% CI -47.6 – 433.8, $n = 5$) shown no change. (**Figure 24 E**).

Morphological studies have revealed there was no changes observed in the area of astrocytes maintained in either serum or serum-free media and

exposed to the TIC when compared to vehicle control. With the area of ($33.26.0 \pm 3.42 \mu\text{m}^2$, $p = 0.9382$, 95% CI 23.74 – 42.77, $n = 5$) vs vehicle control ($30.31 \pm 3.5 \mu\text{m}^2$, 95% CI 20.54 – 40.9, $n = 5$), while the serum-free was ($27.84 \pm 9.9 \mu\text{m}^2$, $p = 0.9906$, 95% CI 0.35 – 55.33, $n = 5$) when compared to the vehicle control ($26.71 \pm 6.7 \mu\text{m}^2$, 95% CI 8 – 45.3, $n = 5$, **Figure 25 C**).

Similarly, no changes in the perimeter of the cells maintained in either serum, or serum-free medium when exposed to TIC. With the perimeter of cells in serum ($23.90 \pm .8252 \mu\text{m}$, $p = 0.9085$, 95% CI 21.61 – 26.19, $n = 5$) vs vehicle control ($21.68 \pm 1.24 \mu\text{m}$, 95% CI 18.22 – 25.14, $n = 5$), while in serum-free was ($20.20 \pm 3.2 \mu\text{m}$, $p > 0.9999$, 95% CI 11.05 – 29.35, $n = 5$) when compared to vehicle control ($19.98 \pm 2.28 \mu\text{m}$, 95% CI 13.65 – 26.31, $n = 5$, **Figure 25 D**).



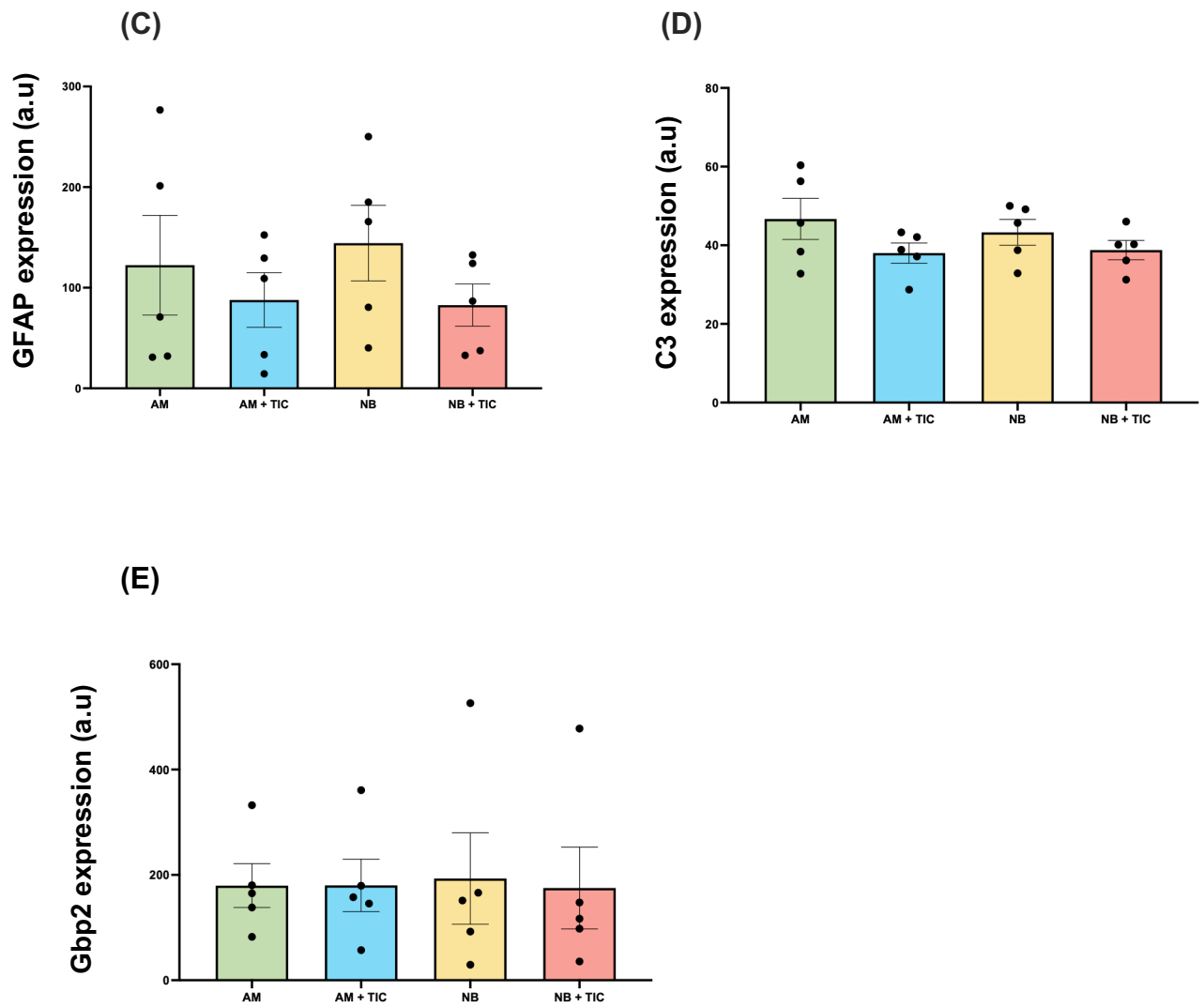


Figure 24: Extended culture duration and tic exposure have no impact on reactive markers in astrocytes. Representative images illustrating no changes in C3 expression in (A) serum media and (B) serum-free media over 7-day period and in response to TIC treatment. Bar charts summarising the effects of TIC on GFAP expression (C) serum, (D) serum-free, C3 expression (E) serum, (F) serum-free and Gbp2 expression (G) Serum, (H) serum-free, where (AM) serum and (NB) serum-free media. Data expressed is mean $\pm$ S.E.M. for $n = 5$, statistical significance was determined using unpaired t-test. Scale bar 10 μ m.

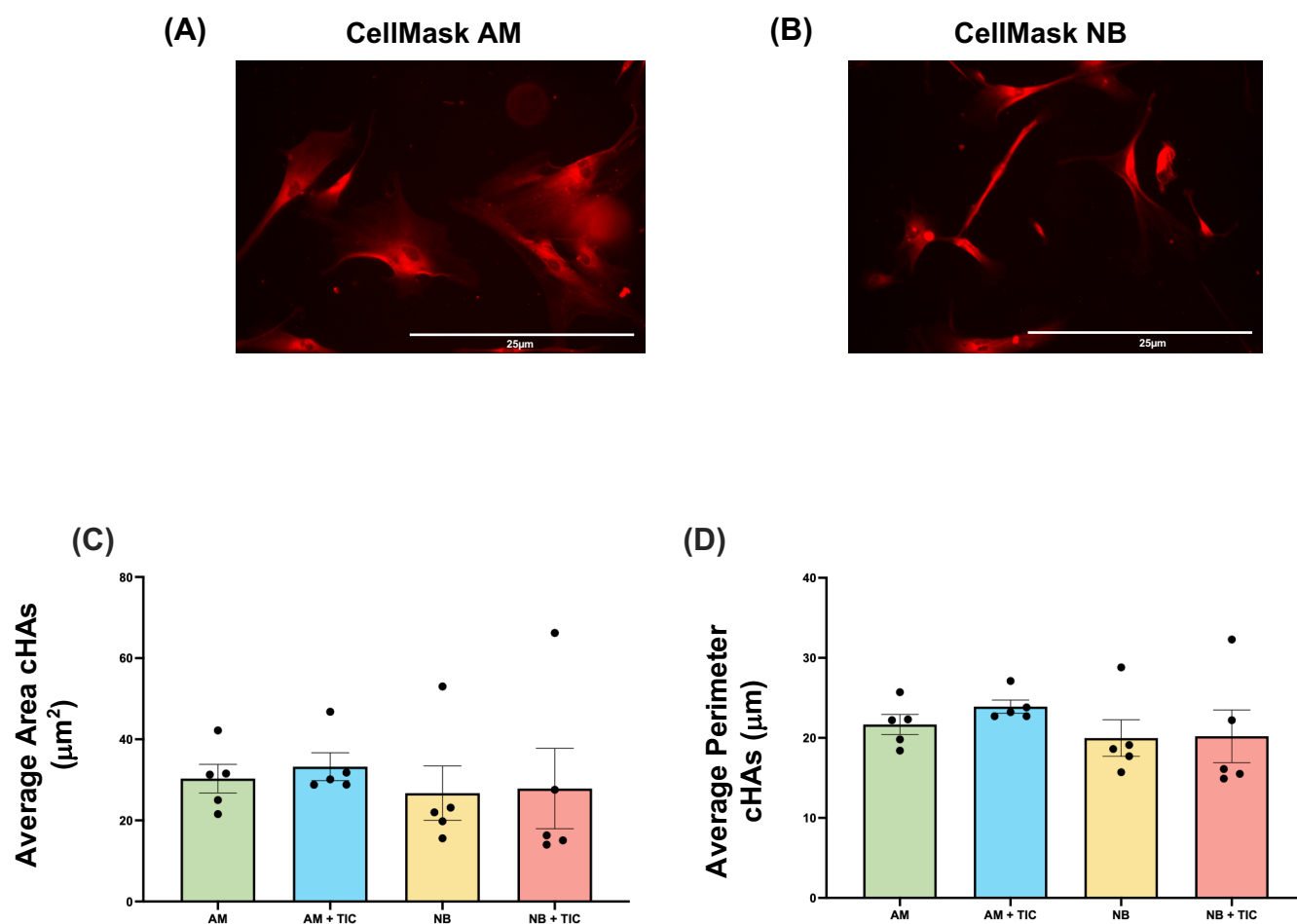


Figure 25: Seven-Day Culture and TIC Treatment Show No Effect on Human Astrocyte Morphology. Representative images of human astrocyte stained with CellMask™ in (A) serum and (B) Serum-free media in response to TIC treatment. Bar charts summarising the effects of TIC on area in (C) serum, (D) serum-free and perimeter in (E) serum, (F) serum-free, where (AM) serum media and (NB) serum-free media. Data expressed is mean $\pm$ S.E.M. for $n = 5$, statistical significance was determined using unpaired t-test. Scale bar 25 μm.

4.4 Understanding phagocytic activity in commercial human astrocytes.

4.4.1 Introduction

The phagocytic activity of glial cells is a critical part of the immune response wherein the cells detect, by formations of actin-based membrane protrusions and engulf and digest large particles such as synaptosomes, pathological protein aggregates, including amyloid-beta ($A\beta$), antibody-opsonized particles, (Jaumouillé & Waterman, 2020). This step is necessary for retaining normal brain activity, but any dysregulation in this phagocytic process leads to exacerbation of CNS pathologies. Phagocytic activity (Ni et al., 2025) is a well-studied neuroimmune mechanism of microglia (Lee et al., 2024; Neher et al., 2011; Neniskyte et al., 2011; Ni et al., 2025; Paolicelli et al., 2011; Zähringer et al., 2025). Microglia, are the brain-resident macrophage, and their phagocytic activity can be both protective and deleterious in nature (Vilalta & Brown, 2018). It is known to prune excess synapses to help in brain development (Lee et al., 2024; Paolicelli et al., 2011; Zähringer et al., 2025), but when activated by inflammatory stimulation can lead to phagocytosis and death of healthy viable neurons (Neher et al., 2011; Neniskyte et al., 2011; Ni et al., 2025). However, astrocytes are also phagocytic but this is a less explored area than microglial phagocytosis, and has limited information on its roles in pathophysiological conditions in the CNS (Yang et al., 2023a), but emerging studies suggest this is present in both human and rodents, potentially playing a compensatory phagocytic role when microglia are impaired or absent to perform phagocytic activity (Konishi et al., 2020; Negro-

Demontel et al., 2024; Yang et al., 2023a). For example, under normal healthy conditions, astrocytes are relatively quiescent and do not engage in phagocytosis, however, under experimental conditions, studies involving models of microglial depletion such as PLX3397 treatment or genetic ablation (Konishi et al., 2020b) or models of impaired microglia such as *Irf8*-deficient mice with compromised phagocytic capacity, have shown an upregulation in astrocytic phagocytic receptors which actively engulf debris, synaptic material and apoptotic cells that are generally cleared by microglia (Konishi et al., 2022a; Zhou et al., 2022). In the given microenvironment astrocytes sense the dysfunction or the absence of microglia, and their inability to clear the debris. Thereby the astrocytic phagocytosis represent tissue level damage repair to ensure CNS homeostasis (Konishi et al., 2020b, 2022a; Zhou et al., 2022).

Beyond this compensatory role during microglial dysfunction, astrocytic phagocytosis also contributes to physiological synaptic refinement and the maintenance of optimal CNS homeostasis in response to neuronal activity and experience-dependent plasticity. This phagocytic response is mediated through specific astrocytic receptors that recognize phosphatidylserine or target synapse signals. Studies have indicated the engulfment of synapses via the MEGF10 and MERTK astrocytic phagocytosis pathways, through recognising the phosphatidylserine presented by target debris (Chung et al., 2013a; Zhuang et al., 2023). Also, evidence have demonstrated that human and rodent astrocytes when subjected to external stimuli such including bacterial toxins and inflammatory mediators, help phagocytose cellular debris

(Wakida et al., 2018), apoptotic cells (Damisah et al., 2020), and synapses (Chung et al., 2013a).

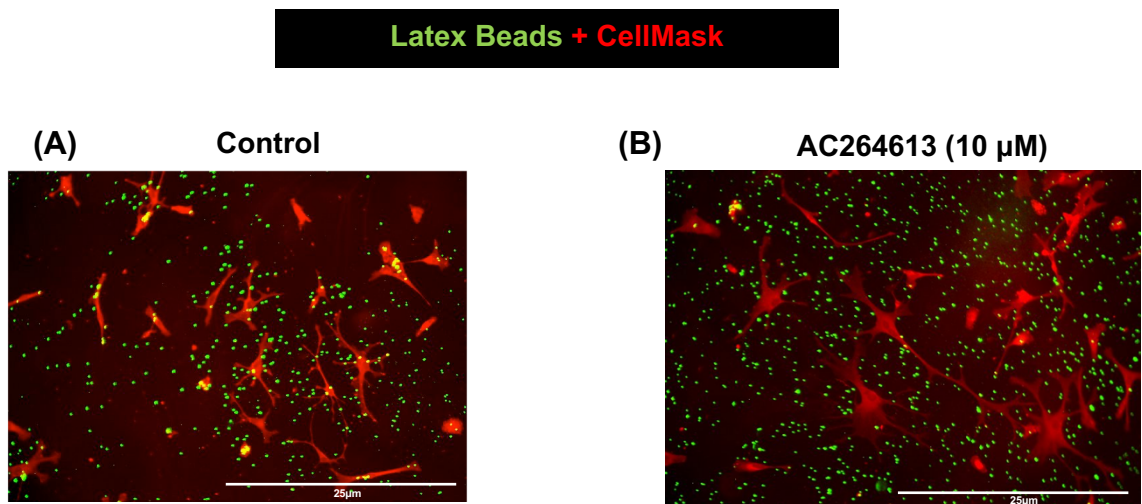
Overall, under physiological conditions, astrocytes are involved in maintaining CNS homeostasis throughout the early life development to adulthood, and phagocytic capacity of astrocytes is influenced by age (Kalmár et al., 2001). While the microglial phagocytosis is induced by key regulators including molecules C1q (Nizami et al., 2019), and inflammatory mediators such as IL-1 β and interferon- γ (IFN- γ) (Rivera-Escalera et al., 2019), and LPS a bacterial endotoxin (dos Santos et al., 2025), the regulation of astrocyte phagocytosis by endogenous immune mediators remains less understood. The first evidence that IL-1 β enhances astrocytic phagocytosis was reported by (Kalmár et al., 2001), who demonstrated that treatment with IL-1 β increased the uptake of latex beads by cultured primary rat astrocytes.

Given that recent studies have indicated astrocyte reactivity modulates glial immune functions including phagocytosis (Gildea & Liddelow, 2025; Gomes et al., 2024; Li et al., 2024; Zhang et al., 2025), we hypothesized that PAR1 and PAR2 activators will modulate astrocytic phagocytosis in cultured human astrocytes. Furthermore, in TIC stimulated cultured human astrocytes phagocytosis is altered.

4.5 Result

4.5.1 PAR1 and PAR2 do not modulate phagocytosis in commercial human astrocytes.

To assess the effects of PAR1 and 2 activators on the phagocytic activity in commercial human astrocytes, we initially exposed cHAs to PAR1 and PAR2 activators, to examine whether this altered their phagocytotic uptake of fluorescently labelled latex beads. The fluorescence values of cells treated with the PAR2 activators AC264613 (10 μ M; 162391 ± 77418 a.u., $p = 0.9224$, 95% CI -36619 – 361401, $n = 6$) and SLIGKV-NH2 (5 μ M; 135009 ± 26958 a.u., $p = 0.9992$, 95% CI -44165 – 181411, $n = 6$), as well as the PAR1 activator TFLLR-NH2 (3 μ M; 68623 ± 24369 a.u., $p=0.7158$, 95% CI 65712 – 204306, $n = 6$), showed no significant alteration in phagocytic activity in cHAs when compared to the control (128127 ± 43876 a.u., 95% CI 65484 – 190769, $n = 6$), as assessed by the phagocytosis assay after one hour of incubation (Figure 27E).



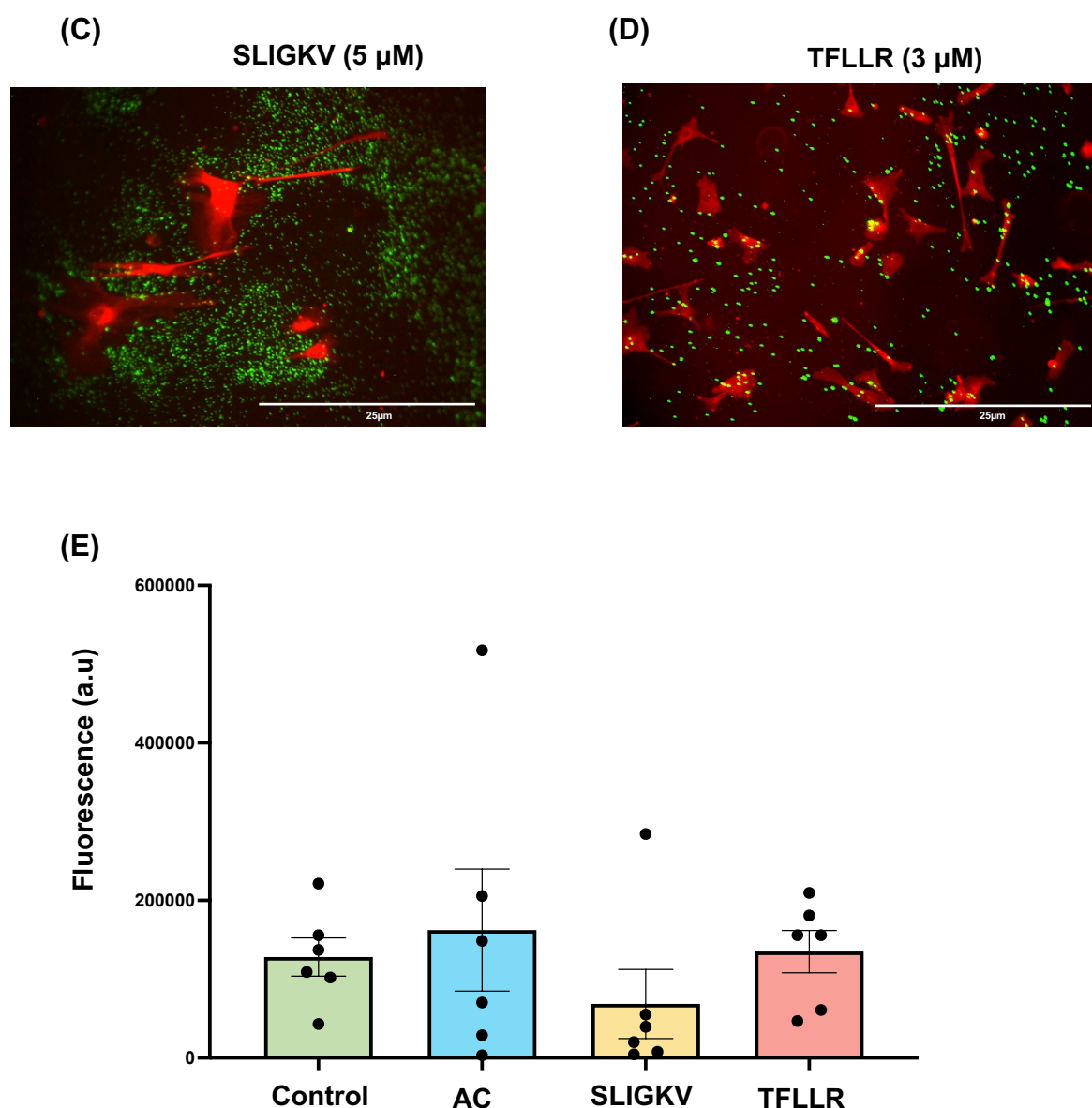


Figure 26: No modulation of phagocytic activity in cultured human astrocytes by PAR activators. Representative fluorescent images of cHAs stained with CellMask™ (red) show no uptake of latex beads (green) in (A) control and when treated with PAR2 activators (B) AC264613 (10 μ M), (C) SLIGKV-NH₂ (5 μ M), and PAR1 activator (D) TFLLR-NH₂ (3 μ M) (E) Bar graph representing the corrected total cell fluorescence values to control cells. Data are presented as mean $\pm$ standard error of the mean (SEM), with one-way Anova followed by Tukey's post-hoc test used for statistics. Scale bar 25 μ m.

4.5.2 TIC exposure had no effect on the commercial human astrocyte phagocytic activity.

Similarly to assess the effects of inflammatory cytokines on the phagocytic activity in cHAs, we initially exposed cHAs to TIC to examine whether this altered their phagocytotic uptake of fluorescently labelled latex beads. Where the fluorescence values of cells when exposed to TIC cocktail (116893 ± 34670 a.u , $p = 0.3559$, 95% CI 27771 – 206015, $n = 6$), when compared to vehicle control (155807 ± 19256 a.u , $p = 0.3559$, 95% CI 106309 – 205-305, $n = 6$) show no significant alteration in phagocytic activity in cHAs. (Figure 28 **C** and **D**).

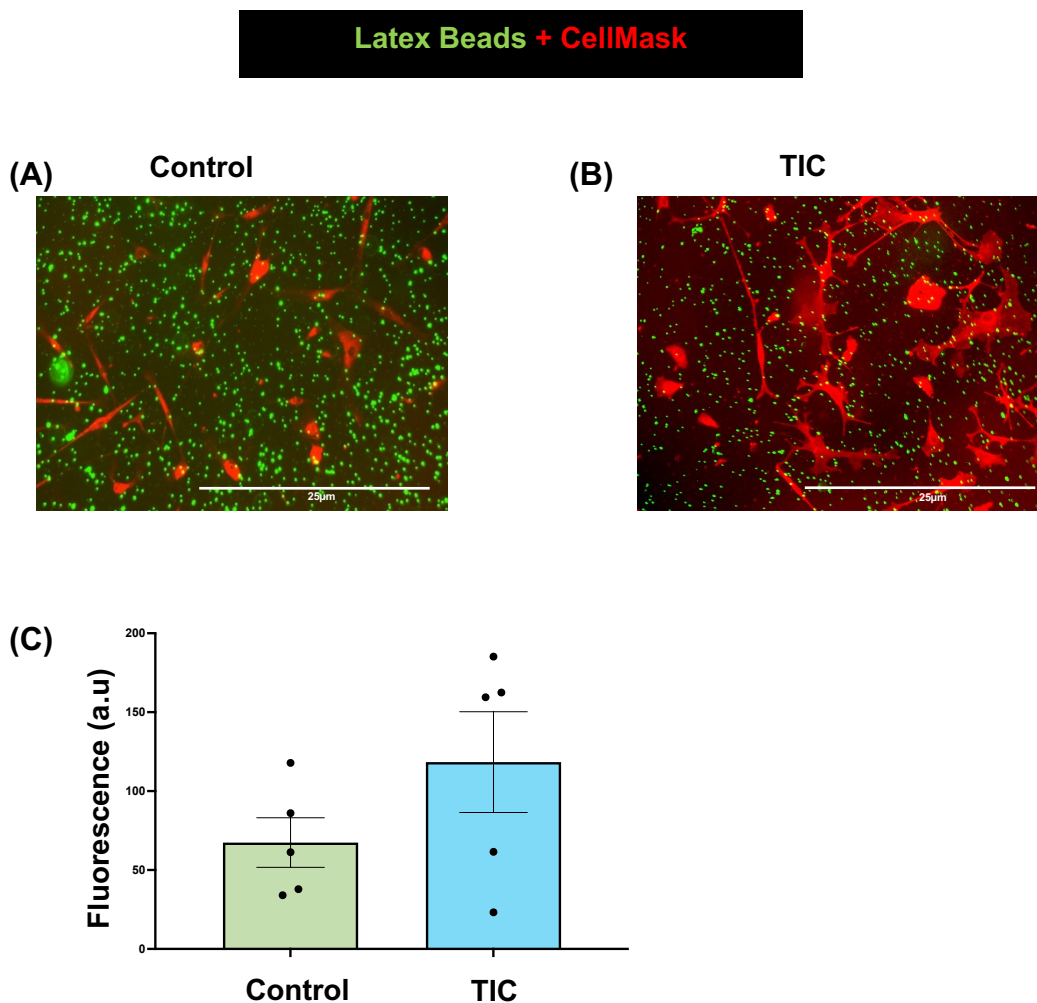


Figure 27: Inflammatory cytokines do not modulate phagocytic activity in cultured human astrocytes. Representative fluorescent images of cHAs stained with CellMask™ (red) show no uptake of latex beads (green) in **(A)** control compared to **(B)** inflammatory cytokines (TIC) **(C)** Bar graph representing the corrected total cell fluorescence values to control cells. Data are presented as mean $\pm$ standard error of the mean (SEM), statistical significance was determined using unpaired t-test. Scale bar 25 μ m.

4.6 DISCUSSION

The study revealed that astrocytic reactivity exhibited no significant changes across various experimental conditions namely, media conditions, following the application of PAR activators, or upon exposure to inflammatory cytokines. These results indicate that astrocytic responses remained consistent irrespective of cell variations, media composition, or external treatments, underscoring the robustness and stability of astrocytic behaviour under the experimental conditions tested. These observations provide valuable insights into astrocytic function and establish a foundation for further investigation into their regulatory mechanisms in diverse physiological and pathological contexts.

4.6.1 Astrocyte reactivity unaffected by media conditions.

In the present study, we investigated the impact of two distinct media conditions serum-containing and serum-free on astrocytic reactivity, as assessed through the expression of key markers including GFAP, C3, and Gbp2. At the 24-hour time point, individual astrocytes cultured in serum-

containing media exhibited elevated expression levels of GFAP, C3, and Gbp2 compared to those in serum-free conditions. However, when expression was averaged across regions of interest, statistical analyses revealed no significant differences in GFAP or C3 levels between the two media types. In contrast, average Gbp2 expression remained significantly higher in the serum-containing media. Extending the culture period to 7 days yielded similar patterns, with no significant differences observed in average GFAP or C3 expression across the media conditions. Notably, however, average Gbp2 expression was higher in the serum-free media at this later time point, suggesting a potential time-dependent shift in the responsiveness of this marker to media composition.

These findings highlight that while individual cellular responses can demonstrate variability in reactivity markers, but in broader trends when averaged cellular expression per culture they may not always reach statistical significance, potentially due to several underlying factors. One key contributor to the lack of significance in GFAP and C3 expression could be the inherent heterogeneity among astrocytes, even within a commercially sourced population (Guttenplan & Liddelow, 2019b; Liddelow et al., 2017a; Sofroniew & Vinters, 2010). Astrocytes are known to exhibit diverse subtypes and functional states, which may lead to variable reactivity profiles that are diluted when averaged across multiple regions of interest. This averaging approach, while necessary for capturing overall trends, may mask subtle, cell-specific differences that are evident at the individual level (Batiuk et al., 2020; Escartin et al., 2021; Liddelow et al., 2017a; Liddelow & Barres, 2017).

Additionally, the high passage numbers of the astrocytes used in this study likely played a role in dampening responsiveness. Higher passages are associated with progressive dedifferentiation, senescence, or altered gene expression patterns in glial cells, which could attenuate media-induced changes in reactivity markers and contribute to the observed non-significance (Bang et al., 2021; Freitag et al., 2022; Pertusa et al., 2007; Salminen et al., 2011). Furthermore, the morphological changes observed during the 24 hour and 7 day of maintenance of cells in different media conditions have shown reduction in both cellular area and perimeter and have shown compact morphology for commercial astrocytes maintained in serum-free media compared to those in serum-containing conditions (**Figure 26 A&B**). These align with the previous studies which highlight that serum contains various factors which may induce astrocyte reactivity causing hypertrophy and morphological changes (Liddel, 2019b; Prah et al., 2019). Conversely, the compact morphology of commercial astrocytes observed under serum-free conditions mimic the characteristic quiescent astrocyte morphology *in vivo* (Prah et al., 2019; White et al., 2024b). Therefore, the observed reduction in area and perimeter under serum-free conditions indicates a transition towards a less reactive state, which is critical for maintain astrocytic functions in brain health (Rumman et al., 2015; Sofroniew, 2020; Yao & Fu, 2020).

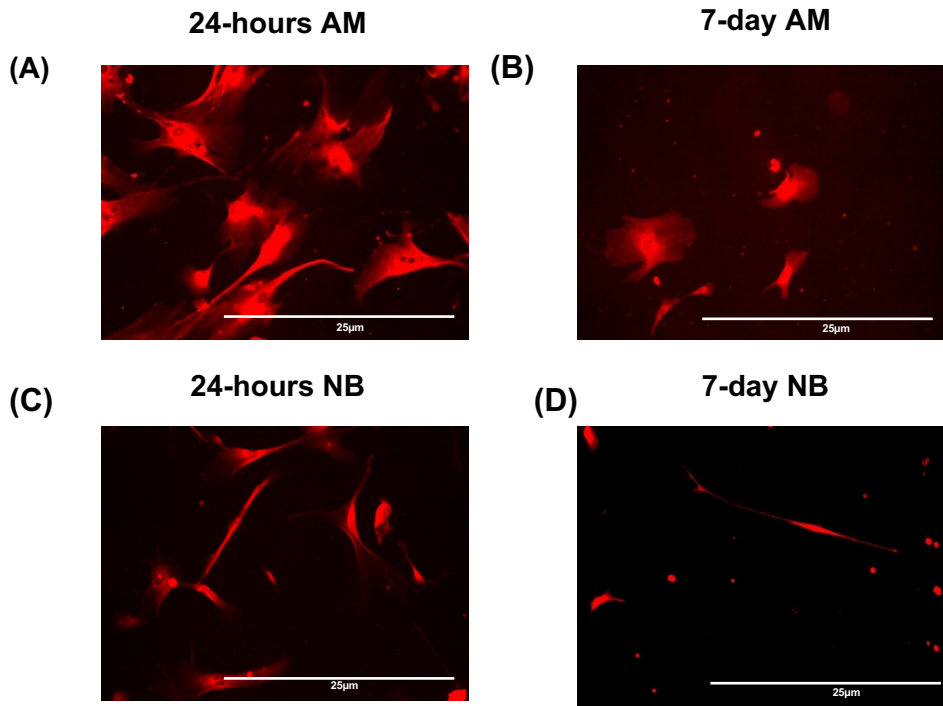


Figure 28: Serum cultivated astrocytes demonstrate fibroblast-like morphology. Representative images of (A) 24-hours, (B) 7-day serum-cultured commercial astrocytes which demonstrate fibroblast-like morphology with enlarged nucleus. (C) 24-hours (D) 7-day serum-free cultured astrocytes exhibit compact morphology with long and thin branching processes. Scale bar 25µM.

4.6.2 PAR activation shows differential effect on modulating astrocytic reactivity in human astrocytes.

In this study, we examined the potential of PAR activation to modulate astrocyte reactivity in commercial astrocytes maintained under two distinct media conditions: serum-containing and serum-free. Astrocytes were treated with PAR activators for 24 hours and 7 days of media conditioning to assess time-dependent effects on reactivity markers, GFAP, C3, and Gbp2.

In serum-containing media, treatment with the PAR1 agonist TFLLR-NH2 and the PAR2 agonist SLIGKV-NH2 significantly elevated GFAP staining intensity

compared to vehicle controls, suggesting that PAR1 and PAR2 signalling pathways promote a reactive phenotype in astrocytes under these conditions. Similarly, in serum-free media, the PAR2 agonists AC264613 and SLIGKV-NH₂ induced GFAP upregulation, indicating that PAR2 activation is particularly effective in eliciting reactivity in astrocytes. This is in persistent increase in GFAP expression after treatment with PAR1 and PAR2 agonists (TFLLR-NH₂, SLIGKV-NH₂, AC264613) across different media conditions links to the role played by signalling pathway in inducing astrocyte reactivity (Chen et al., 2022c; Nicole et al., 2005b).

Secondly, in C3 expression, we observed a significant increase in serum-free media following AC264613 treatment, but no statistically significant elevation in serum media with any PAR activators. C3 upregulation in reactive astrocytes is linked to inflammatory pathways (Liddelow, 2019b; Liddelow & Barres, 2017). The selective increase in serum-free conditions may stem from the lower basal reactivity in this media. In serum media, the lack of significance could be attributed to already elevated baseline. Also, C3 levels can be due to serum-induced reactivity, which might saturate the response further agonist effects (Gomes et al., 2024; Guttenplan & Liddelow, 2019b). Additionally, the biological variability in commercial astrocyte cultures may contribute to the absence of statistical significance, as subtle changes could be masked by inter-culture differences (Matias et al., 2019; Sharif & Prevot, 2012). However, Gbp2 expression showed no significant increase in either serum or serum-free media following PAR activator treatment, despite its association with inflammatory reactive astrocytes in conditions like blood-brain barrier

disruption or neurodegeneration (H. Kim et al., 2022), this could be due to the variability, in cultures, or baseline activation of commercial cells.

Also, astrocyte morphology, assessed by cell area and perimeter, remained unchanged in both media types after PAR treatment, contrasting with some reports of PAR1-mediated stellation reversal or proliferation in rodent models (Chen et al., 2022d). Reactive astrocytes often exhibit hypertrophy and process ramification in response to injury, but these changes are context-dependent (Liddelow & Barres, 2017) and may not manifest with mild or acute stimuli like our peptide agonists. Possible reasons for the lack of morphological alterations include the 24-hour treatment duration being insufficient for detectable cytoskeletal remodelling, species-specific differences (human vs. rodent astrocytes) (Chen et al., 2022d; Foo et al., 2011), or the absence of additional stress that typically amplifies such responses in vivo.

However, no significant increase in GFAP, C3 and Gbp2 expression were observed in cells conditioned in either of the media type and treated with PAR activators over the time point of 7 days. One plausible reason could be that PAR receptors have a unique activation via the G-protein coupling which irreversible proteolytic cleavage, and receptor internalization, degradation, and downregulation of downstream signalling components (Adams et al., 2011; Ramachandran et al., 2012). Therefore, prolonged or repeated exposure to PAR activators may trigger desensitization mechanisms and might reduce the cellular response over this extended time point (Soh & Trejo, 2011). Secondly, our experimental conditions involved serum and serum-free media which

provide different metabolic and trophic environmental support. This might fundamentally interfere and alter the astrocyte phenotype arising from formulations of the media (Prah et al., 2019; Sánchez-Juan et al., 2024; White et al., 2024a) and could have induced a degree of baseline activation in these cells masking the PAR-induced effects (Foo et al., 2011; Guttenplan & Liddelow, 2019a). Finally, there were no significant changes in morphology observed over the period of 7 day with PAR treatment in cells maintained in either of the media. the possible reason for this observation could be that the astrocytes cultured are *in vitro* differ from the ones observed *in vivo* where the astrocytic morphology is complex while the one *in vitro* astrocytes adopt on a stellate or a polygonal morphology on a substrate (Foo et al., 2011), which causes the loss of native architecture in these astrocytes. Due to this, there is a need of stronger stimuli that could influence morphological changes in these cells (Moshayedi et al., 2014). Previous studies have shown the study of morphology over shorter time courses or have looked at certain specific dynamics than a complete transformation (Buddenkotte et al., 2005; Li et al., 2022). Our study of 7 day experimentation period provides insight that PAR activation does not cause sustained morphological changes in a standard culture condition.

In conclusion, our findings suggest that PAR activators have differential effects on astrocyte reactivity and morphology under distinct media conditions. This can possibly contribute to our understanding of the astrocyte reactivity, with its implications in neuroinflammatory and neurodegenerative conditions where reactive astrocytes play a critical role (Burda & Sofroniew, 2014; Escartin et

al., 2021; Kumar et al., 2023; Lawrence et al., 2023; Liddelow et al., 2017a). Moreover this work allows us to further look in to signalling pathways activated by PARs to modulate astrocyte function and lays a foundation to develop targeted interventions to understand astrocyte reactivity in neurological diseases.

4.6.3 Culture conditions exert differential effects on astrocytes to inflammatory treatments.

The results from the current study investigated the effect of the inflammatory cytokine cocktail (TIC; TNF, IL-1 β , and C1q) on the commercial astrocytes maintained in different culture media and assessed for the expression of reactivity markers GFAP, C3, and Gbp2 and cellular morphology in both 24 hour exposure time and 7 day exposure time.

The increase in expression of GFAP in serum containing media but not in serum-free media upon exposure of astrocytes to TIC for 24 hours is contradictory to the previous studies that have reported to have seen an increase in GFAP expression in cells maintained in serum-free conditions (Hasel et al., 2021; Liddelow et al., 2017a), but aligns with studies reported that cells maintained in serum containing media are exposed to various growth factors (Elsharkasi et al., 2025; White et al., 2024a) which might interfere and modulate various signalling pathways, and increase the basal reactivity in these cells leading to increased GFAP expression (Guttenplan & Liddelow, 2019b; Prah et al., 2019; White et al., 2024a; Yao & Fu, 2020).

Adding on, the expression of C3 has shown to be higher in serum-free media, which aligns with the previous studies (Clarke et al., 2018; Liddelow et al., 2017a; Prah et al., 2019) that have shown a significant increase in C3 expression, while the expression of C3 in cells cultured in serum media has been insignificant which points us to the direction of serum components could inhibit C3 upregulation (Prah et al., 2019; White et al., 2024a), possibly through modulation of interferon-responsive elements or suppression of pro-inflammatory signalling (Hasel et al., 2021; Hong et al., 2016; Liddelow & Barres, 2017; Prakash et al., 2024; Zamanian et al., 2012a).

Another reactive marker that which is associated with reactive astrocyte is Gbp2. In this study the expression of Gbp2 has remained unaltered in both the media conditions. Previous research has indicated that the Gbp2 is induced in reactive astrocytes near inflamed vasculature via TNF-STAT3 signalling (H. Kim et al., 2022). The human astrocyte studies indicate that TIC elicits only modest induction of the marker when compared to combinations involving IFN- γ , which synergizes with TNF to strongly upregulate Gbp2 and other interferon-responsive genes (Kobayashi et al., 2025). The absence of IFN- γ in our TIC cocktail along with serum induced baseline reactivity could likely account for the null effect in expression of Gbp2.

Morphologically 24 hour of TIC treatment on cells maintained in serum and serum-free media revealed no significant changes in area or perimeter, which is contradictory to previous studies which have shown changes in astrocyte morphology including hypertrophy and process extension (Escartin et al., 2021; Liddelow & Barres, 2017; Pekny & Pekna, 2014; Sofroniew & Vinters,

2010) upon TIC treatment, the possible reason could be that morphological changes observed in reactive astrocytes *in vivo* generally develop over an extended period of time involving neighbouring cells and their interaction with astrocytes with three dimensional extracellular matrix (Lazic et al., 2022; Pekny & Pekna, 2014; Schiweck et al., 2018). But in a two dimensional culture system, there could be lack of cues needed to guide structural reorganisation (Wilhelmsson et al., 2006). Furthermore, this lack in morphology might suggest cytokine exposure times playing a role in bringing morphological changes (Sheng et al., 2011; van Kralingen et al., 2013) in the cells used in our experiments.

Also, the analysis of astrocytes cultured for 7 days in serum and serum-free media conditions and exposed to inflammatory cytokines (TIC) revealed no distinct reactive or morphological profiles which is in contrast with various other studies (Hasel et al., 2021; Hyvärinen et al., 2019b; Liddelow & Barres, 2017; Sheng et al., 2011; van Kralingen et al., 2013; Wilhelmsson et al., 2006). This may be due to multiple interconnected factors which have been recognised in astrocyte biology. The cytokine treatment along with 7 day culture period positions astrocytes in an altered baseline state, which in an *in vitro* culture is known to induce these phenotypic changes due to the influence of serum in the media (Guttenplan & Liddelow, 2019a; White et al., 2024a). Furthermore, this baseline activation also could cause to saturate signalling pathways such as NF- κ B or JAK/STAT3 which have known to play a role in astrocyte reactivity (Ageeva et al., 2024; Ben Haim et al., 2015) which further limits the capacity to induce morphological or functional changes in reactive markers to any TIC

exposure. Secondly, previous literature has pointed towards cytokine-induced activation peaks within 24-48 hours (Tomioka et al., 2023), which increases the NF- κ B activation or glutamate uptake alterations in response to TNF- α and IL-1 β (Ageeva et al., 2024; Hyvärinen et al., 2019b), reviewing this we assume that in our experimental setup of 7 day media conditioning and exposure to TIC might have failed to capture the increase in cytokine-induced reactivity, thus causing desensitisation in response to TIC and leading to no net observable differences in reactive marker expression or change in morphology. Thirdly, our approach is however comprehensive, yet might have failed to capture sensitivity of the subtle changes that TIC might have caused without undergoing major changes in morphology or in reactive markers as the TIC cocktail do not activate the pan-reactive pathways (Liddelow et al., 2017b; Pekny & Pekna, 2014; Zamanian et al., 2012b).

Overall, this study has demonstrated that *in vitro* reactive astrocyte modelling can be challenging due to lack of 3D matrix organisation, cellular environments and the components of the media which might elevate the basal activation in astrocytes and this may limit the ability to activate pathways or induce reactivity in commercial astrocytes. It has also provided a direction for future studies where it is critically important to choose the controlled media composition that could be potentially sensitive to subtle changes.

4.6.4 Phagocytic activity remained unaltered in commercial human astrocytes upon exposure to PAR1&2 activators and TIC treatment.

The current study investigates a key function of astrocytes, specifically their phagocytic activity, which plays a critical role in maintaining brain homeostasis (Chung et al., 2013b; Jaumouillé & Waterman, 2020; Jones et al., 2013; Kalmár et al., 2001). Our study revealed no changes in the human astrocyte phagocytosis activity when pretreated with PAR1 & 2 activators or inflammatory cytokines TIC and then exposed to fluorescently-labelled latex beads to visualize phagocytosis. Under standard culture conditions, the presence of serum in the media can alter the astrocytic phenotype (White et al., 2024) which might downregulate the receptor and intracellular trafficking machinery necessary for particle uptake, coupled with the previous study (Kalmár et al., 2001; Yang et al., 2022), which demonstrated reduced ability of cultured astrocytes to intake fluorescently-labelled latex beads (Bocchini et al., 1988; Kalmár et al., 2001; Yang et al., 2022).

In addition, to the culture conditions, another reason for inferior uptake could be the short exposure time, to the fluorescent beads. Studies have typically utilised one hour incubation periods (Gedam et al., 2023; Martins et al., 2022; Yang et al., 2023b), indicating that this duration may be standard but may be insufficient duration for robust ingestion of particles. Unlike microglia, astrocytes may require extended exposure to ingest particles. A recent study has demonstrated cultured astrocytes phagocytosed synapses at much slower rate than microglia. Wherein phagocytosis by microglia occurred around 60 mins but required about 48 hours in astrocytes (Byun & Chung, 2018; Chung

et al., 2013c; Jung & Chung, 2018; Tzioras et al., 2023a). Moreover, astrocytic phagocytosis occurs via the MEGF10 and MERTK phagocytic pathways, which recognize phosphatidylserine presented by target debris (Chung et al., 2013a; Zhuang et al., 2023), demonstrating the selective nature of astrocytic phagocytosis. Under reactive states the phagocytosis ability is reduced due to the downregulation of MEGF10 and MERTK receptors (Konishi et al., 2022b) underlining the role of functional state on phagocytic activity.

Finally, treatment of cultured human astrocytes with inflammatory cytokines showed no change on the phagocytic activity in these cells. Inflammatory cytokine exposure primes astrocytes towards a reactive state and which cause reduced phagocytic activity (Pinto-Benito et al., 2022; Ponath et al., 2017; Sinha et al., 2021; Tzioras et al., 2023b; van Kralingen et al., 2013; Shirley Yang et al., 2023b). Research has also indicated that decreased phagocytic activity, could also be due to the presence of serum in the culture media, making astrocytes adopt baseline reactivity (Prah et al., 2019; White et al., 2024). Thereby further subjecting them to inflammatory cytokines would not cause a phase shift in these cell lines causing any increase in phagocytic activity (Prah et al., 2019; White et al., 2024; Shirley Yang et al., 2023b; Yao & Fu, 2020).

In conclusion, our experiments demonstrated no changes in astrocyte phagocytic activity on cultured human astrocytes when exposed to PAR1 or PAR2 activators and also when exposed to inflammatory stimulus (TIC) reflecting on multiple factors. This underscores the necessity to use physiologically relevant phagocytic substrates and experimental conditions to

recapitulate *in vivo* environment to investigate astrocyte phagocytic mechanisms. Future studies to examine the effect of PAR activation on MEGF10/MERTK expression and to study phagocytosis in astrocytes engaging these receptor pathways.

CHAPTER 5

Results

Modulation of astrocytic reactivity and phagocytic activity in iPSC-derived astrocytes

5. Characterizing Astrocyte Reactivity in iPSC-Derived- Astrocytes.

5.1 Introduction

iPSCs represent a powerful technological tool for studying human neural biology. Through differentiation protocols spanning 4–8 weeks, iPSCs can be efficiently differentiated into glial populations, including both glial progenitor cells (GPCs) and mature astrocytes (Fossati, 2023), as well as other cell types such as neurons (Pistollato et al., 2017; Shi et al., 2017), oligodendrocyte progenitor cells (OPCs), mature oligodendrocytes (Zeldich & Rajkumar, 2024), and microglia (Hasselmann & Blurton-Jones, 2020). This approach provides a platform to study human glial cells *in vitro*, circumventing the significant limitations associated with primary astrocytes, or immortalised cell lines (Gildea & Liddelow, 2025; Liddelow, 2019; Liddelow & Barres, 2017), such as batch to batch variability affecting phenotype and reproducibility (Cerneckis et al., 2024), senescence is another issue due to continuous divisions or passages affecting the experiments (Yi et al., 2023), and finally a phenotypic drift due to exposure to extended culture time and to serum (Lange et al., 2012). iPSC-derived glial cultures exhibit substantial cellular heterogeneity, containing both immature glial progenitor cells representing earlier developmental stages in the astrocytic lineage and mature astrocytes (Hedegaard et al., 2020; R. Santos et al., 2017a). GPCs are characterized by expression of progenitor-associated markers such as GFAP and vimentin, whereas mature astrocytes express a more complete range of astrocytic markers including S100 β (McComish et al., 2024a), AQP4

(Thormann et al., 2024), and ALDH1L1 (Apostolou & Stadtfeld, 2018; O'Toole et al., 2024).

Recent investigations have leveraged iPSC-derived astrocyte models to investigate reactive phenotypes and their pathological implications (Clayton & Liddelow, 2025; Hedegaard et al., 2020; McComish et al., 2024b; Patani et al., 2023; Szabo et al., 2021; Zeldich & Rajkumar, 2024). Exposure of differentiated iPSC-derived astrocytes to a cocktail of pro-inflammatory cytokines, induces a reactive astrocyte state characterized by profound alterations in gene expression, inflammatory mediator production, and metabolic function. (Escartin et al., 2021; Liddelow & Barres, 2017). These TIC-induced reactive phenotypes have been observed in tissue from several CNS diseases and are implicated in the pathogenesis of various neurodegenerative diseases, such as Alzheimer's disease (Liddelow, 2019; Qin et al., 2023; Wilkinson & El Khoury, 2012), multiple sclerosis (Mohamadi & Borhani-Haghighi, 2023; Ponath et al., 2018) , and amyotrophic lateral sclerosis (Yang et al., 2024). These iPSC-derived models does provide invaluable platforms for understanding and elucidating molecular mechanisms underlying astrocyte reactivity and evaluating potential therapeutic interventions targeting pathological reactive states.

Finally, the iPSC-derived GPCs employed in this investigation were generously provided by Dr. Charlie Arber's laboratory at University College London (UCL). Dr. Arber's group has established a collection of control iPSC lines derived from healthy donors, encompassing four distinct control lines: Control 1 (male), Control 2 (male), Control 3 (male) (Arber et al., 2020), and

WT (female) (Arber et al., 2019). These genetically diverse control lines enable comparative analysis across sex and individual genetic backgrounds.

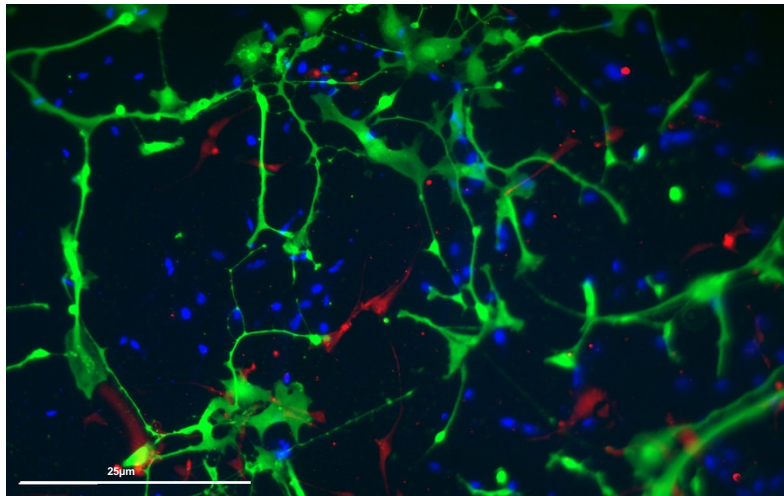
The work presented in this chapter, has aimed to investigate and understand astrocyte reactivity and phagocytic activity under inflammatory conditions utilising WT cell line (female) iPSC-derived astrocytes as our experimental model. This cell line was selected because it was the most robust and reproducible in culture, whereas other lines presented technical difficulties during maintenance.

5.1 Results

5.1.1 Characterisation and identification of GPC and iPSC-derived astrocytes.

Before conducting comprehensive reactivity studies in iPSC-derived astrocytes, we first performed immunofluorescence staining to identify and characterize the distinct cellular populations within GPCs and differentiated cultures. Immunostaining utilised well-characterized molecular markers enabled us to distinguish glial progenitor cells from mature astrocytes, confirming the composition and developmental stage distribution of our iPSC-derived glial cultures. This foundational characterization established the cellular basis for subsequent reactivity analyses and ensured accurate interpretation of marker expression changes in specific cell populations (Figure 30 A and B). Using immunostaining (see section 2.2.6) GPCs stained and terminal differentiated matures astrocytes were stained for GFAP, MAP2.

(A) GFAP +MAP2+ DAPI



(B) GFAP +MAP2+ DAPI

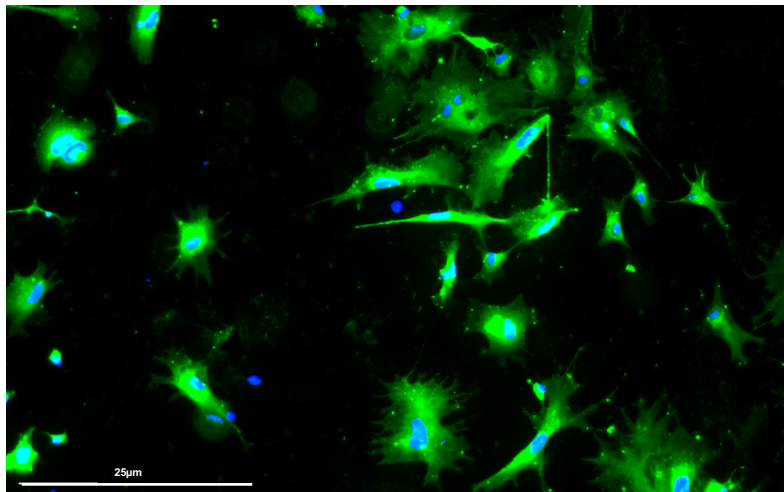


Figure 29: Immunostaining of GPCs and iPSC-derived astrocytes. Representative images of (A) GPCs (DIV14) stained with GFAP (green), MAP2 (red) and DAPI (blue) and (B) iPSC-derived astrocytes (DIV 14) stained with GFAP (green), DAPI (blue), and MAP2. Scale bar 25 μ m.

5.1.2 Transcriptomic analysis of iPSC-derived astrocytes.

To comprehensively investigate the molecular aspect of reactive astrocytes derived from iPSCs, we analysed transcriptomic data generously provided by Dr. Charlie Arber's laboratory at University College London (UCL). This dataset provides high-resolution gene expression profiling that enables systematic understanding of the transcriptional signatures associated with astrocyte reactivity, allowing the identification of key regulatory pathways and molecular drivers of the reactive astrocyte phenotype in iPSC-derived systems.

The transcriptomic data analysis revealed significant and distinct alterations in gene expression profiles between vehicle control and TIC conditions. Analysis of GFAP transcript levels has shown as reduction upon TIC treatment (10850 ± 2442 , $p = 0.001$, $n = 12$) compared to its vehicle control (38628 ± 6765 , $n = 12$, Fig 30 A).

Similarly analysis of reactivity marker C3 transcript levels which were almost undetectable in the vehicle control (15.17 ± 5 , $n = 12$) while a robust increase was observed in TIC-treated samples (17639 ± 2603 , $p = 0.0001$, $n = 12$, Fig 30 B). Similarly, Gbp2 mRNA expression was near baseline in vehicle controls (357 ± 64.9 , $n = 12$) and significantly increased following TIC treatment samples (11554 ± 1075 , $p = 0.0001$, $n = 12$ Fig 30 C).

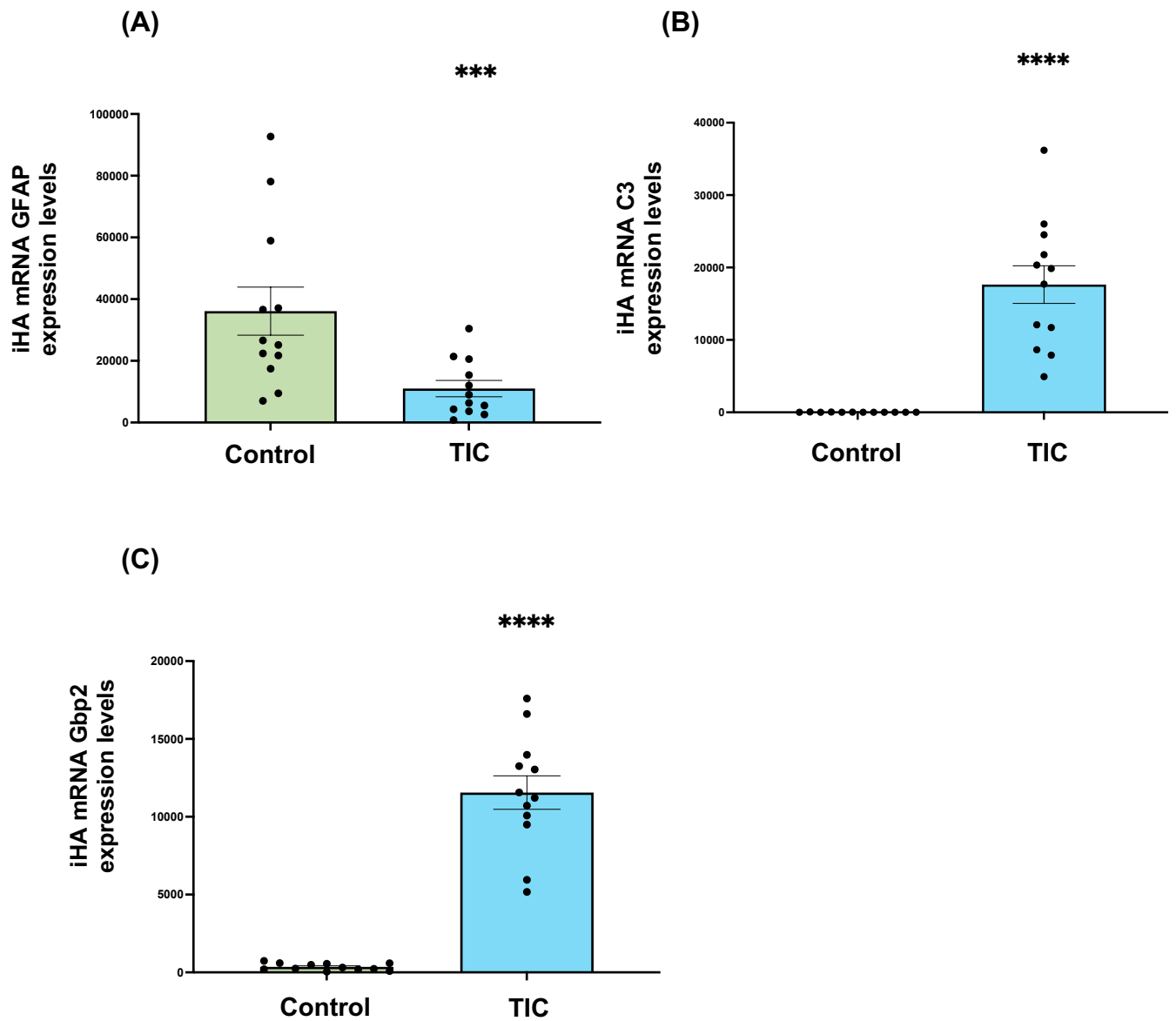


Figure 30: Transcriptomic signatures of TIC-induced astrocyte reactivity. Bar graph showing reduced **(A)** GFAP mRNA expression in TIC stimulated iPSC-derived astrocytes, **(B)** increased C3 mRNA expression in TIC stimulated iPSC-derived astrocytes and **(C)** increased Gbp2 mRNA expression in TIC stimulated iPSC-derived astrocytes, *** = $p < 0.0010$, **** = $p < 0.0001$ versus vehicle controls, determined by an unpaired student t-test.

5.1.3 TIC treatment does not alter immunostaining profiles in iPSC-derived astrocytes.

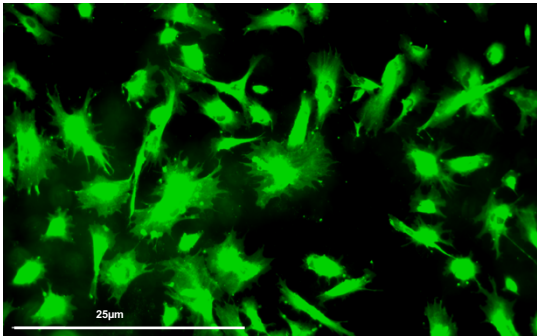
Following the analysis of the transcriptomic data, we incorporated immunostaining to validate and visualize the observed molecular findings. Using the transcriptomic profiles as a reference, specific markers that showed significant expression differences in TIC stimulated iPSC-derived astrocytes were selected for immunostaining. This approach allowed us to assess whether the observed transcriptional changes were reflected at the protein level and to confirm cell-type-specific phenotypic characteristics in line with the transcriptomic results. In our experiments we stimulated both GPCs and iPSC-derived astrocytes with TIC and performed immunocytochemistry with the reactivity markers GFAP, C3 and Gbp2.

The immunostaining revealed that iPSC-derived astrocytes when exposed to TIC treatment have no change in reactive marker expressions. GFAP expression in TIC treated mature astrocytes (64.53 ± 6.4 a.u, $p = 0.1423$, 95% CI 46.6- 82.4, $n = 5$, **Figure 31A**) did not significantly differ when compared to vehicle control (84.97 ± 10.4 a.u, 95% CI 55.95 – 114, $n = 5$). Similarly, C3 expression also show no change in expression when stimulated with TIC (56.96 ± 6 a.u, $p = 0.8879$, 95% CI 40.15 – 73.76, $n = 5$) compared to control (55.82 ± 4.9 a.u 95% CI 42 – 69.6, $n = 5$, **Figure 31B**). Finally, Gbp2 expression also have shown no change in expression (108.8 ± 13.8 a.u, $p = 0.5644$, 95% CI 70.3 – 147.4, $n = 5$, **Figure 31C**) compared to its control (98.69 ± 9.4 a.u, 95% CI 72.4 – 124.9, $n = 5$). Furthermore, morphologically, TIC stimulation did not induce changes in area or perimeter, with the area

($86.63 \pm 29.12 \mu\text{m}^2$, $p = 0.7257$, 95% CI 5.72- 167.5, $n = 5$, **Figure 32 C**), and perimeter ($15.91 \pm 3.74 \mu\text{m}$, $p = 0.6383$, 95% CI 5.52 – 26.3, $n = 5$, **Figure 32 D**) versus the control area ($101.4 \pm 28.46 \mu\text{m}^2$, 95% CI 22.41 – 180.4, $n = 5$) and perimeter ($18.77 \pm 4.48 \mu\text{m}$, 95% CI 6.3 – 31.2, $n = 5$).

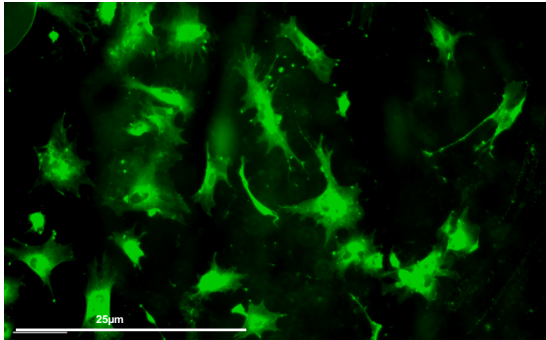
(A)

Control GFAP

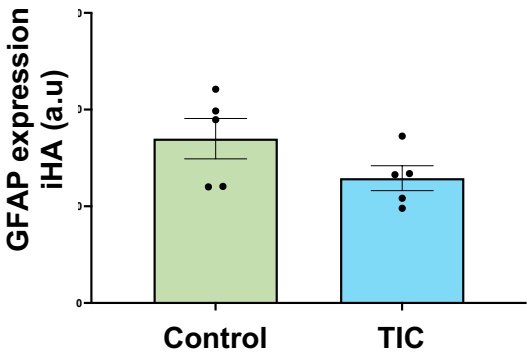


(B)

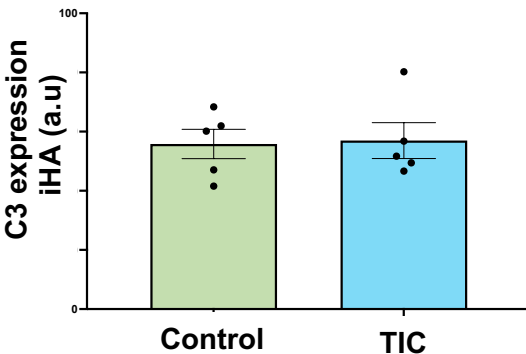
TIC GFAP



(C)



(D)



(E)

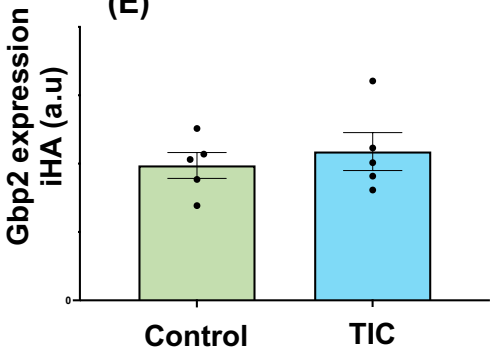


Figure 31: TIC stimulation does not alter expression of reactive markers in iPSC-derived astrocytes. Representative immunofluorescence images depicting GFAP expression in (A) control mature astrocytes and (B) mature astrocytes stimulated with TIC. Bar graphs showing relative expression levels of (C) GFAP, (D) C3, and (E) Gbp2 in control and TIC-stimulated mature astrocytes. Data are expressed as mean $\pm$ SEM, and statistical significance was determined using unpaired t-test. Scale bar 10 μ m.

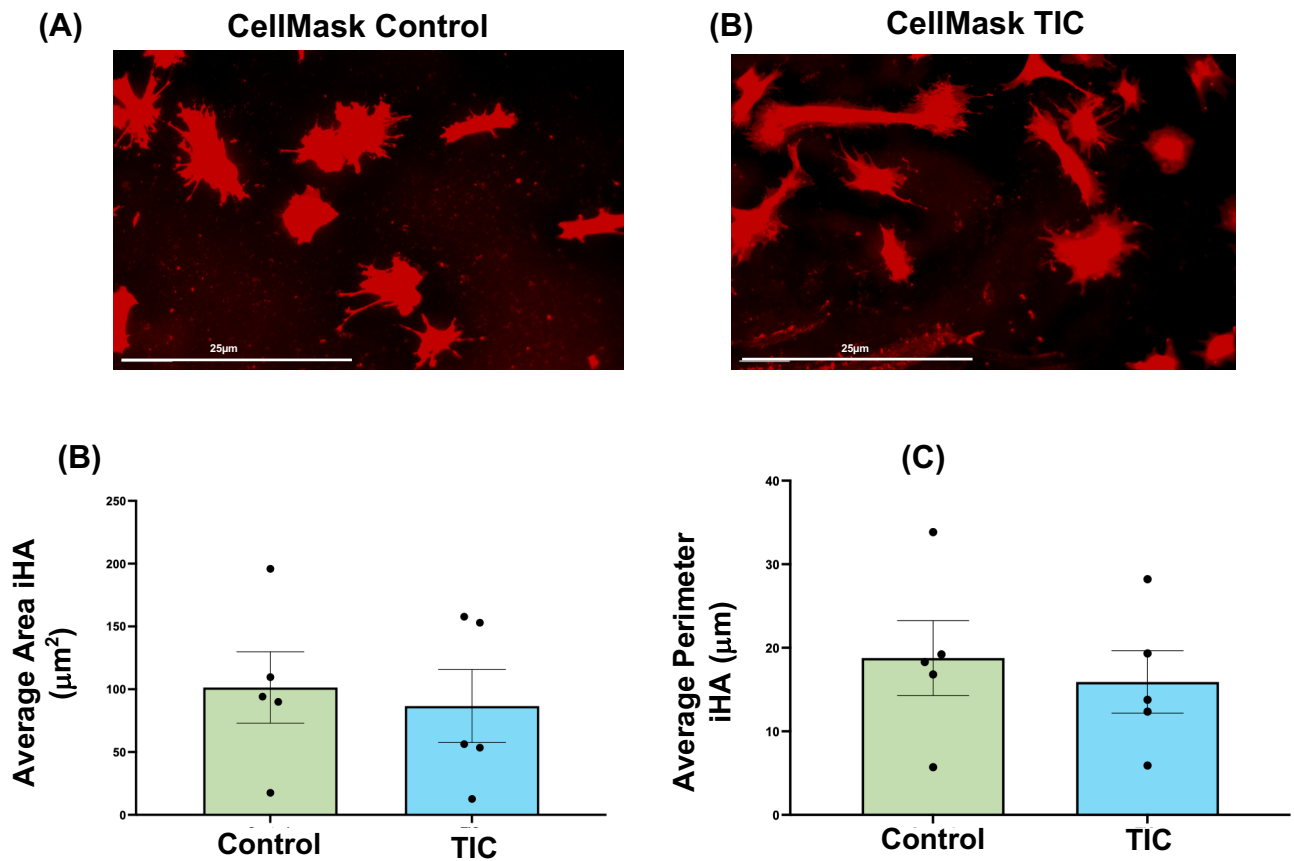


Figure 32: TIC stimulation does not alter cellular morphology in iPSC-derived astrocytes. Representative immunofluorescence images of matured astrocytes stained with CellMaskTM in (A) Control (B) TIC stimulated mature astrocytes. Bar graph summarizing (C) average area (D) average perimeter in TIC stimulated matured astrocytes. Data are expressed as mean $\pm$ SEM, and statistical significance was determined using unpaired t-test. Scale bar 25 μ m.

5.1.4 No effect of TIC on Molecular and Morphological Alterations in Glial Progenitor Cells

To further investigate whether similar effects could be seen in less differentiated glial populations, we examined GPCs following TIC stimulation. The primary aim was to determine if TIC exposure could alter their molecular profile or cell morphology.

The comparative analysis of TIC-stimulated GPCs, revealed that GFAP expression remained unchanged between control GPCs (61.72 ± 4.0 a.u, 95% CI 36.9 – 76.9, $n = 5$) and TIC stimulated GPCs, (61.93 ± 4.2 a.u, $p = 0.9212$, 95% CI 50.34 -73.1, $n = 5$, **Fig 33C**). Similarly, C3 expression remained unchanged between control GPCs (68.8 ± 1.6 a.u, 95% CI 64.19 – 73.45, $n = 5$) and TIC stimulated GPCs (63.55 ± 4.3 a.u, $p = 0.9212$, 95% CI 51.43 -75.6, $n = 5$ **Fig 33D**). Finally, Gbp2 expression was unchanged between GPC controls (136.6 ± 21.8 a.u, 95% CI 75.9 -197.4, $n = 5$ **Fig 33 E**) and TIC stimulated GPCs (136.3 ± 18.2 a.u, $p = 0.9750$, 95% CI 85.8 – 186.9, $n = 5$). Finally, morphological studies also have not shown any significant change in either TIC treated GPCs compared to the vehicle controls (**Figure 34A & B**). The area of TIC treated GPCs was ($136.3 \pm 6.482 \mu\text{m}^2$, $p = 0.1661$, 95% CI 118.3 -154.3, $n = 5$, vs control area ($121.8 \pm 6.989 \mu\text{m}^2$, 95% CI 102.4 – 141.2, $n = 5$, **Figure 34C**). While the perimeter of the TIC treated GPCs was ($46.41 \pm 1.38 \mu\text{m}$, $p = 0.2778$, 95% CI 42.58 – 50.25, $n = 5$, vs control perimeter ($44 \pm 1.5 \mu\text{m}$, 95% CI 39.72 – 48.28, $n = 5$, **Figure 34D**).

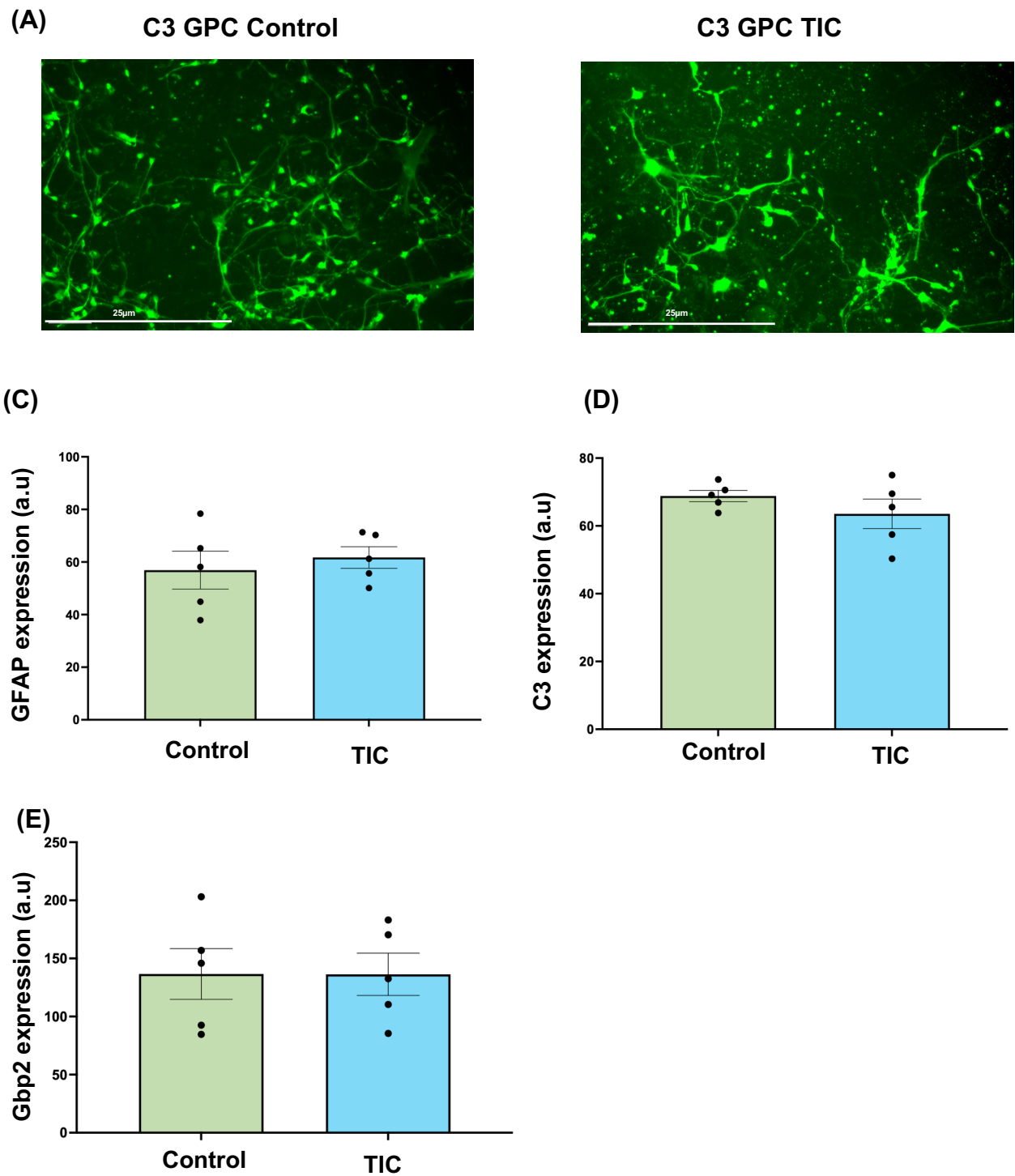


Figure 33: Effects of inflammatory cytokine stimulation on reactive marker expression in glial progenitor cells. Representative immunofluorescence images depicting C3 expression in (A) control GPCs and (B) GPCs stimulated with TIC. Bar graphs showing relative expression levels of (C) GFAP, (D) C3, and (E) Gbp2 in

control and TIC-stimulated GPCs. Data are expressed as mean $\pm$ SEM, and statistical significance was determined using unpaired t-test. Scale bar 25 μ M.

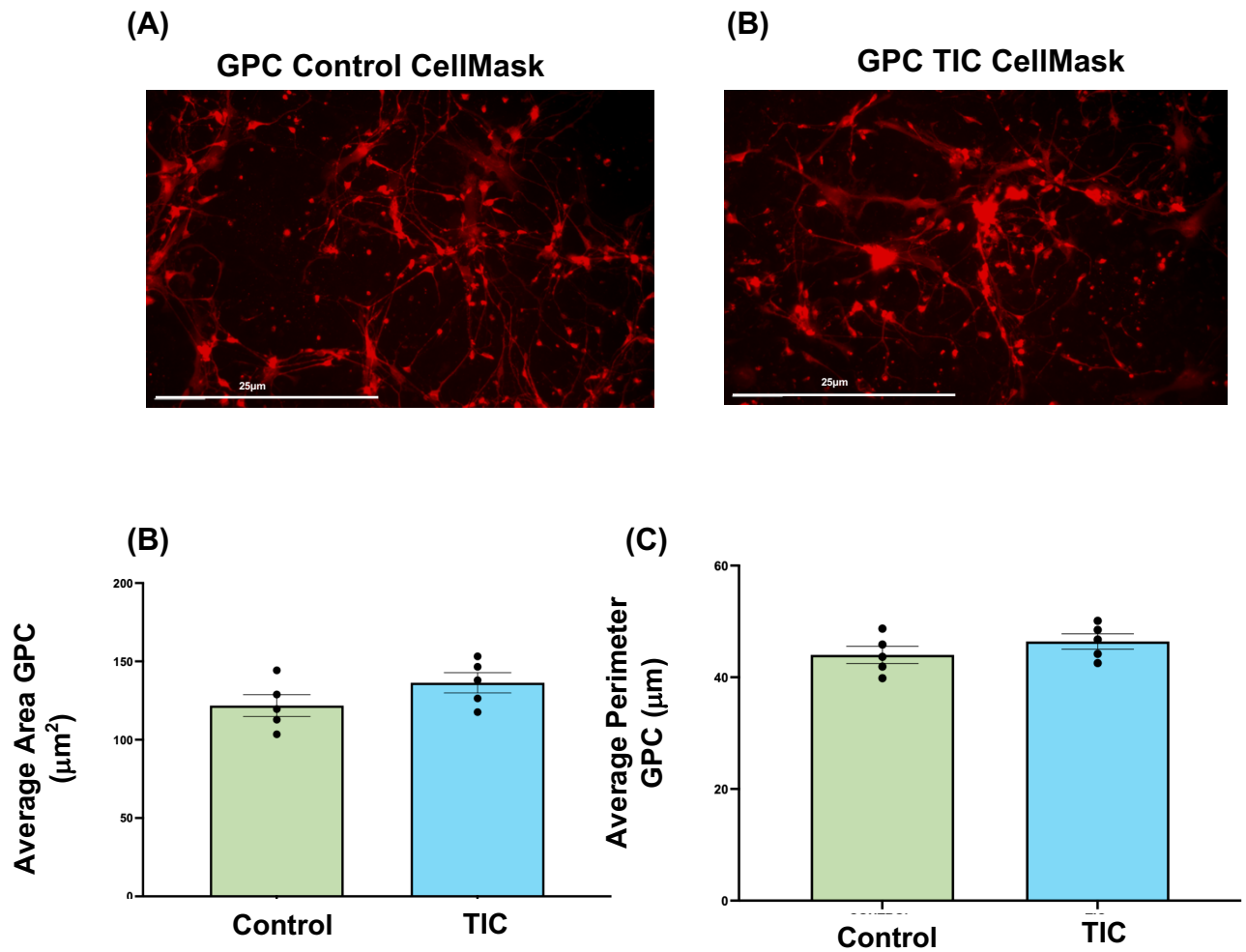
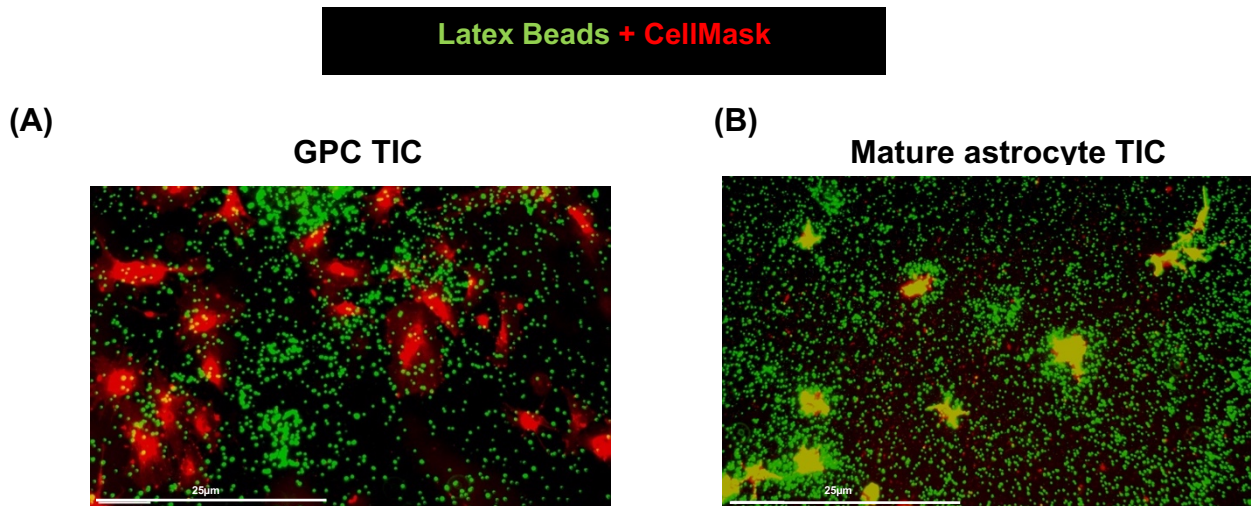


Figure 34: Effects of Inflammatory Cytokine Stimulation on Cellular Morphology in Glial Progenitor Cells. Representative immunofluorescence images GPCs stained with CellMaskTM (A) Control (B) Tic stimulated. Bar graph summarising (C) average Area (D) average perimeter in TIC stimulated GPCs. Data are expressed as mean $\pm$ SEM, and statistical significance was determined using unpaired t-test. Scale bar 25 μ M.

5.1.5 Differential effect of phagocytosis in mature astrocytes and GPCs upon TIC-stimulation

As previously discussed, phagocytosis plays a critical role in maintaining central nervous system homeostasis by enabling the clearance of foreign material, cellular debris, and apoptotic cells (Konishi et al., 2020, 2022; Li & Underhill, 2025). Astrocytes, in particular, exhibit phagocytic activity that is essential for synaptic pruning and the support of neuronal function. TIC stimulated GPCs (150.8 ± 6.0 a.u, $p = 0.0628$, 95% CI $135.2 - 166.5$, $n = 5$, **Figure 35C**) show no effect on phagocytic activity when compared to their control (131.5 ± 6.8 a.u, 95% CI $113.8 - 149.3$, $n = 5$). While there was an increase in phagocytic activity in TIC stimulated mature astrocytes treated with TIC (222.3 ± 8.0 a.u, $p = 0.0001$, 95% CI $200 - 244.5$, $n = 5$, **Figure 35D**) compared to their control (115.6 ± 3.6 a.u, 95% CI $105.4 - 125.8$, $n = 5$).



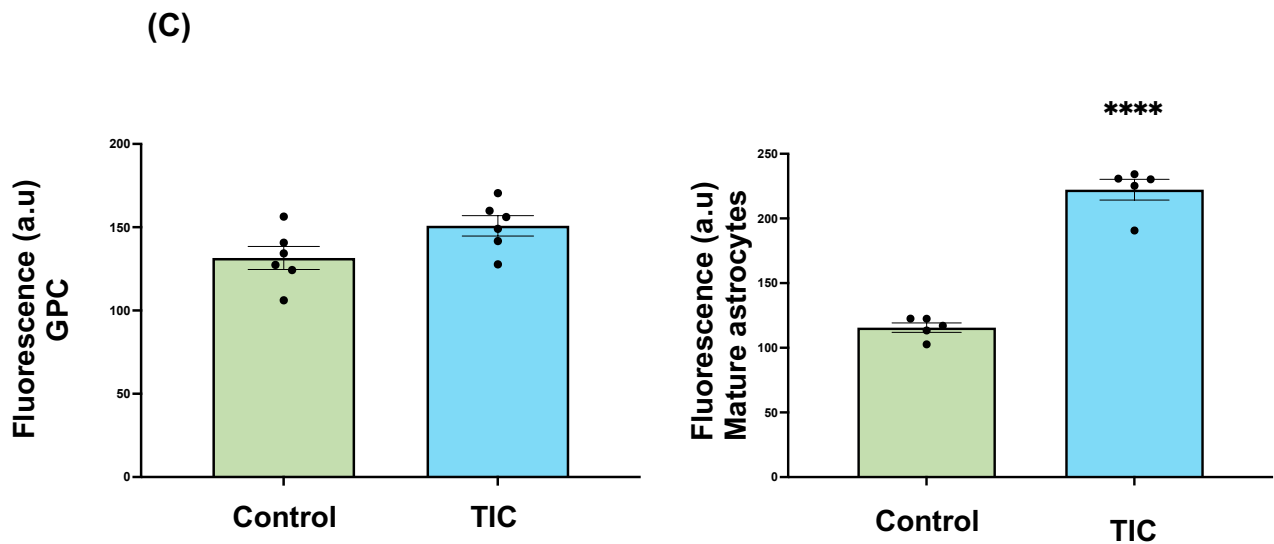


Figure 35: TIC stimulation increases phagocytic activity in mature astrocytes. Representative immunofluorescence images showing phagocytosis in TIC-stimulated (A) GPCs while in (B) Mature astrocytes. cells stained with CellMask™ (red) and latex beads (green) were used to assess phagocytic uptake. In mature astrocytes co-localization of beads with plasma membrane is shown in yellow indicating bead internalization, whereas this co-localisation is absent in GPCs. Bar graphs showing phagocytic activity in TIC-stimulated (C) GPCs and (D) mature astrocytes. Data are expressed as mean $\pm$ SEM, and statistical significance was determined using unpaired t-test. Scale bar 25 μ M.

5.2 Discussion

5.2.1 TIC stimulation upregulates C3 and Gbp2 but not GFAP mRNA expression in iPSC-derived astrocytes.

In our analysis of the transcriptomic dataset generously provided by Dr. Charlie Arber's laboratory, composed of data regarding iPSC-derived astrocytes stimulated with TIC, we aimed to understand the effect of TIC stimulation on astrocyte reactivity. We found there was a robust induction of reactivity, indicated by an increase reactive markers C3 (Prakash et al., 2024) and Gbp2 (Clayton & Liddelow, 2025) whereas GFAP transcript levels were reduced upon TIC stimulation. This is intriguing as previous studies have shown GFAP upregulation as a traditional hallmark of astrocyte reactivity (Clayton & Liddelow, 2025; Gildea & Liddelow, 2025; Liddelow & Barres, 2017; Prakash et al., 2024), but emerging studies have also proven that reactive astrocytes exhibit heterogeneous responses and are context-dependent (Escartin et al., 2021; D. E. Santos et al., 2025). This reduction, in GFAP may indicate, a loss of neuroprotective function, associated with homeostatic astrocytes (Fang et al., 2022; Lawrence et al., 2023). Astrocytes maintain synaptic function, provide metabolic support and preserve BBB integrity. Thus, downregulation of GFAP in TIC stimulated astrocytes may indicate a shift from these supportive role and compromised neuronal health (Lawrence et al., 2023). These neuroprotective roles are dependent on the astrocyte function and morphology, of which GFAP plays an important role as a key cytoskeletal marker. This downregulation of GFAP following TIC stimulation therefore may indicate reduction of supportive roles, reduced glutamate clearance,

compromised BBB function. Reduced GFAP may also indicate that astrocytes are less capable in maintaining homeostasis under diseased or stressed conditions (Leipp et al., 2024; Uguagliati & Grilli, 2024; Verkhratsky et al., 2023). An increase in seen in C3 in TIC stimulated astrocytes indicate critical role played in mediating astrocyte-induced neuronal damage concurrent with previous studies (Clayton & Liddelow, 2025; Gildea & Liddelow, 2025; Liddelow, 2019; Liddelow & Barres, 2017).

Astrocytic C3 helps synapse elimination by opsonizing synapses, via the microglial phagocytosis mediated via CR3/CD11b (Lawrence et al., 2023). However, synaptic pruning is essential for normal development of the brain, but the upregulation of C3 under neuroinflammation and neurodegenerative contexts causes pathological synapse loss and induces neurotoxicity (Chen et al., 2024; Lawrence et al., 2023). Beyond synaptic loss, C3aR signalling disrupts Ca^{2+} homeostasis and induces excitotoxicity (Gedam & Zheng, 2024; Zhang et al., 2022) with sustained C3 elevation leads to chronic microglial activation and inflammatory cascades driven by NF- κ B-mediated transcription of complement and pro-inflammatory genes in response to TIC cytokines (Lawrence et al., 2023; Lian et al., 2015). This aligns with previous studies indicating that TIC-treated astrocytes exhibit neurotoxic phenotypes marked by the loss of neuroprotective functions. (Clayton et al., 2024; Kim et al., 2022; Liddelow & Barres, 2017; Nakano-Kobayashi et al., 2023; Uguagliati & Grilli, 2024). Finally, increase in Gbp2 transcripts, indicates the activation of interferon-responsive pathways. Gbp2 is a type I interferon-stimulated gene and is known to participate in innate immune responses and studies have

shown it to be a reliable marker to identify reactive astrocytes (Clayton et al., 2024). Upregulation of Gbp2 indicates the interferon regulatory pathways driving gene expression related to, immune cell recruitment, inflammatory signalling and antigen presentation (Kim et al., 2022; Nakano-Kobayashi et al., 2023; Taylor et al., 2022). However, this mechanism is important for antiviral defence, but under chronic activation it promotes neuroinflammation causing synaptic dysfunction and neurodegeneration. This upregulation is in agreement with previous studies (Clayton et al., 2024; Kim et al., 2022; Liddel & Barres, 2017; Nakano-Kobayashi et al., 2023; Uguagliati & Grilli, 2024).

Overall, the analysis has highlighted the robust and reproducible nature of TIC-induced reactive astrocytes, offering a valuable platform to study molecular mechanisms to understand astrocyte-mediated neurotoxicity and to identify potential therapeutic targets.

5.2.2 No change in reactive marker expression upon TIC stimulation in iPSC-derived astrocytes and GPCs.

The current findings have shown no changes in the reactive markers GFAP, C3 and Gbp2 upon TIC stimulation in mature astrocytes which is in contrast to the already established studies in iPSC-derived astrocytes (Hyvärinen et al., 2019; Jurga et al., 2021), and human stem cells (McComish et al., 2024c), where cytokine cocktail induces neurotoxicity. This observation in our study warrants careful consideration into cell culture heterogeneity, complex nature of *in vitro* culturing, also the translation of mRNA into proteins and could reflect on the study being underpowered or relating to antibody sensitivity which fails to capture subtle phenotypic changes. Cellular heterogeneity is one critical factor that can be taken into consideration, within iPSC-derived astrocyte cultures. Although, various differentiation protocols may yield enriched astrocytes, but TIC stimulation may preferentially activate only a few cells and this can lead to dilution of detectable changes within the population (Z. Li et al., 2025; McComish et al., 2024d). Another important aspect that can relate to this negative result is the fact that transcriptomic data have revealed transient increases in mRNA levels for GFAP, C3, and Gbp2, but these changes did not translate into detectable protein via immunofluorescence. This is in contrast to previous studies which proteomically profiled various reactive markers including GFAP and C3 (Prakash et al., 2024) and Gbp2 (Clayton et al., 2024). Finally, mature astrocytes, do not show any change in morphology upon TIC stimulation, this could reflect on the fundamental limitations of the

2D culture system and also on the sensitivity limitations of immunofluorescence methods.

Moreover, our study also incorporated whether GPCs will show any response to TIC stimulation. GPCs show no change in either reactive markers nor in morphology, which raises an important questions about the developmental-stages, heterogeneity within glial lineage of cell populations and cellular maturation. GPCs are an intermediate developmental stage cells (Apostolou & Stadtfeld, 2018; Llorente et al., 2021) and express low levels of marker observed in mature astrocytes, and may lack the full complementary signalling system that is needed for responding to TIC stimulation (R. Santos et al., 2017b). This could also relate to the line variability which might display heterogeneous responses to TIC stimulation (Li et al., 2025; Llorente et al., 2021). GPCs show no change in morphology upon TIC stimulation, which could reflect on their development stage as progenitors (Gomes et al., 2024; Prakash et al., 2024).

5.2.3 TIC stimulation increases phagocytosis in matured astrocytes but not in GPCs

Our study demonstrates that TIC stimulation increases phagocytic activity in mature astrocytes (Konishi et al., 2022; Li & Underhill, 2025) while no change in phagocytic activity was observed in GPCs, reflecting on their developmental stage and functional responses to inflammatory signals (Chung et al., 2013; Jaumouillé & Waterman, 2020; Konishi et al., 2020). The development of GPCs into matured astrocytes involves the acquiring of morphological complexity, specialized transcriptional signatures and fully developed phagocytic receptors such as MEGF10 and MERTK (Chung et al., 2013; Jaumouillé & Waterman, 2020; Konishi et al., 2020). Mature astrocytes also possess inflammatory signalling pathways including complete cytokine receptor systems, all of these enable them to respond to the TIC signals into enhanced phagocytic function (Nizami et al., 2019). In contrast GPCs remain in a relatively undifferentiated state and express progenitor markers and lack this molecular system needed for phagocytosis and lack cytokine receptor and downstream signalling components (Li et al., 2025; McComish et al., 2024d; Santos et al., 2017b).

These differential responses indicate that phagocytosis is not present in all astrocyte lineage cells, rather is developed during the maturation stage, where the cells develop the necessary machinery to perform these specialised functions. This is important for understanding phagocytic function in both health and disease condition (Li et al., 2025; McComish et al., 2024d; Santos et al., 2017b; Schuurmans et al., 2024).

5.3 Conclusion

In conclusion, this chapter has aimed study, understand and characterise astrocyte reactivity using GPCs and iPSC-derived astrocytes, as an *in vitro* model. While the transcriptomic analysis revealed a robust induction of reactive markers C3 and Gbp2 following inflammatory TIC stimulation but the canonical marker GFAP has shown a reduction in transcript level indicating heterogeneous and complex astrocyte phenotype. However, these transcriptomic changes have not shown to translate in to altered protein expression or any change in morphology observed using immunostaining techniques, indicating probable limitations in translating mRNA changes to functional protein levels in these models under the condition of our experiments.

Moreover, mature astrocyte and but not GPCs have demonstrated increased phagocytic activity upon TIC treatment underscoring functional maturation which is developmental – dependent. These results also highlight the need and the challenges of iPSC-derived astrocyte models for studying reactivity and their roles in neuroinflammation and neurodegeneration, with an emphasis on the heterogeneity, maturation stage, and the sensitivity of methodology in interpreting data.

Overall, the experiments conducted throw light on the molecular and functional aspects of astrocyte reactivity to understand and develop therapeutic target for neurodegenerative and neuroinflammatory mechanisms.

CHAPTER 6

General Discussion,

limitations and future

directions

6. General Discussion

6.1. Discussion

Astrocytes are essential and dynamic components of the central nervous system (CNS), contributing to its structural integrity, functional maintenance, and immune regulation. Far beyond their historical characterization as passive support cells, astrocytes are now recognized as active participants in synaptic modulation, metabolic support, and neuroimmune interactions. Under conditions of stress or injury, these cells undergo a process known as reactive astrogliosis, which can either protect neurons and promote repair or, conversely, contribute to neuroinflammation and degeneration. Understanding the molecular mechanisms that govern this duality is therefore critical to unravelling the complexities of CNS homeostasis and pathology.

In this thesis, I focus on the signalling pathways that regulate astrocyte reactivity, with particular attention to protease-activated receptor (PAR) signalling. Among these, protease-activated receptor 2 (PAR2) has emerged as a molecule of interest due to its complex and context-dependent influence on astrocyte function. Activation of PAR2 can trigger both beneficial and detrimental responses, highlighting its potential as a therapeutic target in neurological diseases where inflammation plays a central role.

To explore this, I employed both commercial human astrocytes and human induced pluripotent stem cell (iPSC)-derived astrocytes, integrating advances in stem cell technology with rigorous molecular and functional characterization. The study standardized culture conditions, applied inflammatory cytokines and PAR-specific activators, and utilized imaging and immunocytochemical

approaches to assess cellular signalling, morphology, and marker expression. In addition, the phagocytic activity of astrocytes was examined to better understand their role in maintaining neural health and responding to injury or disease.

By comparing these cellular models and delineating the mechanisms of PAR2-mediated astrocyte reactivity, this research aims to provide deeper insights into how astrocytes contribute to neuroinflammation and neuroprotection alike. Ultimately, the findings contribute to the knowledge that positions human astrocytes not merely as support cells, but as key players and potential therapeutic targets in CNS disorders.

6.2. Chapter 3

Chapter 3 explored the expression and functional role of protease-activated receptor 2 (PAR2) in primary rat hippocampal cultures and for the first time in commercial human astrocytes. Using calcium imaging, it was confirmed that PAR2 activation induces significant calcium signalling exclusively in astrocytes, not neurons, suggesting a predominant glial role for PAR2 in neurophysiology. The study also detailed signalling mechanisms, using calcium imaging and western blotting showing that PAR2-induced calcium signalling operates via the phospholipase C (PLC)-IP3 pathway without modulating MAPK signalling in human astrocytes.

(A) Summary of Findings

- PAR2 and PAR1 activation caused robust elevation in intracellular $[Ca^{2+}]_i$ in astrocytes but not neurons, highlighting cell-type-specific expression and signalling.
- PAR2 agonists (trypsin, SLIGKV-NH₂, AC264613) elevated Ca^{2+} in commercial human astrocytes, confirming for the first time functional PAR2 presence.
- PLC inhibitor U73122 abolished PAR2-induced Ca^{2+} rises, establishing the Gq-PLC-IP₃ axis as the key pathway.
- Activation of PARs did not significantly modulate MAPK or ERK1/2 phosphorylation in commercial human astrocytes under tested conditions, contrasting with some previous rodent studies (Greenwood & Bushell, 2010; Viorel et al., 2024; Wang et al., 2002).

(B) Limitations

- Lack of neuronal responses to PAR activation may reflect culture composition or maturity.
- Absence of data on PAR3 and limited receptor subtype scope.
- In vitro culture conditions may not capture the full complexity of in vivo signalling and cellular interactions.

(C) Future Directions

- Extended analysis of PAR-mediated signalling pathways, including non-canonical and long-term effects in astrocytes.

- Explore receptor crosstalk and roles of other PAR subtypes in neuronal-glial communication.
- Investigate PAR2 roles in pathological contexts such as neuroinflammation and neurodegeneration.

6.3. Chapter 4

This chapter examined how PAR1 and PAR2 activation, serum presence or absence, and inflammatory cytokine cocktail TIC (TNF- α , IL-1 β , C1q) affect astrocyte reactivity markers (GFAP, C3, Gbp2) and morphology in commercial human astrocyte cultures. Both short (24h) and long (7-day) treatments under serum-containing and serum-free conditions were applied to assess functional plasticity.

(A) Summary of Findings

- PAR1 and PAR2 activators induced significant transient increases in GFAP expression in serum-containing media, while C3 was selectively upregulated in serum-free media in response to PAR2 activators.
- Gbp2 expression remained largely unchanged across all treatments and conditions, indicating relative stability of this marker.
- TIC treatment induced no significant morphological or reactivity marker changes in astrocytes under serum-free conditions; a modest increase in GFAP was noted under serum-containing media, likely due to basal activation.

- No significant changes in cell area or perimeter were observed following PAR activation or TIC treatment in any condition, suggesting limited morphological plasticity in this setting.

(B) Limitations

- Commercial astrocytes' high passage number may reduce responsiveness.
- Two-dimensional culture and serum presence may mask subtle reactive phenotypes.
- Lack of inclusion of pan-reactive or interferon-related cytokines may limit induction of full reactive states.

(C) Future Directions

- Employ three-dimensional models and co-culture with microglia or neurons to better mimic in vivo conditions.
- Integrate transcriptomic and proteomic profiling to identify subtle reactive signatures.
- Extend cytokine panels to include interferons or other modulators relevant to disease states.

6.4. Chapter 4 – Phagocytosis

Chapter 4 investigated how PAR1 and PAR2 activation and inflammatory cytokines affect phagocytic activity in cultured human astrocytes. Fluorescent latex bead uptake assays were used as a surrogate measure of phagocytosis.

(A) Summary of Findings

- PAR1 and PAR2 activators (TFLLR-NH₂, AC264613, SLIGKV-NH₂) did not significantly alter the uptake of latex beads by human astrocytes.
- Similarly, TIC stimulation failed to modulate astrocyte phagocytic activity.
- iPSC-derived astrocytes also demonstrated no change in phagocytic capacity upon TIC or PAR activation, supporting a limited functional shift under these experimental conditions.

(B) Limitations

- The one-hour incubation period for phagocytosis assays may not be sufficient to observe incremental phagocytic activity in astrocytes, which operate slower than microglia.
- Use of latex beads rather than physiologically relevant substrates (e.g., synaptic debris, apoptotic cells) may limit translational relevance.
- Baseline activation state due to serum-containing media could suppress dynamic range for detecting phagocytic changes.

(C) Future Directions

- Utilize disease-relevant, physiological substrates and longer assay durations to better capture astrocytic phagocytosis.
- Explore expression changes in phagocytic receptors MEGF10 and MERTK under different stimuli.
- Incorporate live imaging and co-culture with microglia to delineate coordinated glial phagocytic responses.

6.5 Chapter 5

Chapter 5 investigated how inflammatory cytokines TIC (TNF- α , IL-1 β , C1q) affect astrocyte reactivity in iPSC-derived astrocytes. Fluorescent latex bead uptake assays were used as a surrogate measure of phagocytosis, in iPSC-derived astrocytes.

(A) Summary of Findings

- Transcriptomic analysis of astrocyte reactivity, revealed significant and distinct alterations in gene expression in GFAP, C3 and Gbp2 and between vehicle control and TIC-stimulated conditions.
- Heterogeneous astrocyte phenotype: The reduction in GFAP paired with upregulation of C3 and Gbp2 demonstrates that reactive astrocytes exhibit context-dependent, heterogeneous responses rather than uniform reactivity patterns.
- No significant changes in immunostaining: Despite robust transcriptomic changes, immunofluorescence revealed no significant alterations in reactive marker protein expression.
- No morphological changes: TIC stimulation did not induce changes in cell area or perimeter, suggesting limited morphological plasticity in 2D culture conditions.
- GPCs showed no response to TIC stimulation across all measured parameters: No changes in - GFAP, C3, or Gbp2 expression or in morphological alterations.

- Mature astrocytes demonstrated increased phagocytosis upon TIC stimulation: Phagocytic activity in TIC-stimulated mature astrocytes was significantly elevated compared to controls.
- GPCs showed no phagocytic response: TIC-stimulated GPCs showed no significant enhancement of phagocytic activity when compared to controls.

(B) Limitations

- Human iPSC-derived astrocyte cultures contain mixed populations of glial progenitor cells and more mature astrocytes, many of which may retain immature or foetal-like features, leading to variable and often attenuated responses to inflammatory stimulation such as TIC.
- Use of two-dimensional monocultures may fail to recapitulate the three-dimensional architecture, extracellular matrix composition, and neuron–microglia–endothelial interactions that critically shape astrocyte reactivity and phagocytic function in vivo.
- A clear discordance was observed between robust TIC-induced changes at the transcriptomic level and the absence of detectable protein or morphological alterations using immunofluorescence and short phagocytosis assays, highlighting limited sensitivity, and substrate relevance of the assays employed.
- Experiments were largely performed in a single control iPSC line under one cytokine paradigm, so the findings may not fully capture line-to-line variability, sex and genetic background effects, or the broader spectrum of astrocyte phenotypes relevant to diverse neurodegenerative diseases.
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(C) Future Directions

- Develop three-dimensional brain organoids or co-cultures incorporating iPSC-derived astrocytes with neurons, microglia, and endothelial cells to recapitulate in vivo cellular interactions, extracellular matrix complexity, and blood-brain barrier dynamics that influence astrocyte reactivity and phagocytic function.
- Integrate single-cell transcriptomics, proteomics, and live-cell imaging with physiologically relevant phagocytic substrates, and extended time-course analyses to resolve mRNA-protein discordance, identify transient reactive signatures, and quantify phagocytic receptor dynamics (MEGF10, MERTK).

6.6. Conclusions

This research highlights the complex and context-dependent nature of astrocyte reactivity, emphasizing that protease-activated receptor signalling, particularly through PAR2, exerts distinct effects on cellular signalling, gene expression, and function in human astrocytes. The findings demonstrate that PAR2 primarily activates calcium-dependent pathways via the PLC-IP3 axis without significant MAPK involvement and that its activation modulates reactivity markers under specific culture conditions but does not influence morphology or phagocytic capacity. These results underscore the nuanced and tightly regulated roles of PAR signalling in astrocyte biology and point to the necessity of advanced, physiologically relevant models to fully elucidate

astrocyte behaviour in neuroinflammatory and neurodegenerative contexts, where they represent both a key effector and a promising therapeutic target.

CHAPTER 7

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