

Neuroinflammation – the Trojan Horse of Neurodegeneration:

Dissecting Regional, Genetic and Therapeutic
Modulation of Astrocyte Reactivity in Alzheimer's
Disease



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Declaration and statement of plagiarism

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work. The unpublished and/or published work of others has been duly acknowledged in the text wherever included.

Professor Maeve Caldwell was involved in study design, analysis and interpretation of data. Professor Eric Downer was involved in the study design, analysis and interpretation of data (Chapter 5).

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Summary

Neuroinflammation is more widely accepted to play a cardinal role in the onset and progression of neurodegenerative disease. Yet despite extensive evidence, its contribution is still often underestimated, thought to operate more subtly than classical pathological hallmarks. In this thesis, neuroinflammation is conceptualised as a “Trojan Horse” of neurodegeneration, an insidious process that infiltrates early, amplifies cellular dysfunction and accelerates downstream pathology long before clinical symptoms emerge. To investigate this idea, human iPSC-derived astrocytes were assessed for their response to Alzheimer’s disease-relevant stressors and the potential of cannabinoids to modulate these inflammatory pathways was explored.

Using human iPSC-derived astrocytes, reproducible models of astrocyte reactivity were established and an idea that regional identity shapes inflammatory behaviour was explored. Cortical (CTX) and ventral midbrain (VM) astrocytes displayed distinct basal profiles and differential sensitivity to IL-1 α , TNF α +IL-17A and TNF α +IL-1 α +C1q (TIC) stimulation, with region-specific secretion of mediators such as CXCL10, IL-6, RANTES and GM-CSF, as well as a wide array of inflammatory and metabolic genes. This regional divergence suggests that astrocytes from different brain areas contribute to neuroinflammatory landscapes in unique ways. Furthermore, we elucidated that APOE genotype significantly modifies these responses in CTX astrocytes, with APOE4 cells exhibiting heightened inflammatory sensitivity and altered transcriptional signatures. A β ₁₋₄₀ and A β ₁₋₄₂ oligomers induce species- and dose-dependent changes in viability and cytokine release, highlighting astrocytic vulnerability to Alzheimer’s disease-associated stressors.

The therapeutic component of this work demonstrates that cannabinoids (CBD, THC and their combination) effectively dampen IL-1 α -induced inflammatory signalling in CTX astrocytes. Cannabinoids reduce NF κ B nuclear translocation, suppress cytokine and chemokine release and downregulate multiple inflammation-associated genes. Mechanistic interrogation using PPAR γ agonist/antagonist and CB1 receptor antagonist reveals that these effects are

mediated through coordinated modulation of NF κ B-, PPAR γ - and CB1-linked pathways. By contrast, the NLRP3 inhibitor MCC950 shows only minimal activity, reflecting limited inflammasome competence in fully-differentiated human astrocytes.

Together, these findings provide new insight into the molecular drivers of human astrocyte reactivity, reveal region-, APOE- and A β -dependent susceptibilities relevant to neurodegeneration, and identify cannabinoid-responsive pathways with therapeutic promise. This work supports the notion that neuroinflammation, though often underestimated, acts as a Trojan Horse in neurodegenerative disease, quietly shaping pathology from within, and underscores the importance of human iPSC-based models for developing astrocyte-targeted interventions.

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Abbreviations

2-DG 2-deoxyglucose

α -syn alpha synuclein

A

AA ascorbic acid

AD Alzheimer's disease

ALS amyotrophic lateral sclerosis

AMM Astrocyte Maintenance Media

AMM-BL Astrocyte Maintenance Media supplemented with BMP4 and hLIF

AMM-EL Astrocyte Maintenance Media supplemented with EGF and hLIF

APC astrocyte progenitor cells

APOE Apolipoprotein E

APOE2 Apolipoprotein E ϵ 2

APOE3 Apolipoprotein E ϵ 3

APOE4 Apolipoprotein E ϵ 4

APP amyloid precursor protein

Astros mature hiPSC-derived astrocytes

ATP adenosine triphosphate

A.U. Arbitrary Units

B

BACE1	β -secretase; Beta-site Amyloid Precursor Protein Cleaving Enzyme 1
BBB	blood-brain barrier
BDNF	brain-derived neurotrophic factor
BMP4	bone-morphogenic protein 4

C

C1q	complement component 1
CB ₁	cannabinoid receptor type 1
CB ₂	cannabinoid receptor type 2
CBD	cannabidiol
CD49f	Cluster of Differentiation 49f/ITGA6
cDNA	complementary DNA
CNS	central nervous system
CSF	cerebrospinal fluid
CTX	cortical
Cx43	Connexin 43
CXCL10	C-X-C motif chemokine ligand 10

D

DAMPs	damage-associated molecular patterns
DMSO	dimethyl sulfoxide

E

E	embryonic day
EAAT1	Excitatory Amino Acid Transporter 1
ECAR	Extracellular Acidification Rate
EDTA	ethylenediaminetetraacetic
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
EMA	European Medical Agency
EN1	Engrailed-1

F

FAD	Familial Alzheimer's Disease
FOXA2	Forkhead Box Protein A2
FOXP1	Forkhead Box Protein G1

G

GDNF	glial cell-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GPCR	G protein-coupled receptors
GWAS	genome-wide association studies

H

HD	Huntington's disease
hLIF	human leukaemia inhibitory factor

I

ICC	immunocytochemistry
IFN	interferon
IL-1 α	interleukin 1 alpha
IL-6	interleukin 5
iPSC	induced pluripotent stem cells
IRF	interferon regulatory factor
ITGA6	Integrin alpha 6/CD49f

L

LDN	LDN-193189
LMX1A	LIM Homeobox Transcription Factor Alpha 1
LOAD	late-onset Alzheimer's disease

M

Max	maximum
MDB	membrane desalting buffer
Min	minimum
MS	Multiple Sclerosis

N

NFκB	Nuclear Factor kappa-light-chain enhancer of activated B cells
NMM	Neuro Maintenance Media
NPC	neural progenitor cells
NRF2	Nuclear Factor Erythroid 2-related factor 2
NSAIDs	non-steroidal anti-inflammatory drugs

O

OTX2	Orthodenticle Homeobox 2

P

PBS	phosphate buffered saline
PD	Parkinson's disease
PFA	paraformaldehyde
Pio	Pioglitazone hydrochloride
PRRs	pattern-recognition receptors
P.U.	Pixel Units

R

RAGE	Receptor for Advanced Glycation End-products
RT	room temperature
RT-qPCR	Reverse-Transcription quantitative Polymerase Chain Reaction
RQ	relative quantification

S

S100 β	S100 Calcium-Binding Protein B
SAD	Sporadic Alzheimer's Disease
SB	SB431542
scRNA-seq	single-cell RNA sequencing
SEM	Standard Error of the Mean
SHH	sonic hedgehog
SR14	SR141617

T

TBR1	T-Box Brain Transcription Factor 1
THC	Δ 9-tetrahydrocannabinol
TIC	TNF α +IL-1 α +C1q stimulation cocktail
TLR	toll-like receptor
TNF α	tumour necrosis factor alpha
T007	T0070907
TUJ1	β -Tubulin III

V

VM	ventral midbrain

Chapter 1. Introduction

1.1. Neurodegeneration and the Central Role of Neuroinflammation

Neurodegenerative diseases represent one of the greatest global health challenges of the 21st century. Conditions such as Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and Huntington's disease (HD) are characterised by the progressive loss of specific neuronal populations, which lead to cognitive, motor and behavioural decline. Although each neurodegenerative disorder presents with distinct clinical and pathological hallmarks, including extracellular β -amyloid ($A\beta$) plaques and intracellular tau tangles in AD, α -synuclein (α -syn) inclusions in PD, TDP-43 or SOD1 aggregates in ALS and huntingtin mutation in HD, they share several unifying features, including synaptic dysfunction, mitochondrial impairment, disrupted intra- and extracellular homeostasis, as well as chronic inflammation (Heemels, 2016; Wilson *et al.*, 2023). These processes suggest that neurodegeneration may arise not solely from intrinsic neuronal dysfunction, but also from maladaptive interactions with the surrounding glial and immune environment.

Historically, neurodegenerative research has focused on pathogenic proteins and the neurons they affect. However, recent advances in genetics, transcriptomics and cell biology research have shifted this perspective, revealing that neuroinflammation is not merely a downstream consequence of neuronal death, but is, in fact, a central driver of disease onset and progression (Heneka *et al.*, 2015; Heppner, Ransohoff and Becher, 2015; Wilson *et al.*, 2023). Evidence across multiple neurodegenerative disorders now indicates that inflammatory and neuroimmune changes appear years, if not decades, before the neuronal loss becomes evident and the disorder is symptomatic. For example, in AD, microglial activation, astrocytic reactivity and cytokine dysregulation are detectable in early preclinical stages and their presence strongly correlates with future cognitive decline (Calsolaro and Edison, 2016). In PD, innate immune activation precedes α -synuclein pathology and some of the genetic risk factors, such as LRRK2, act partly through inflammatory pathways, such as the NF κ B pathway (Tansey and Goldberg,

2010; Russo, Bubacco and Greggio, 2022). Similarly, in ALS, the altered glial-neuronal crosstalk and neuroimmune activation contribute to the vulnerability of motor neurons long before the clinical onset (Brettschneider *et al.*, 2014). These observations collectively challenge the classical neuron-centric view of neurodegeneration.

Neuroinflammation refers to a prolonged or dysregulated activation of the brain's immune system, primarily involving astrocytes and microglia. Unlike acute inflammation, which is necessary for host defence and tissue repair, chronic neuroinflammation is characterised by sustained cytokine production, unresolved oxidative stress, impaired phagocytosis in astrocytes and impaired synaptic stripping in microglia, as well as maladaptive metabolic changes within glial cells (Ransohoff, 2016), characteristic of glial activation. This persistent activation can exacerbate neuronal dysfunction by disrupting synaptic homeostasis, compromising trophic support and promoting excitotoxicity. Notably, neuroinflammation interacts with classical pathogenic processes, such as A β aggregation and α -syn deposition, which accelerates their spread, amplifies toxicity and shapes the selective neuronal vulnerability, including cortical neurons in AD and dopaminergic neurons within the substantia nigra in PD, respectively. Thus, inflammation is increasingly recognised not as a secondary by-product but as an enabling environment that both responds to and fuels neurodegenerative pathology.

Recent genetic discoveries further support this integrative model. Genome-wide association studies (GWAS) have identified numerous risk genes for neurodegenerative diseases that converge on immune pathways, glial function or lipid metabolism. For example, in AD, many of the strongest risk genes, including APOE, TREM2, CLU, CD33 and CR1, are mainly expressed in astrocytes and microglia (Lambert *et al.*, 2013; Kunkle *et al.*, 2019). These genes regulate processes such as cholesterol transport, phagocytosis, complement activation and inflammatory signalling, thus highlighting the importance of glial biology in shaping disease susceptibility. Similar patterns emerge in PD, where immune and lysosomal genes contribute to disease risk, and in ALS, where glial and immune pathways are

implicated in both sporadic and familial cases (Brown and Al-Chalabi, 2017; Billingsley *et al.*, 2018). Collectively, these findings indicate that genetic risk for neurodegeneration is strongly mediated through non-neuronal cells.

Astrocytes, in particular, have emerged in recent years as central regulators of the neuroinflammatory landscape. Traditionally viewed as supportive cells, astrocytes are now understood to play crucial roles in neurotransmitter recycling, immune signalling, potassium buffering, metabolic support, synaptogenesis and vascular regulation. Their strategic position between synapses, blood vessels and microglia places them at the intersection of neuronal and immune dynamics. When challenged by injury, pathogens or protein aggregates, astrocytes undergo a process termed “reactivity” or “reactive astrogliosis”, which involved complex and context-dependent transcriptional, morphological and metabolic changes (Pekny and Pekna, 2014; Escartin *et al.*, 2021). While reactive astrocytes can initially provide neuroprotection by isolating damage and releasing trophic factors, chronic reactivity can lead to maladaptive outcomes, including excessive cytokine release, impaired glutamate clearance, mitochondrial dysfunction and promotion of synaptic loss (Liddelow and Barres, 2017). The dual nature of astrocyte reactivity underscores the need to distinguish protective responses from those that are pathological.

The concept that neuroinflammation acts as a “Trojan Horse” in neurodegeneration is particularly relevant in this context. Like the mythological Trojan Horse that infiltrated Troy under the guise of a harmless offering, neuroinflammation can be covert in its early stages, subtle enough to evade detection while enabling the accumulation and propagation of damaging processes. Initially triggered by physiological immune surveillance or mild stress, it may appear adaptive or benign. However, once chronic inflammation is established, it silently undermines neuronal resilience, facilitates toxic protein spread, impairs metabolic homeostasis and diminishes synaptic integrity (Amor and Woodroffe, 2014). By the time neuronal death becomes clinically evident, the inflammatory machinery has already orchestrated widespread changes within the neural vicinity. Therefore, the Trojan Horse metaphor encapsulates the central premise of the present thesis; that

neuroinflammation is an underestimated and early mechanistic driver of neurodegeneration, embedded with the very processes it appears to follow.

Understanding neuroinflammation as an early and active participant in neurodegeneration also reframes therapeutic priorities. Historically, treatments have focused on removing protein aggregates or directly protecting neurons. Yet clinical trials targeting A β deposition in AD, for example, have produced limited cognitive benefits, suggesting that removing aggregates may not be sufficient once neuroinflammatory circuits are established (Long and Holtzman, 2019; Zhang *et al.*, 2024). In contrast, targeting glial pathways, whether by modulating cytokine signalling, regulating lipid metabolism, enhancing phagocytic capacity or promoting metabolic resilience, offers a promising avenue for altering disease trajectories before irreversible neuronal loss occurs. Indeed, the therapeutic landscape is gradually shifting toward glial-focused interventions, underscoring the need for mechanistic insight into astrocyte and microglial biology.

In this growing paradigm, human stem cell models provide an unprecedented opportunity to study neuroinflammation in a controlled, disease-relevant context. Traditional rodent models, although invaluable, often fail to recapitulate human-specific immune pathways, APOE biology, or astrocyte transcriptional profiles (Zhou *et al.*, 2020). Advances in human induced pluripotent stem cells (iPSC) have enabled the generation of patient-specific or genetically engineered astrocytes, microglia and neurons that retain human genetic architecture, and are able to express the specific gene of interest. These models allow the examination of glial-neuronal interactions, genetic risk factors and inflammatory signalling with a fidelity not achievable in animal systems. For diseases such as AD, where APOE genotype and A β interactions are particularly human-specific, iPSC-derived astrocytes represent a critical tool for understanding pathogenic mechanisms.

In summary, neurodegeneration is best understood as a dynamic interplay between neurons, glia, immune pathways and genetic factors. Neuroinflammation, once viewed as secondary, is now recognised as a principal contributor to disease initiation and progression. The metaphor of neuroinflammation as a Trojan Horse

captures the duality of this process being seemingly reactive yet deeply enabling of pathology. By investigating astrocyte biology, APOE genotype, regional identity and responses to pro-inflammatory factors and A β , this thesis aims to elucidate the early mechanisms by which neuroinflammation shapes neurodegeneration and to identify potential routes for therapeutic intervention.

1.2. Astrocyte Biology and Regional Heterogeneity

Astrocytes are the most abundant glial cell type in the human brain and perform a wide array of functions essential for neural circuit development, homeostasis, synaptic signalling and immune regulation. Once dismissed as passive support cells, astrocytes are now recognised as highly specialised and dynamic regulators of central nervous system (CNS) physiology. Their influence extends from neurotransmitter clearance and ion homeostasis to metabolic coupling, blood-brain barrier (BBB) maintenance and the modulation of inflammation. In the context of neurodegeneration and neuroinflammation, astrocytes play a central role in determining neuronal fate. Understanding astrocyte biology is therefore fundamental to deciphering the mechanisms that underlie neuroinflammatory states and their contribution to neurodegenerative disease.

1.2.1. Astrocyte Development and Regional Patterning

Astrocytes arise during CNS development from radial glia, which initially function as neural progenitor cells before transitioning into gliogenic lineages. This gliogenic switch is tightly regulated by temporal cues, transcription factors and extrinsic signals, including Notch, JAK/STAT and BMP pathways (Molofsky and Deneen, 2015). Regional identity is established early in development through morphogen gradients that pattern the neural tube along anterior-posterior and dorsal-ventral axes. Key morphogens include WNT, FGFs, BMPs and sonic hedgehog (SHH), which regulate the expression of transcriptional genes that specify forebrain, midbrain, hindbrain and spinal cord domains (Hochstim *et al.*, 2008; Schitine *et al.*, 2015; Markey *et al.*, 2023).

These early patterning mechanisms give rise to regionally distinct pools of neural progenitors defined by canonical transcription factors. For example, FOXG1 governs

cortical identity, while LMX1A, FOXA2 and EN1 mark ventral midbrain lineages associated with dopaminergic neuron development (Andersson, Sandberg and Lendahl, 2011). Astrocytes derived from these progenitors inherit aspects of the regional transcriptional profile, creating domain-specific molecular signatures that persist into adulthood. This early developmental imprinting contributes to the heterogeneity observed across astrocyte populations and shapes their functional specialisation within different neural circuits.

1.2.2. Functional Roles of Astrocytes in the Healthy Brain

Astrocytes support neuronal and synaptic function through an extensive repertoire of physiological activities, many of which are essential for maintaining CNS homeostasis. They play a central role in neurotransmitter clearance through high expression of the glutamate transporters, EAAT1 and EAAT2, with EAAT2 alone accounting for approximately 90% of glutamate uptake in the human CNS, thus preventing excitotoxicity and maintaining synaptic signalling (Danbolt, 2001). Astrocytes also regulate extracellular potassium and other ions via channels such as Kir.4, stabilising membrane excitability during periods of high neuronal activity. Their metabolic functions are also vital, with astrocytes providing neurons with energy substrates, e.g. lactate, and store the brain's only glycogen reserves, which can be mobilised during metabolic stress (Magistretti and Allaman, 2015). Structurally, astrocytic end-feet cover over 90% of CNS vasculature and contribute to the maintenance of BBB integrity by regulating blood flow and endothelial-pericyte communication (Abbott, Rönnbäck and Hansson, 2006). Additionally, astrocytes shape synaptic development and plasticity by secreting synaptogenic molecules (e.g. glypicans) crucial for formation, maturation and functioning of synapses, and by actively pruning synapses through phagocytic pathways (Chung *et al.*, 2013). Finally, astrocytes are key regulators of neuroimmune function, expressing pattern-recognition receptors, producing cytokines and chemokines and modulating microglial activation. Collectively, these functions position astrocytes as key integrators of neural stability and highlights their substantial contribution to the pathological transitions observed during neuroinflammatory states.

1.2.3. Evidence for Astrocyte Diversity and Regional Heterogeneity

Astrocytes exhibit significant heterogeneity in their morphology, gene expression, electrophysiological properties and functionality. This diversity is regionally patterned, reflecting developmental origins and local microenvironmental cues. Astrocytes in the cortex display a protoplasmic morphology with dense branching networks, whereas fibrous astrocytes in the white matter possess longer, less complex processes. However, these regional differences extend beyond classical grey vs white matter distinctions; hippocampal astrocytes, cerebellar Bergmann glia and striatal astrocytes each show distinct spatial architectures and domain organisation (Chai *et al.*, 2017).

The single-cell RNA sequencing (scRNA-seq) identified region-specific astrocyte subtypes with unique molecular signatures. Studies demonstrate distinct transcriptional profiles in cortical, hippocampal, midbrain and cerebral astrocytes, involving differences in neurotransmitter transporters, cytokine receptors, metabolic genes and ion channels. Some region-specific differences in gene expression include higher expression of EAAT1 in the cerebellum and high expression of the GABA transporter genes in the striatum, as well as differences in the expression of Kir4.1 between the cortex, the midbrain and the spinal cord (Batiuk *et al.*, 2020; Lozzi *et al.*, 2020), which further illustrates the depth of functional diversity across astrocyte populations.

Astrocytes originating from different brain regions also exhibit distinct metabolic profiles. For example, midbrain astrocytes have displayed enhanced antioxidant pathways and higher basal glutathione levels, likely reflecting the oxidative environment associated with dopaminergic neurotransmission, while cortical astrocytes prioritise synaptic regulation and glutamate cycling (Zhang *et al.*, 2014; Chai *et al.*, 2017; Morales *et al.*, 2017).

Emerging evidence also suggests that astrocytes from distinct brain regions respond differently to inflammatory stimuli. Cortical astrocytes exhibit robust Nuclear Factor kappa-light-chain enhancer of activated B cells (NFkB) activation and upregulation of cytokines such as IL-6 and CXCL10 following TNF α or IL-1 α

exposure. In contrast, ventral midbrain astrocytes display a more antioxidant-based reactive profile, which could potentially offer greater protection to dopaminergic neurons (Clarke *et al.*, 2021; Hasel *et al.*, 2021).

Together, these intrinsic regional differences will influence the susceptibility to neurodegenerative pathology, synaptic regulation, metabolic stress responses, reactive oxygen species (ROS) handling, cytokine release and neurotoxic and neuroprotective reactive phenotypes. Regional heterogeneity of astrocytes is therefore not only an anatomical distinction but a crucial determinant of disease mechanisms.

1.2.4. Implications of Astrocyte Heterogeneity for Neurodegeneration

Astrocyte heterogeneity has significant implications for understanding selective vulnerability in neurodegenerative diseases. Different CNS regions show stereotyped patterns of neurodegeneration. For example, the cortex and hippocampus are early targets in AD, whereas the ventral midbrain, particularly the substantia nigra pars compacta, is primarily affected in PD. These patterns reflect not only neuronal vulnerability, but also region-specific differences in astrocyte molecular profiles, stress responses and ability to maintain homeostasis (Matias, Morgado and Gomes, 2019; Zimmer, Orr and Orr, 2024).

In AD, the neocortex and hippocampus exhibit early and pronounced astrocyte reactivity, with transcriptomic and histopathological studies showing region-dependent astrocyte states that track the progression of A β and tau pathology (Grubman *et al.*, 2019; Mathys *et al.*, 2019; Habib *et al.*, 2020). Cortical astrocytes in AD are exposed to elevated levels of IL-1 α , TNF α and C1q released by microglia, which drive their neurotoxic reactive phenotypes characterised by impaired glutamate transport, altered synaptic support, mitochondrial dysfunction and reduced antioxidant capacity (Liddelow *et al.*, 2017; Zimmer, Orr and Orr, 2024). Genetic risk factors such as Apolipoprotein E ϵ 4 (APOE4) further bias astrocytes toward pro-inflammatory and metabolically compromised states even at baseline, thus amplifying cytokine production, complement activation and oxidative stress in

vulnerable cortical and hippocampal regions (Lin *et al.*, 2018; Fernandez *et al.*, 2019; Liu *et al.*, 2021; Arnaud *et al.*, 2022; Zimmer, Orr and Orr, 2024). These regionally and genetically tuned astrocyte responses likely contribute to the early involvement of cortical and hippocampal circuits in AD.

In PD, neurodegeneration is concentrated in dopaminergic neurons of the ventral midbrain, which operate in a highly oxidative environment due to dopamine metabolism and heavy mitochondrial load (Dias, Junn and Mouradian, 2013). Astrocytes in the substantia nigra play a crucial protective role by buffering oxidative stress and mitigating dopamine oxidation-induced toxicity. When these astrocyte antioxidant mechanisms are impaired, dopaminergic neuron vulnerability increases, thus accelerating degeneration (Kuter, Olech and Głowacka, 2018; Segura-Aguilar *et al.*, 2022). Although these astrocytic properties may initially protect dopaminergic neurons from ROS, overwhelming inflammatory or metabolic stress, for example driven by α -syn aggregation, genetic mutations or chronic neuroinflammation, can compromise astrocyte function. This leads to energy failure, oxidative damage and excitotoxicity, thus amplifying neurodegeneration in a region with limited neuronal redundancy (Booth, Hirst and Wade-Martins, 2017; Sonninen *et al.*, 2020).

In ALS, astrocytes in spinal cord and motor cortex undergo profound reactive changes and can become directly neurotoxic to motor neurons, driven by impaired glutamate clearance, disrupted metabolic support and the release of toxic, pro-inflammatory mediators (Gomes *et al.*, 2020; Provenzano *et al.*, 2023; Stoklund Dittlau and Van Den Bosch, 2023; Valori *et al.*, 2023). Similarly, in HD, astrocytes in the striatum exhibit disease-associated transcriptional states characterised by mitochondrial dysfunction, altered glutamate homeostasis and impaired metabolic support, all of which contribute to the selective vulnerability of medium spiny neurons and regionally-restricted neural circuit failure (Al-Dalahmah *et al.*, 2020; Paryani *et al.*, 2024).

Overall, regional astrocyte heterogeneity, shaped by developmental patterning, local neuronal circuitry and genetic background, emerges as a fundamental principle for explaining why specific CNS regions and neuronal populations are

preferentially affected across different neurodegenerative diseases (Matias, Morgado and Gomes, 2019; Zimmer, Orr and Orr, 2024).

1.3. Astrocyte Reactivity and Neuroinflammatory Pathways

Astrocyte reactivity is a fundamental component of the CNS response to injury, infection and neurodegenerative processes. Rather than representing a single uniform state, astrocyte reactivity encompasses a spectrum of transcriptional, morphological and functional changes including hypertrophy, upregulation of glial fibrillary acidic protein (GFAP) and vimentin, altered calcium dynamics and shifts in cytokine production (Escartin *et al.*, 2021). Modern transcriptomic studies demonstrate that reactive states are highly heterogeneous and context-dependent, challenging earlier binary classifications such as A1/A2 (Batiuk *et al.*, 2020; Clarke *et al.*, 2020).

A central driver of astrocyte reactivity is activation of inflammatory signalling pathways. The NFκB pathway plays a crucial role in integrating signals from cytokines such as IL-1α, toll-like receptors (TLRs) and damage-associated molecular patterns (DAMPs), which ultimately induce transcription of pro-inflammatory mediators including IL6, CXCL10 and CCL5 (Nelson *et al.*, 2004; Hayden and Ghosh, 2008; Liu *et al.*, 2017). NFκB activation results in nuclear translocation of p65/p60 complexes and widespread transcriptional rewiring that amplifies inflammatory signalling in astrocytes and other glia. In parallel, the JAK/STAT pathway, particularly STAT3, is crucial for astrocyte responses associated with glial scar formation, tissue repair and chronic inflammatory states (Herrmann *et al.*, 2008; Sofroniew, 2020). STAT3 activation downstream of the IL-6 family of cytokines can drive sustained transcriptional reprogramming in reactive astrocytes.

Astrocytes also mount type I interferon (IFN) responses, mediated by interferon-stimulated genes regulated through interferon regulatory factor (IRF) transcription factors. This antiviral-like response can be triggered by protein aggregates, mitochondrial stress or microglial cytokines and is increasingly recognised as a defining feature of disease-associated astrocyte states (Zamanian *et al.*, 2012; Clarke *et al.*, 2020). Another hallmark of reactivity is upregulation of components of

the complement cascade, particularly C3, which mediates microglial activation, synapse tagging and region-specific neuronal vulnerability (Stevens *et al.*, 2007; Hong *et al.*, 2016; Liddel *et al.*, 2017).

These signalling pathways have profound consequences for neuronal stability and circuit function. Reactive astrocytes exhibit impaired glutamate and potassium buffering, compromising synaptic transmission and predisposing neurons to excitotoxicity (Rossi *et al.*, 2008; Olsen *et al.*, 2015). They undergo metabolic reprogramming, often shifting toward glycolysis and reducing oxidative phosphorylation capacity, which diminishes their ability to support neuronal energy demands (Allaman, Bélanger and Magistretti, 2011; Bélanger, Allaman and Magistretti, 2011). Reactive astrocytes also influence blood–brain barrier integrity, modulate microglial activation and secrete neurotoxic or neuroprotective mediators depending on context (Haim and Rowitch, 2017; Sofroniew, 2020). Over time, these processes contribute to synaptic dysfunction, impaired plasticity and progressive neurodegeneration. A summary of the neuroinflammatory pathways and processes involving astrocyte reactivity can be found in Figure 1.3.1 below.

Together, these pathways illustrate that astrocyte reactivity is not a secondary or passive feature of CNS disease but an active and dynamic process that shapes disease onset, progression and regional susceptibility. Understanding the mechanisms governing these reactive states, particularly in human astrocytes, is essential for identifying therapeutic strategies to modulate neuroinflammation in neurodegenerative disorders.

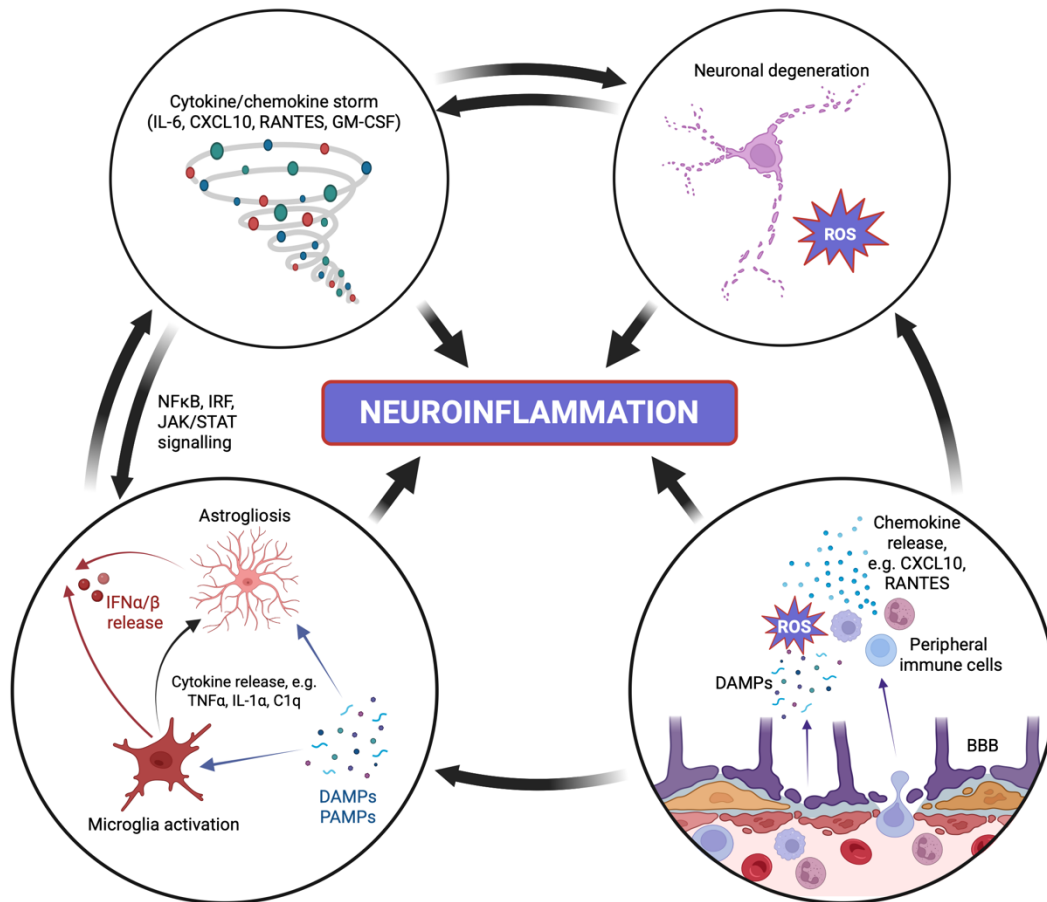


Figure 1.3.1 Vicious cycle of neuroinflammation in neurodegeneration.

Neuroinflammation is sustained by reciprocal interactions between neurons, glial cells, and peripheral immune components. BBB dysfunction promotes the recruitment of peripheral immune cells into the central nervous system, thus increasing ROS production and the release of chemokines and DAMPs. This release of inflammatory mediators activates resident microglia and astrocytes. Activated microglia release pro-inflammatory cytokines (e.g., TNFα, IL-1α, C1q), driving astrogliosis, the release of interferons (IFNα/β) and further inflammatory signalling. These processes are mediated through key inflammatory pathways, including NF-κB, IRF, and JAK/STAT signalling. Reactive glial cells amplify inflammation through the release of cytokines and chemokines, contributing to a cytokine/chemokine storm (e.g., IL-6, CXCL10, RANTES, GM-CSF). Together, these interconnected processes form a self-perpetuating feedback loop that exacerbates neuroinflammation and accelerates neuronal dysfunction and degeneration. Figure created in BioRender (<https://www.biorender.com>).

1.4. Alzheimer's Disease

Alzheimer's disease (AD) is a chronic progressive neurodegenerative disease that is characterised by significant memory loss (dementia), as well as cognitive and behavioural decline. Although the concept of dementia has been known for hundreds of years, AD was first documented as a disorder by a German psychiatrist and neuropathologist, Aloisius "Alöis" Alzheimer, in 1907 (Alzheimer, 1907; for the English translation of the article, use Stelzmann, Norman Schnitzlein and Reed Murtagh, 1995). However, in-depth research on the mechanisms involved in the pathogenesis of AD only began in the 1970s (Bondi, Edmonds and Salmon, 2017).

With AD as the leading cause of dementia worldwide, accounting for approximately 70% of cases (Prince *et al.*, 2015; Fiest *et al.*, 2016), it does not come as a surprise that the World Health Organisation (2012) called dementia "a public health priority" due to the ever-increasing numbers of diagnoses. Indeed, according to Alzheimer's Disease International, there were 46.8 million people living with dementia in 2015. This number is predicted to double every 20 years, reaching staggering numbers of 74.7 million by 2030 and 131.5 million by 2050 (Prince *et al.*, 2015). Since 2020, AD has been the seventh-leading cause of death, and for years now, it has also been the leading cause of disability and morbidity in older adults in the U.S. (Alzheimer's Association, 2022). With the constantly increasing life expectancy, this only further exacerbates the severity of the problem, and the burden AD has on society, healthcare and the economy worldwide.

The formation of amyloid β ($A\beta$) neuritic plaques extracellularly and tau neurofibrillary tangles (NFTs) intracellularly are thought to be the main hallmarks of AD. However, nowadays, neuroinflammation, which causes astrocyte and microglia activation, is also being recognised as a highly contributing factor to the pathogenesis of AD (Calsolaro and Edison, 2016; Lane, Hardy and Schott, 2018), as seen in Figure 1.4.1. below. Other hypotheses of AD pathophysiology also include the mitochondrial cascade and oxidative stress (Kumar and Singh, 2015). Together, these features of AD cause neurodegeneration resulting in synaptic and neuronal loss, as well as significant brain atrophy (Lane, Hardy and Schott, 2018). Memory

impairment is usually the first symptom of Alzheimer's disease (Hyman *et al.*, 1984), with many other symptoms, such as behavioural and cognitive decline, following as the disease progresses.

The majority of AD cases are sporadic, but <0.5% of cases are of the familial form of AD (fAD), which is genetic in origin. In the case of fAD, the onset of the disease usually occurs earlier, between 30-50 years old, than in the sporadic AD (Bateman *et al.*, 2011). Sporadic AD, also often referred to as late-onset AD (LOAD), is most likely caused by an interplay of genetic and environmental factors, with approximately 70% of the risk being attributed to genetics (Lane, Hardy and Schott, 2018).

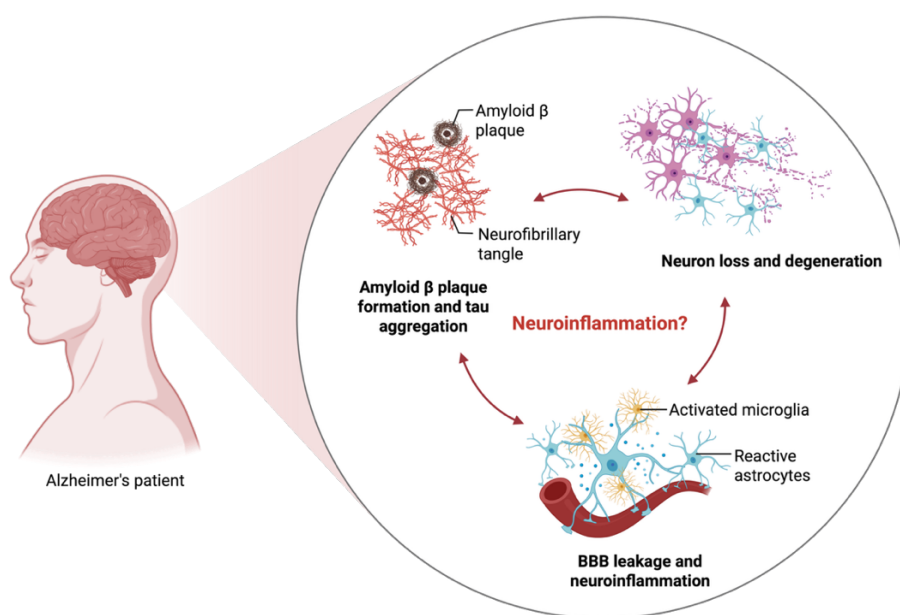


Figure 1.4.1 Interactions between classical Alzheimer's disease pathology and neuroinflammatory processes.

Alzheimer's disease is characterised by extracellular amyloid- β ($A\beta$) plaques and intracellular neurofibrillary tangles composed of hyperphosphorylated tau, which together initiate synaptic dysfunction and neuronal degeneration. These pathological features trigger activation of microglia and reactive transformation of astrocytes, promoting the release of pro-inflammatory cytokines, complement components and reactive oxygen species. Concurrent disruption of the blood-brain barrier (BBB) enhances inflammatory cell infiltration and metabolic stress. Neuroinflammation both responds to and accelerates $A\beta$ and tau pathology, forming a self-reinforcing loop that contributes to progressive neuronal loss. Illustration created in BioRender (<https://www.biorender.com>).

1.5. APOE in Astrocytes: Genetics, Function and Relevance to Alzheimer's Disease

APOE is the most influential genetic factor for LOAD, and its effects extend across multiple biological domains including lipid metabolism, neuroinflammation, synaptic stability and amyloid processing. In the healthy CNS, astrocytes are the primary producers of APOE, controlling the lipidation state and availability of APOE-containing lipoproteins. The strong association between APOE genotype and AD risk has led to a major focus on how APOE shapes astrocyte function and contributes to disease vulnerability. Understanding the interplay between APOE genotype, astrocyte biology and AD pathology is therefore essential for clarifying mechanisms of neurodegeneration and identifying therapeutic targets.

1.5.1. APOE Genetics and Isoforms

Human APOE exists in three common isoforms, APOE2, APOE3 and APOE4, that differ by single amino acid substitutions at positions 112 and 158. These small structural differences produce profound changes in protein conformation, receptor binding properties and lipidation efficiency (Mahley and Huang, 2012). APOE3 is the most prevalent isoform and is considered the “neutral” allele. APOE2 confers relative protection against AD, whereas APOE4 is associated with a dose-dependent increase in risk. Individuals carrying one APOE4 allele have approximately a 3-fold increased risk, while those with two alleles have up to a 12-fold increased risk of developing AD. APOE4 also accelerates the age of onset and exacerbates amyloid deposition, tau pathology and neuroinflammatory responses (Holtzman, Herz and Bu, 2012; Liu *et al.*, 2013). These genotype-dependent effects highlight APOE as a major determinant of selective vulnerability in neurodegenerative disease.

1.5.2. APOE Function in the Healthy Brain

In the CNS, APOE is central to lipid and cholesterol homeostasis. Astrocytes synthesise cholesterol and package it into APOE-containing lipoproteins, which are delivered to neurons through receptors such as LDLR and LRP1. These lipid

particles are crucial for synaptogenesis, membrane repair and dendritic spine maintenance, particularly during development and periods of heightened synaptic activity (Holtzman, Herz and Bu, 2012; Liu *et al.*, 2013). APOE also influences synaptic plasticity by regulating lipid composition at synaptic membranes and supporting receptor trafficking and spine stability (Bu, 2009).

A critical role of APOE in AD relates to its involvement in A β metabolism. APOE binds A β and facilitates its clearance through receptor-mediated uptake, proteolytic degradation, and glymphatic drainage. APOE isoforms differ in their efficiency, with APOE4 demonstrating markedly impaired lipidation and reduced capacity to promote A β clearance compared with APOE2 and APOE3 (Castellano *et al.*, 2011). APOE also participates in immune regulation by modulating toll-like receptor signalling, NF κ B activation thresholds and cytokine release (Maezawa *et al.*, 2006; Zhang, Wu and Wu, 2011; Fernández-Calle *et al.*, 2022), thus underscoring its role as a key integrator of metabolic and inflammatory pathways in the CNS.

1.5.3. APOE4-Dependent Astrocyte Dysfunction

A growing body of evidence shows that APOE4 enhances inflammatory signalling in human astrocytes. Human iPSC-derived astrocytes expressing APOE4 adopt a more pro-inflammatory state than APOE3 cells, characterised by the activation of NF κ B signalling pathways and the upregulation of inflammatory mediators (Arnaud *et al.*, 2022). This shift in transcriptional state increases the sensitivity of astrocytes to inflammatory cues and primes them to amplify neuroinflammatory responses. More broadly, reactive astrocytes upregulate cytokines such as IL-6 and display altered immune signalling and complement activity, processes that contribute to synaptic dysfunction and neuronal stress in neurodegenerative disease (Price, Johnson and Norris, 2021). Although the direct link between APOE4 and specific chemokines or complement proteins remains incompletely defined in human astrocytes, current evidence indicates that APOE4 lowers the threshold for inflammatory activation and promotes a cellular environment that favours chronic neuroinflammation.

APOE4 also induces metabolic and mitochondrial deficits, characterised by reduced oxidative phosphorylation, increased glycolysis, impaired NRF2 antioxidant responses and elevated oxidative stress (Lin *et al.*, 2018). These alterations weaken the astrocyte's ability to support neuronal energy demands and maintain redox homeostasis. Finally, APOE4 impairs A β clearance by reducing receptor-mediated uptake, slowing lysosomal degradation and limiting the ability of astrocytes and microglia to remove extracellular A β (Castellano *et al.*, 2011). Together, these dysfunctions produce a metabolically stressed, pro-inflammatory and less supportive astrocyte phenotype that contributes directly to AD pathogenesis.

1.5.4. APOE Expression and Plasticity in Disease

Although astrocytes are the predominant source of APOE in the healthy brain, APOE expression becomes more widely distributed across cell types in disease contexts. Microglia upregulate APOE as part of the TREM2–APOE pathway, a transcriptional program associated with dysfunctional, disease-associated microglia in multiple neurodegenerative disorders (Krasemann *et al.*, 2017). Oligodendrocytes also express APOE, with single-cell transcriptomic studies demonstrating APOE upregulation in oligodendrocyte populations in demyelinating diseases such as multiple sclerosis (Zhang *et al.*, 2014; Jäkel *et al.*, 2019). This dynamic, cell-type-specific regulation underscores APOE's role as a shared regulator of glial and neuronal stress responses and its involvement in coordinating inflammatory and metabolic adaptations during neurodegeneration.

1.5.5. APOE and A β : Convergent Pathways

APOE and A β intersect at multiple points in the AD pathogenic cascade. APOE4 not only impairs A β clearance but also promotes A β aggregation and increases neuronal susceptibility to A β toxicity (Castellano *et al.*, 2011; Liu *et al.*, 2013). Because APOE4 astrocytes display heightened inflammatory and oxidative responses, A β exposure drives stronger cytokine release, mitochondrial dysfunction and metabolic stress in APOE4-expressing cells than in APOE2 or APOE3 astrocytes. These interactions create a pathological feedback loop where

impaired clearance leads to greater A β accumulation, which further activates glia, thus driving inflammation and causing synaptic damage.

1.5.6. APOE Genotype Effects in Human iPSC-derived Astrocytes

Human iPSC-derived astrocytes provide a powerful platform for investigating APOE genotype effects with human specificity. Isogenic APOE2/2, APOE3/3 and APOE4/4 iPSC lines allow controlled comparisons without inter-individual genetic variability. Studies using these models demonstrate that APOE4 astrocytes exhibit reduced cholesterol secretion and impaired APOE lipidation (Tcw *et al.*, 2017), reduced glutamate uptake and heightened secretion of IL-8, TIMP-2 and CCL5 (Jones *et al.*, 2017), increased oxidative stress, mitochondrial fragmentation and exaggerated NF κ B activation (Lin *et al.*, 2018). In co-culture systems, APOE4 astrocytes provide less metabolic and trophic support to neurons, leading to reduced synaptic activity and increased vulnerability to stress (Fleeman *et al.*, 2023). In contrast, APOE2 astrocytes display enhanced lipidation, anti-inflammatory transcriptional signatures and improved neurotrophic functions, aligning with their protective association in human genetics (Li *et al.*, 2020; Brookhouser *et al.*, 2021; de Leeuw *et al.*, 2022).

Overall, the APOE genotype profoundly shapes astrocyte biology, influencing lipid metabolism, inflammatory reactivity, glutamate homeostasis and metabolic resilience. APOE4 drives a shift toward a pro-inflammatory, metabolically compromised state that reduces astrocytic support for neurons and amplifies A β pathology. APOE2, in contrast, confers resilience by enhancing lipidation and dampening inflammatory responses.

1.6. β -amyloid and Astrocyte Interactions

A β accumulation is a defining pathological hallmark of AD and one of the earliest detectable biochemical alterations in the disease process. Although historically viewed primarily as a direct toxin to neurons, it is now clear that A β exerts profound effects on glial cells, including astrocytes. Astrocytes interact with A β at multiple levels: they contribute to its clearance, respond to its presence through inflammatory, oxidative and metabolic pathways, and can amplify downstream

neurotoxicity (Allaman, Bélanger and Magistretti, 2011; Ries and Sastre, 2016; Singh *et al.*, 2024). These effects are modulated by factors such as APOE genotype, developmental origin, inflammatory environment and metabolic state. Understanding how astrocytes detect, internalise and respond to A β is therefore essential for interpreting early pathogenic events in AD.

1.6.1. A β Production, Aggregation and Toxic Species

A β peptides are generated through sequential cleavage of the amyloid precursor protein (APP) by β -secretase (BACE1) and the γ -secretase complex. This process yields several A β species, among which A β_{1-40} and A β_{1-42} are the most abundant. A β_{1-40} is produced in larger quantities, while A β_{1-42} is more hydrophobic and more prone to aggregation, forming the core of amyloid plaques (Selkoe and Hardy, 2016). A β aggregation occurs along a continuum from monomers to soluble oligomers, protofibrils, fibrils and eventually plaques. Among these, soluble oligomers are widely regarded as the most synaptotoxic species and are strongly associated with impairments in synaptic plasticity and neuronal signalling (Benilova, Karran and De Strooper, 2012). These oligomeric forms can also activate glial cells, including astrocytes, long before plaque deposition becomes evident.

1.6.2. Functional Differences Between A β_{1-40} and A β_{1-42}

A β_{1-40} and A β_{1-42} differ significantly in their biophysical and biological properties, which affects their distribution and pathogenic roles in AD. A β_{1-40} is more soluble and is strongly associated with cerebral amyloid angiopathy, while A β_{1-42} is more aggregation-prone and enriched within neuritic plaques in AD brain tissue (Selkoe and Hardy, 2016). Consistent with these biochemical differences, plaque-level analyses of human AD tissue reveal distinct A β_{1-42} -rich plaque cores and differential plaque morphologies compared with deposits containing higher proportions of A β_{1-40} (Enzlein *et al.*, 2024).

Functionally, A β_{1-42} exhibits greater neurotoxicity than A β_{1-40} , with numerous *in vitro* studies demonstrating enhanced synaptic dysfunction, calcium dysregulation, and neuronal loss following exposure to A β_{1-42} oligomers or fibrils (Narayan *et al.*, 2014; Aguirre-Rueda *et al.*, 2015). A β_{1-42} is also a more potent inducer of glial activation,

eliciting stronger astrocytic and microglial inflammatory and transcriptional responses than A β ₁₋₄₀ in primary culture systems (Zhu *et al.*, 2022). Nevertheless, both isoforms contribute to AD pathology, and many in vitro studies employ A β ₁₋₄₀ and A β ₁₋₄₂, often including a range of different aggregation states, to characterise astrocyte reactivity and glial-mediated mechanisms of neurodegeneration (Narayan *et al.*, 2014; Matuszyk *et al.*, 2022).

1.6.3. Astrocyte Responses to A β Exposure

Astrocytes respond dynamically to A β , with early responses often being protective while chronic exposure eventually leads to dysfunction. Astrocytes internalise A β via receptors such as LRP1 and scavenger receptors and can degrade it through lysosomal pathways. However, prolonged A β exposure leads to intracellular accumulation, lysosomal impairment and oxidative stress, as well as impaired degradation capacity (Söllvander *et al.*, 2016). Soluble A β oligomers activate pattern-recognition receptors such as TLR4 and Receptor for Advanced Glycation End-products (RAGE), triggering NF κ B signalling and promoting secretion of cytokines including IL-1 β , IL-6, TNF α (Johnstone, Gearing and Miller, 1999; White *et al.*, 2005; LaRocca *et al.*, 2021). These mediators contribute to microglial recruitment, synaptic dysfunction and neuronal vulnerability.

A β exposure can also induce complement activation in astrocytes. Increased expression of complement component C3 is a hallmark of the neurotoxic reactive astrocyte phenotype described in AD and other neuroinflammatory conditions (Liddelow *et al.*, 2017). Complement activation contributes to synapse loss and amplifies inflammatory cascades. A β additionally induces oxidative and metabolic stress in astrocytes, resulting in increased ROS production, reduced mitochondrial membrane potential and impaired adenosine triphosphate (ATP) generation (Abramov, Canevari and Duchon, 2004; Sarkar *et al.*, 2014). These metabolic disturbances overwhelm astrocytic antioxidant defences, including glutathione-dependent pathways. In rodent studies, chronic A β exposure can also reduce astrocytic expression or surface localisation of GLT-1/EAAT2, impairing glutamate uptake and increasing excitotoxicity risk (Matos *et al.*, 2008; Scimemi *et al.*, 2013).

However, the evidence for this mechanism in humans, including iPSC-derived astrocytes, is much more limited and requires more in-depth investigation.

Although A β induces inflammatory and oxidative responses, multiple studies show that A β alone does not fully recapitulate astrocyte reactivity observed in AD brain tissue. Instead, microglia-derived cytokines, particularly IL-1 α , TNF α and C1q, act synergistically with A β to induce robust reactive phenotypes (Ferrari-Souza *et al.*, 2025). This complementary signalling reflects *in vivo* complexity that astrocytes rarely encounter A β without accompanying inflammatory cues.

1.6.4. The Interplay Between A β , APOE and Neuroinflammation

The APOE genotype profoundly shapes astrocyte responses to A β . APOE4 enhances A β aggregation, reduces clearance efficiency and increases inflammatory activation of astrocytes and microglia (Castellano *et al.*, 2011; Yamazaki *et al.*, 2019). Human iPSC-derived APOE4 astrocytes accumulate intracellular A β more readily and exhibit impaired lysosomal degradation, exaggerated cytokine responses and increased oxidative stress, in contrast to APOE2 and APOE3 astrocytes, which show more efficient A β uptake and degradation (Tcw *et al.*, 2017). These genotype-dependent differences contribute to the markedly increased AD risk in APOE4 carriers.

1.7. Therapeutical Modulation of Neuroinflammation in Neurodegenerative Diseases

Despite the growing recognition that neuroinflammation plays a decisive role in Alzheimer's disease progression, current therapeutics have only modest effects on glial-driven inflammatory signalling. This has led to increasing interest in the endocannabinoid system, a neuromodulatory network with broad influence over immune responses, synaptic activity and cellular metabolism. Cannabinoids, including CBD, THC and various receptor-specific modulators, engage multiple molecular pathways in astrocytes and microglia, offering a mechanistically diverse approach to regulating inflammation. As such, cannabinoid-based interventions

represent a promising yet underexplored therapeutic avenue, particularly in the context of APOE- and A β -related astrocyte dysfunction.

1.7.1. Current Therapeutic Approaches for Alzheimer's Disease

Pharmacological treatments for AD are still largely symptomatic. Cholinesterase inhibitors, such as donepezil, rivastigmine and galantamine, are widely used in mild to moderate AD and work by prolonging the action of acetylcholine at synapses (Birks, 2006; Birks, Chong and Grimley Evans, 2015). Memantine, an NMDA receptor antagonist, is prescribed in moderate to severe AD to reduce glutamate-mediated excitotoxicity (Reisberg *et al.*, 2003).

More recently, anti-amyloid monoclonal antibodies have introduced the first disease-modifying therapeutic class. Aducanumab was granted accelerated FDA approval based on amyloid plaque reduction, although clinical benefit remains debated (Alexander *et al.*, 2021; Planche and Villain, 2021). Two newer antibodies, lecanemab and donanemab, have shown statistically significant slowing of cognitive and functional decline in early AD (Mintun *et al.*, 2021; Sims *et al.*, 2023; van Dyck *et al.*, 2023). However, treatment is complicated by amyloid-related imaging abnormalities (ARIA), extreme cost of therapeutic research and development, and limited long-term data.

These limitations have shifted attention toward non-amyloid targets, including tau-directed therapies, synaptic stabilisers, metabolic modulators and immunomodulatory strategies aimed at microglia and astrocytes (Alexander *et al.*, 2021). As neuroinflammation is increasingly recognised as a major driver of disease progression, therapeutic modulation of glial responses has become a central research focus.

1.7.2. Therapeutic Targeting of Neuroinflammation in Neurodegenerative Diseases

Neuroinflammation is now recognised as a major contributor to the onset and progression of neurodegenerative diseases, including AD, PD and ALS. As a result, multiple therapeutic strategies have been developed to modulate inflammatory

signalling, glial activation and immune-mediated synaptic dysfunction. Early efforts focused on broad anti-inflammatory agents such as non-steroidal anti-inflammatory drugs (NSAIDs). Although observational studies initially suggested a protective effect, large randomised clinical trials of naproxen, celecoxib and related compounds failed to demonstrate cognitive benefit in AD and sometimes increased adverse events, indicating that broad cyclooxygenase inhibition is insufficient or poorly timed as a disease-modifying approach (Kiraly, Foss and Giordano, 2023; Melchiorri *et al.*, 2023). More recent therapeutic efforts have shifted toward targeting specific immune pathways implicated in AD pathology. One major focus is the NLRP3 inflammasome, a cytosolic sensor activated by A β , tau and mitochondrial damage. Preclinical studies show that NLRP3 inhibition reduces A β deposition, improves synaptic function and attenuates microgliosis, and early-phase clinical trials in other inflammatory conditions have demonstrated good tolerability of selective inhibitors such as dapansutrile (OLT1177) (Barczuk *et al.*, 2022; Kiraly, Foss and Giordano, 2023). Dapansutrile has now progressed to phase 2 testing in PD, marking one of the first applications of NLRP3 inhibition in human neurodegeneration, although AD trials have not yet begun.

Microglia-directed therapies represent another major therapeutic avenue. TREM2 agonist antibodies, such as AL002, aim to enhance microglial activation states associated with A β clearance and synaptic protection. Preclinical studies demonstrated improved microglial response and reduced pathology, and phase 1b trials confirmed pharmacodynamic activity in humans. However, the subsequent phase 2 INVOKE-2 trial did not achieve its primary clinical endpoint in early AD, illustrating the complexity of translating microglial modulation into functional outcomes (Kiraly, Foss and Giordano, 2023; van Dyck *et al.*, 2023). Additional microglial regulators, including CSF1R inhibitors and Bruton's tyrosine kinase (BTK) inhibitors, are under investigation, though most remain at preclinical or early-phase stages and are not yet applied directly to AD. Complement pathway inhibition has also gained traction given the pivotal role of C1q- and C3-mediated synapse loss in AD. Several complement inhibitors, including anti-C1q (ANX005) and C3 inhibitors, have entered clinical development in neurodegenerative and neuroinflammatory

diseases. Safety of long-term complement blockade is already established in peripheral disorders such as paroxysmal nocturnal haemoglobinuria and cold agglutinin disease, but CNS-specific efficacy remains to be demonstrated (Zelek and Tenner, 2025).

Collectively, these approaches illustrate both the promise and the challenges of targeting neuroinflammation. While glial pathways can be modulated, no anti-inflammatory agent has yet produced consistent disease-modifying benefit in AD. Current evidence suggests that therapeutic success may require precision targeting of specific pathways (e.g., NLRP3, TREM2, complement), appropriate disease staging and combination treatments. In parallel, neuroinflammatory pathways are increasingly being explored as sources of fluid biomarkers for patient stratification and therapeutic monitoring. Biomarkers associated with astrocyte and microglial activation, including GFAP, YKL-40 and soluble TREM2, show promise as indicators of glial reactivity, disease progression and treatment response in AD and related neurodegenerative disorders (Falcon *et al.*, 2019; Pereira *et al.*, 2021; Lista *et al.*, 2024). This growing landscape underscores the need for additional mechanistic work in human glial models and provides rationale for evaluating cannabinoid-based and receptor-specific immunomodulatory compounds, such as CBD, THC, PPAR γ agonists and antagonists and NLRP3 inhibitors, as explored in later chapters of this thesis.

1.7.3. Cannabinoids and Neuroinflammation

The endocannabinoid system (ECS) includes endogenous ligands (anandamide, 2-AG), metabolic enzymes and cannabinoid receptors CB $_1$ and CB $_2$. CB $_1$ is one of the most abundant G protein-coupled receptors (GPCRs) in the CNS. It is predominantly neuronal but also present on astrocytes, where it modulates intracellular Ca $^{2+}$ and gliotransmission (Navarrete and Araque, 2008). CB $_2$, although expressed at low levels in the healthy CNS, is strongly upregulated in activated microglia in response to neuroinflammatory stimuli, including A β accumulation, pro-inflammatory cytokines, neuronal injury and oxidative stress, where it can shift microglia toward anti-inflammatory states (Atwood and Mackie, 2010; Stella, 2010; Cassano *et al.*, 2017). Cannabinoids, particularly phytocannabinoids, also interact

with PPAR γ , TRPV1, 5-HT1A and GPR55, offering multiple routes for modulating glial signalling (Pertwee, 2008; Iannotti *et al.*, 2014).

CBD has minimal psychoactive properties and weak affinity for CB $_1$ /CB $_2$, but influences inflammation and oxidative stress through alternative pathways. In A β -treated mouse models, CBD reduces microglial and astrocyte activation, decreases pro-inflammatory cytokines and enhances neurogenesis (Esposito *et al.*, 2006, 2007, 2011). These effects are PPAR γ -dependent, as the addition of the PPAR γ antagonist GW9662 severely diminishes CBD's anti-inflammatory actions (Esposito *et al.*, 2011). CBD additionally suppresses NF κ B activation, reduces IL-1 β , IL-6 and TNF α , and limits ROS while supporting mitochondrial function (Campos *et al.*, 2016). Through TRPV1 and other ion channels, CBD modulates Ca $^{2+}$ homeostasis and regulates mitochondrial dynamics (Iannotti *et al.*, 2014).

THC, a partial agonist of CB $_1$ and CB $_2$, reduces presynaptic neurotransmitter release through CB $_1$ and modulates astrocytic Ca $^{2+}$ waves (Navarrete and Araque, 2008)(Covelo *et al.*, 2021). CB $_2$ activation by THC or CB $_2$ -selective agonists suppresses microglial activation, lowers cytokine production and reduces oxidative stress (Ehrhart *et al.*, 2005). In AD and inflammatory models, THC reduces A β -induced neurotoxicity and inflammatory signalling (Ramírez *et al.*, 2005). However, high doses may produce opposite, potentially pro-inflammatory effects, reflecting a narrow therapeutic window.

CBD can act as a negative allosteric modulator of CB $_1$, reducing THC-induced psycho-activity (Laprairie *et al.*, 2015). However, co-administration of CBD and THC may produce complementary anti-inflammatory actions through CB $_2$, PPAR γ and NF κ B pathways (Cui Sun *et al.*, 2025).

PPAR γ agonists such as pioglitazone hydrochloride reduce pro-inflammatory signalling, inhibit NF κ B and enhance mitochondrial function in AD models (Nicolakakis *et al.*, 2008). T0070907, a PPAR γ antagonist, blocks PPAR γ -dependent signalling and is widely used to validate PPAR γ -mediated mechanisms in astrocytes and microglia (Ji *et al.*, 2018).

CB₁ receptor antagonism provides a crucial tool for dissecting cannabinoid signalling in neuroinflammation. SR141716 (rimonabant) is a selective CB₁ antagonist and inverse agonist that increases intracellular cAMP and enhances excitatory transmitter release by blocking constitutive CB₁ signalling (Sim-Selley, 2003; Pertwee, 2005; Bisogno and Maccarrone, 2014). Because CB₁ is Gi/o-coupled, receptor activation normally suppresses adenylate cyclase activity and limits cAMP production. In contrast, CB₁ antagonism elevates cAMP levels, promoting protein kinase A (PKA) and CREB signalling, pathways that interact closely with inflammatory transcriptional factors including NFκB (Kendall and Yudowski, 2017). Cannabinoid-mediated modulation of CREB and NFκB signalling is thought to contribute to the suppression of pro-inflammatory cytokine expression and oxidative stress responses in glial cells (Chiurchiù, Leuti and Maccarrone, 2015; Chiurchiù, 2016). Consistent with this, CB₁ activity in glial cells generally dampens inflammatory responses through suppression of NFκB and MAPK pathways. Therefore, blocking CB₁ with SR141716 can shift astrocytes and microglia toward a more pro-inflammatory state, increasing cytokine production and reducing the anti-inflammatory efficacy of cannabinoids (Chiurchiù, Leuti and Maccarrone, 2015; Chiurchiù, 2016; Kiraly, Foss and Giordano, 2023). Experimental studies show that SR141716 reliably abolishes CB₁-dependent anti-inflammatory effects of THC and can partially block broader cannabinoid responses involving Ca²⁺ regulation and oxidative signalling (Pertwee, 2005; Chiurchiù, Leuti and Maccarrone, 2015).

The NLRP3 inflammasome is a critical regulator of IL-1β and IL-18 maturation. MCC950 selectively inhibits NLRP3 ATPase activity and prevents inflammasome formation (Coll *et al.*, 2015). In Aβ-driven models, MCC950 reduces IL-1β release, lowers amyloid burden, limits microglial activation and improves cognition (Heneka *et al.*, 2013; Dempsey *et al.*, 2017).

1.8. Modelling Alzheimer's Disease

Modelling AD remains one of the greatest challenges in neurobiology, owing to the disorder's multifactorial nature and the profound species-specific differences in brain structure, immune signalling and amyloid biology. Traditional approaches

have been invaluable for uncovering broad pathological principles, but fail to reproduce key aspects of human disease, particularly those involving astrocyte function, A β aggregation, APOE genotype effects and neuroinflammatory responses. Human post-mortem tissue provides crucial pathological insight, but represents end-stage disease and cannot reveal early mechanistic events or permit controlled experimental manipulation. Emerging human-based models such as cerebral organoids, chimeric mouse–human systems and primary human astrocytes offer further translational relevance, but remain limited by variability, accessibility or incomplete maturation. In this context, human iPSC-derived astrocytes have become a powerful and versatile platform, integrating human genetic background, developmental patterning and controlled microenvironmental cues. They uniquely allow the study of regional identity, APOE genotype and amyloid stress within a human-specific, experimentally tractable system, providing a critical bridge between other cell models and the complexity of the human brain.

1.8.1. Most Common Models of Alzheimer's Disease

A wide range of experimental systems have been used to model AD, each offering distinct advantages but also their own limitations. AD research has relied heavily on transgenic mouse models, such as APP/PS1, 5xFAD or TauP301S mice, which recapitulate selected aspects of AD pathology, including amyloid deposition or tau aggregation. However, these models diverge substantially from human disease. Mice rarely exhibit the degree of neuronal loss observed in humans, and amyloid plaque burden does not reliably correlate with cognitive impairment (Drummond and Wisniewski, 2017). Furthermore, mouse A β differs from human A β by three amino acids, reducing its aggregation propensity and limiting its ability to replicate human oligomer toxicity (Benilova, Karran and De Strooper, 2012). Astrocytes and microglia also differ between species at morphological and molecular levels, with human astrocytes being larger, more complex and exhibiting distinct transcriptional signatures, including approximately 2,700 human-enriched genes (Zhang *et al.*, 2016). These differences restrict the translational fidelity of rodent models, particularly in terms of modelling inflammatory and metabolic pathways central to AD.

Other *in vivo* models, including non-human primates, better reproduce human amyloid biology, but are constrained by cost, ethical limitations and lack of available genetic manipulation. Canine cognitive dysfunction offers a naturally occurring model with amyloid deposition, but lacks robust mechanistic tools (Drummond and Wisniewski, 2017).

Given these limitations, human-derived models provide critical opportunities to study AD-relevant processes. Primary human astrocytes offer high biological relevance, but access is limited, samples often come from foetal or peri-operative tissue rather than aged brain, and genetic backgrounds cannot be controlled. Human post-mortem tissue provides invaluable insight into late-stage AD, but cannot reveal the temporal sequence leading to pathology or allow experimental manipulation of genotype or inflammatory conditions (Olah *et al.*, 2018).

Human cerebral organoids provide a 3D multicellular environment, including developing astrocytes, neurons and sometimes microglia. Organoids show spontaneous amyloid accumulation when derived from familial AD iPSC lines (Gonzalez *et al.*, 2018), but limitations in maturation, hypoxic cores, and batch variability constrain their utility for quantitative mechanistic studies. Chimeric mouse–human models, generated by transplanting human glial progenitors into neonatal mouse brains, demonstrate that human astrocytes adopt more complex morphologies and confer enhanced synaptic plasticity compared with mouse astrocytes (Han *et al.*, 2013). However, such chimeras still rely on a rodent microenvironment, limiting their ability to model human-specific inflammatory interactions.

Against this backdrop, human iPSC-derived astrocytes offer a uniquely powerful system. They combine human genetic identity, experimental tractability, and compatibility with high-resolution assays while allowing controlled manipulation of disease-associated genotypes such as APOE4.

1.8.2. Why Use Human iPSC-derived Astrocytes?

Human astrocytes differ from rodent astrocytes in size, complexity, synaptic coverage and immune signalling. They exhibit distinct transcriptional programmes,

including thousands of human-enriched genes related to inflammation, metabolism and synaptic regulation, and show stronger cytokine responses than their rodent counterparts (Zhang *et al.*, 2016; Barbar *et al.*, 2020; Habib *et al.*, 2020; Escartin *et al.*, 2021). These species differences are especially important for modelling AD, where processes such as complement activation, APOE signalling and A β toxicity diverge significantly between rodents and humans. Moreover, human A β oligomers show far greater aggregation propensity and neurotoxicity than rodent A β , making human cells essential for modelling amyloid pathology (Benilova, Karran and De Strooper, 2012).

iPSC-derived astrocytes offer additional advantages. Their human-specific transcriptomes and immune pathways enable more accurate modelling of neuroinflammatory responses. CRISPR-Cas9-generated isogenic APOE2/2, APOE3/3 and APOE4/4 iPSC lines allow direct comparison of genotype effects without confounding genetic background (Zhang *et al.*, 2017). These models have revealed APOE4-specific reductions in cholesterol efflux, impaired A β clearance and increased inflammatory signalling (Tcw *et al.*, 2017), demonstrating the power of iPSC technology for dissecting genotype-dependent mechanisms. Developmental control from pluripotent stem cells to neural progenitors and astrocytes makes it possible to examine stage-specific phenotypes, and the resulting astrocytes reliably respond to pro-inflammatory microglial factors such as IL-1 α and TNF α , as well as A β , and multiple other stimuli, in ways that parallel observations from human tissue (Barbar *et al.*, 2020; Leng *et al.*, 2021).

These cells are also highly compatible with a broad range of mechanistic assays, including qPCR, ELISA, immunocytochemistry and Seahorse extracellular flux analysis, thus enabling detailed and reproducible investigation of metabolic and inflammatory pathways relevant to AD.

1.8.3. Differentiation of iPSCs into Cortical and Ventral Midbrain Astrocytes

Astrocyte differentiation from iPSCs mirrors human neurodevelopment where pluripotent cells undergo neural induction, produce neural progenitor cells (NPCs)

and then differentiate into astrocytes over ~60–90 days, as already discussed in Section 1.2.1. Importantly, NPCs can be regionally patterned, allowing generation of astrocytes with cortical or ventral midbrain identities.

Cortical astrocyte differentiation relies on dual-SMAD inhibition (e.g., SB431542 and LDN-193189) and WNT modulation, producing NPCs expressing PAX6, FOXG1 and EMX1 (Chambers *et al.*, 2009, 2012; Bradley *et al.*, 2019). These NPCs are then further differentiated into astrocyte progenitors using various protocols, such as Serio *et al.* (2013). The resulting astrocytes express canonical markers such as S100 β , GFAP and EAAT1, show characteristic morphology and mount robust inflammatory responses via NF κ B and JAK/STAT pathways. This makes them well suited for modelling A β toxicity and APOE genotype effects, as well as investigating potential anti-inflammatory therapeutics capable of reversing/inhibiting astrocyte reactivity.

Ventral midbrain astrocytes are generated using SB431542, LDN-193189, SHH, CHIR, FGF8, BDNF and ascorbic acid, producing progenitors expressing midbrain markers including LMX1A, FOXA2 and EN1 (Nolbrant *et al.*, 2017). Astrocytes from this lineage retain features relevant to dopaminergic circuitry, enhanced antioxidant capacity, distinct metabolic signatures and region-specific inflammatory responses, thus supporting direct comparison of intrinsic susceptibilities across brain regions.

iPSC-derived astrocytes express canonical astrocyte markers (GFAP, S100 β , ALDH1L1, EAAT1) and exhibit functional characteristics including glutamate uptake, cytokine secretion (e.g., IL-6, CXCL10), calcium signalling and metabolic activity. They mount robust responses to inflammatory stimuli such as TNF α , IL-17A and TIC mixtures, paralleling human neuroinflammatory responses (Tcw *et al.*, 2017; Bradley *et al.*, 2019; MacMahon Copas *et al.*, 2024; McComish *et al.*, 2024). These regionally patterned astrocytes retain intrinsic transcriptional and functional differences, making them ideal for examining regional vulnerability to inflammation, as reported in Chapter 3.

Despite their strengths, iPSC-derived astrocytes also present challenges. They resemble foetal or early postnatal astrocytes, although extended differentiation, established protocols or neuronal co-culture improves maturation. However, variability across differentiation protocols can influence inflammatory and metabolic profiles. Astrocyte-only cultures lack microglia and thus cannot fully model reciprocal cytokine signalling or A1-like activation states, though TIC stimulation provides a useful approximation. Finally, 2D cultures do not recapitulate age-related changes or the long-term dynamics characteristic of AD.

1.9. Knowledge Gaps

Despite major advances in understanding astrocyte biology, neuroinflammation and AD pathogenesis, several crucial gaps remain in our knowledge of how regional identity, genetic risk factors and pathological stimuli interact to shape human astrocyte function. These gaps limit our ability to interpret the selective vulnerability observed in neurodegenerative diseases, constrain mechanistic understanding of APOE genotype effects and impede the development of targeted therapeutic interventions.

1.9.1. Gap 1: Limited Understanding of Regional Astrocyte Heterogeneity in Human Systems

Astrocyte heterogeneity is now recognised as a core principle of CNS organisation, with rodent studies demonstrating region-specific transcriptional and functional identities. However, human astrocyte heterogeneity remains considerably less well defined. Most existing evidence derives from rodent tissue, sparse human post-mortem datasets or studies that group astrocytes into broad, poorly resolved categories. Systematic comparisons of human cortical versus ventral midbrain astrocytes, particularly under inflammatory conditions, are lacking. As a result, several key questions remain unanswered: whether iPSC-derived astrocytes retain their developmental regional identity; whether intrinsic differences in morphology, gene expression and metabolism are preserved *in vitro*; and whether cortical and midbrain astrocytes differ in their inflammatory responsiveness in ways that may

reflect region-specific vulnerability, such as cortical degeneration in AD versus midbrain degeneration in PD.

1.9.2. Gap 2: Unclear How APOE Genotype Shapes Human Astrocyte Reactivity

Although APOE is the strongest genetic risk factor for late-onset AD, the mechanisms by which APOE isoforms influence astrocyte biology remain incompletely understood. Rodent studies have provided foundational insight, yet species differences in APOE sequence, lipidation behaviour, complement signalling, inflammatory responsiveness and amyloid handling limit their translational relevance. Emerging work using human iPSC-derived astrocytes suggests genotype-dependent differences in lipid metabolism, inflammatory signalling and metabolic function, but existing studies are typically limited to one or two genotypes or rely on non-isogenic backgrounds. Major outstanding questions include how APOE genotype influences astrocyte differentiation, baseline inflammatory tone, mitochondrial and glycolytic metabolism and responsiveness to cytokine stimulation.

1.9.3. Gap 3: Limited Understanding of A β -Induced Astrocyte Dysfunction in Human Models

Although A β is a defining pathological feature of AD, its effects on astrocytes, especially early-stage oligomeric forms, remain poorly characterised in human systems. Several limitations hinder current understanding: rodent astrocytes exhibit attenuated responses to human A β oligomers; many studies apply supraphysiological A β concentrations; and A β alone often triggers only weak inflammatory activation, thus complicating interpretation. Few studies directly compare A β_{1-40} and A β_{1-42} , and the interaction between APOE genotype and A β exposure in human astrocytes is not well defined. Critical unanswered questions include whether human astrocytes respond to physiologically relevant A β doses, whether different A β isoforms elicit distinct transcriptional or metabolic responses and whether co-stimulation with cytokines such as IL-1 α better models *in vivo* microenvironments.

1.9.4. Gap 4: Incomplete Understanding of Astrocyte Metabolic Dysfunction in AD and Neuroinflammation

Astrocyte metabolism plays a central role in neuronal support and resilience, yet metabolic profiling of human astrocytes remains limited. Little is known about regional differences in metabolic behaviour, the extent to which APOE genotype influences astrocytic bioenergetics or how A β and inflammatory cytokines alter glycolysis and mitochondrial respiration. Existing work has focused predominantly on neurons, leaving astrocyte-specific metabolic vulnerability poorly characterised.

1.9.5. Gap 5: Lack of Mechanistic Clarity in Cannabinoid Modulation of Human Astrocyte Reactivity

Cannabinoids such as CBD and THC, as well as receptor-specific modulators including PPAR γ agonists/antagonists, CB₁ blockers and NLRP3 inhibitors, exhibit anti-inflammatory properties in glial cells. However, the molecular basis of these effects in human astrocytes remains poorly defined. Key unanswered questions include which receptors are functionally relevant in astrocytes; whether cannabinoid effects primarily operate through PPAR γ , CB₁/CB₂ or other targets; and whether these compounds can modulate astrocyte reactivity induced by pro-inflammatory cytokines. Few studies have explored cannabinoid signalling in human iPSC-derived astrocytes.

1.10. Thesis Rationale and Overarching Aims

Together, these knowledge gaps provide a strong rationale for the experimental work presented in this thesis. Astrocyte reactivity and neuroinflammation do not arise from single factors, but instead emerge from the intersection of regional identity, genetic susceptibility, pathological stimuli and metabolic state. Understanding how these factors interact in human astrocytes is essential for elucidating early mechanisms of neurodegeneration and for informing therapeutic development.

At the core of this thesis is the hypothesis that astrocyte responses to inflammatory and amyloid-related challenges are governed by an interplay between intrinsic regional identity and APOE genotype, and that these responses can potentially be modulated through cannabinoid signalling, PPAR γ activation and inhibition of inflammasome pathways. This hypothesis reflects the integration of three major axes of vulnerability (regional specificity, genetic risk and pathological stimulation) and proposes that their convergence shapes astrocyte reactivity, metabolic resilience and contribution to neurodegenerative processes.

This thesis therefore aims to:

1. characterise regional heterogeneity in human astrocyte inflammatory and metabolic responses;
2. define how APOE genotype and A β -stimulation modulate astrocyte reactivity;
3. investigate cannabinoid and receptor-specific therapeutic pathways;
4. establish mechanistic links across molecular, metabolic and functional outcomes.

By addressing these gaps, the work provides both foundational and translational insight into human astrocyte biology and its relevance to Alzheimer's disease.

Chapter 2. Materials and Methods

2. Cell Culture

2.1. Cell Lines Utilised

The following cell lines were used during the studies described in this thesis. The non-affected sibling 2 (NAS2) iPSC cell line (<https://ebisc.org/EDi002-A>) was derived from human dermal fibroblasts by viral transduction using POU5F1, SOX2, KLF4 and MYC reprogramming factors. This line was generated by the Kunath lab (Devine *et al.*, 2011) at the University of Edinburgh.

The BIONi037 iPSC cell lines are a group of APOE2/2 (<https://ebisc.org/BIONi037-A-2>), APOE3/3 (<https://ebisc.org/BIONi037-A>) and APOE4/4 (<https://ebisc.org/BIONi037-A-4>) lines, which are isogenic. The control (BIONi037-A) line carrying the APOE3/3 gene was generated from the fibroblasts of a healthy woman by electroporation with episomal plasmids containing OCT4, SOX2, KLF4, L-MYC and LIN28 (Nimsanor *et al.*, 2016). The BIONi037-A-2 and BIONi037-A-4 cell lines have undergone homozygous isogenic modification to express the APOE2/2 and APOE4/4 gene respectively.

2.2. Principles of Cell Culture

2.2.1. Aseptic Cell Culture Technique

Cell culture techniques involved in these studies were carried out in sterile laminar flow class II biological safety cabinets (Nuair, USA or Faster, Italy), with aseptic techniques used throughout cell culture. Materials were autoclaved for sterility to avoid microbiological contamination, and all materials entering the safety cabinet were sprayed with 70% ethanol to maintain these sterile conditions. All cells were incubated in a humidified incubator (37°C, 5% CO₂; Binder, Germany).

2.2.2. Cell Cryopreservation

Cells were dissociated from the plate using the appropriate enzymatic dissociation agent, such as ethylenediaminetetraacetic acid (EDTA; 0.5 mM; Thermo Fisher Scientific, USA) for iPSC or Accutase™ (Thermo Fisher Scientific, USA) for NPC and

APC, and then centrifuged [1000 rpm; 3 min; room temperature (RT)]. The cells were resuspended in CryoStor® cell cryopreservation media (Sigma-Aldrich, UK) and added to cryovials. A MrFrosty® storage container stored at -70°C was used to cool down and freeze the cells at 1°C per min, then transferred to liquid nitrogen for long-term storage.

2.2.3. Cell Thawing

To thaw cells, the cryovials were taken out of liquid nitrogen and placed in a water bath at 37°C. Once only a small crystal of ice remained, cells were diluted 1:10 in appropriate media to dilute the dimethyl sulfoxide present in CryoStor® media, then centrifuged (1000 rpm; 3 min; RT).

2.3. iPSC cell culture

2.3.1. iPSC Maintenance

iPSC were cultured on Vitronectin (VTN; 0.5 µg/cm²; Thermo Fisher Scientific, USA) coated 6-well plates (Nunc by Thermo Fisher Scientific, USA). The Essential 8™ Flex (E8) media (Thermo Fisher Scientific, USA) was changed daily as per the Chen *et al.* (2011) protocol. Cells were given extra media for the weekend.

2.3.2. iPSC Passaging

Once the cells reached 80% confluency, they were passaged in the E8 culture system as per the manufacturer's protocol. Used media was removed from the well. The cells were washed once with phosphate buffered saline (PBS; Thermo Fisher Scientific, USA) and once with 0.5 mM EDTA. The cells were dissociated from the plate with EDTA by incubation for 4 minutes at RT. EDTA was removed, and cells were washed once with fresh E8 media to remove any remaining EDTA to prevent issues with adherence. The cells were then detached from the well with fresh E8 media, collected in a falcon tube, and plated on a VTN-coated plate. A 1:5 split ratio was used for the expansion of iPSC cells. Lower densities were used for neural differentiation.

2.4. Neuronal Cell Culture

2.4.1. Cortical Neuralisation

A Dual-SMAD inhibition protocol adapted from several commonly used protocols (Chambers *et al.*, 2009, 2012; Shi, Kirwan and Livesey, 2012; Nistor *et al.*, 2015; Nolbrant *et al.*, 2017) was used to differentiate iPSC into neural progenitors (NPC). To induce NPC differentiation, iPSC were first passaged on a 12-well plate (Nunc by Thermo Fisher Scientific, USA) at a low density using the steps from Section 2.3.2 above, which has to be optimised for each individual cell line. For example, a 1:10 and 1:6 split ratios were used to neuralise the NAS2 and the APOE2 iPSC cell lines, respectively. For each new cell line, a range of densities, such as 1:5-1:10, has to be trialled to optimise it specifically for this cell line. Once the cells have reached the 60-70% confluency in a 12-well plate, these cells can be patterned towards a cortical fate for 10 days in Neural Maintenance Media (NMM) containing 1:1 ratio of Dulbecco's Modified Eagle Medium (DMEM)/F-12 + GlutaMAX and Neurobasal, 1% B27, 1% non-essential amino acids (NEAA), 1% penicillin-streptomycin (Pen/Strep), 0.5% N2, 0.5% GlutaMAX, 75µM β-mercaptoethanol and 5µg/mL insulin, supplemented with LDN193189 (100 nM; Sigma-Aldrich, UK) to inhibit mesoderm differentiation and SB431542 (10 µM; Bio-Techne, USA) to inhibit pluripotency. On day 3 (D3) and D6, laminin (0.1 µg/cm²) was added to the media to aid cell adherence. On D10, SB and LDN were removed from the NMM media. From D10 onward, NPC were maintained in NMM only. On D11, cells were passaged onto poly-L-ornithine (1 µg/cm²; 1h, 37°C; Sigma-Aldrich, UK) and laminin (1 µg/cm²; 2h, 37°C; Thermo Fisher, USA) coated 6-well plates as described in Section 2.4.3. below.

2.4.2. NPC Maintenance

Starting from D11, NPC were cultured on poly-L-ornithine and laminin coated 6-well plates in NMM. Cells were fed every 1-2 days as needed, and extra media was added when the cells were closer to being fully confluent.

2.4.3. NPC Passaging

Once 100% confluency was reached, Y-27632 (10 μ M; Bio-Techne, USA), a Rho-associated protein kinase (ROCK) inhibitor that reduces dissociation-induced apoptosis, or RevitaCell (1X; Thermo Fisher Scientific, USA), a chemically defined cell survival supplement containing anti-apoptotic factors, were added to NPC for 2h before the passage to aid cell survival (Watanabe *et al.*, 2007; de Rus Jacquet, 2019). NPCs were passaged onto poly-L-ornithine and laminin coated 6-well plates. Used media was removed and discarded. Cells were washed once with PBS and then dissociated with Accutase™ by incubation for 5-7 min at RT. Accutase™ was removed, and the cells were lifted with NMM and transferred onto a poly-L-ornithine and laminin-coated 6-well plate at 1:2 split ratio. Y-27632 (10 μ M) or RevitaCell (1X) were added to the cells after passaging to further improve post-dissociation survival. Brain-derived neurotrophic factor (BDNF; 20 ng/mL), glial cell-derived neurotrophic factor (GDNF; 20 ng/mL) and ascorbic acid (AA; 0.2 ng/mL), which support the survival of neuronal cells can also be used for long-term maintenance of iPSC-derived NPCs.

2.4.4. Plating for Characterisation

To characterise NPCs, Y-27632 (10 μ M) or RevitaCell (1X) were added to 100% confluent cells for 2h. These cells were dissociated as described in Section 2.4.3 above, detached and centrifuged (1000 rpm; 3 min; RT). The supernatant was removed, and the cell pellet was resuspended in NMM media, supplemented with Y-27632 (10 μ M) or RevitaCell (1X). Cells were seeded on UV-sterilised, poly-L-ornithine and laminin-coated glass coverslips at a density of 100 μ L of cell suspension per coverslip. Cells were incubated (37°C; 5% CO₂) for at least 1h to allow them to adhere to the coverslips. Each well was then topped up with NMM (400 μ L/well). Figure 2.1 below shows a timeline of neural progenitor cell differentiation protocol.

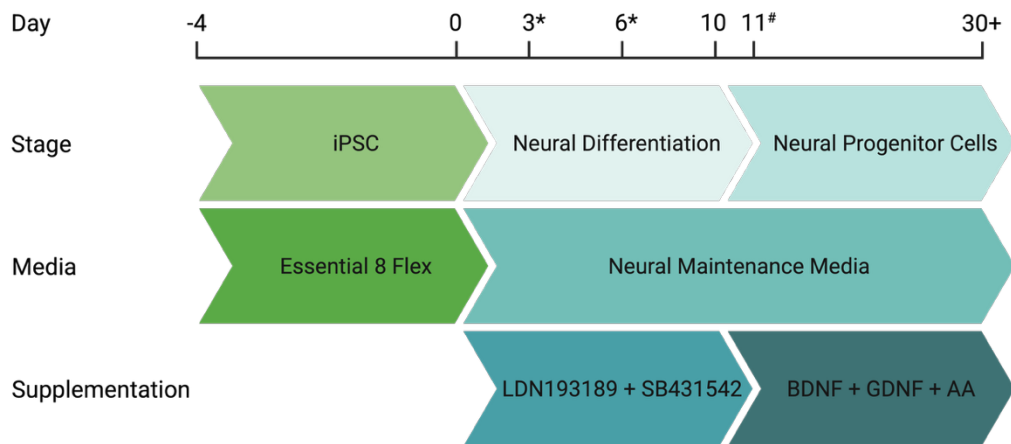


Figure 2.4.1 Cortical neuralisation timeline.

*iPSC can be used to differentiate into NPC in a 10-day protocol by using NMM media supplemented with SB and LDN. The addition of laminin on D3 and D6 (marked as *), keeping the first passage until D11 (marked as #), as well as the supplementation with BDNF, GDNF and AA, all contribute towards a successful differentiation of iPSC-derived NPC and their long-term maintenance. Figure created in BioRender (<https://www.biorender.com>).*

2.5. Astrocyte Cell Culture

2.5.1. APC Differentiation

From D30, NPC can be patterned toward a glial fate as per the Serio (2013) protocol. Once NPC were confluent, NMM media was removed and the well was washed with PBS. Astrocyte maintenance media (AMM), also referred to as ‘Serio’ containing Advanced DMEM/F-12 (Thermo Fisher Scientific, USA), 1% GlutaMAX, 1% NEAA, 1% Pen/Strep, 1% N2, and 0.2% B27, was supplemented with human leukaemia inhibitory factor (hLIF; 20 ng/mL; Sigma-Aldrich, UK) and epidermal growth factor (EGF; 20 ng/mL; Sigma-Aldrich, UK) and added to the cells. EGF is a potent driver of glial differentiation, while hLIF is a strong mitogen which maintains cell proliferation in a long-term culture (Serio *et al.*, 2013). 72 h after the initial addition of Serio, cells were passaged onto Geltrex-coated (0.5X; 2h; 37°C; Thermo Fisher Scientific, USA) 6-well plates as described in Section 2.5.3 below. Figure 2.2 below shows a timeline of astrocyte progenitor cell (APC) differentiation.

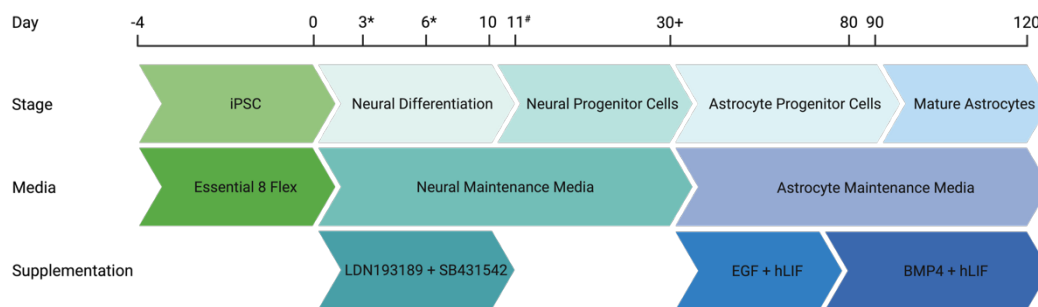


Figure 2.5.1 Astrocyte differentiation timeline.

APC can be derived from NPC by the addition of Serio media supplemented with EGF (20 ng/mL) and hLIF (20 ng/mL). Once they reach D80, APC can be terminally differentiated to mature astrocytes by plating them in Serio supplemented with bone morphogenic protein 4 (BMP4; 20 ng/mL) for 7 days, then replaced with Serio only for 72h before experiments. APC and mature astrocytes are optimal for expansion and use in experiments up to D120. Figure created in BioRender (<https://www.biorender.com>).

2.5.2. APC Maintenance

iPSC-derived APC were expanded on Geltrex-coated 6-well plates in AMM. APC were fed every two days by removing the media and replacing it with fresh AMM. The double volume of media was given for weekend breaks.

2.5.3. APC Passaging

APC were passaged when cells reached 100% confluency. A 6-well plate was coated with 0.5X Geltrex. Media was removed from the well, cells were washed with PBS and incubated in Accutase (3-4 min; RT). Accutase was removed, and APC were detached with AMM, collected in a falcon tube and centrifuged (1000 rpm, 3 min; RT). The supernatant was discarded, and the cells were resuspended in AMM supplemented with EGF and hLIF (AMM-EL). Resuspended APC were then transferred onto a Geltrex-coated 6-well plate at a 1:4 ratio. RevitaCell (1X) or Y-27632 (10 μ M) was added to the cells after each passage to aid the cell survival (Watanabe *et al.*, 2007).

2.5.4. Plating and Terminal Differentiation

APC were plated on 24-well plates from D80 for terminal differentiation and treatment. APC were dissociated as described in Section 2.5.3 above, detached with AMM media and centrifuged (1000 rpm; 3min; RT). The supernatant was removed, and the pellet was resuspended in AMM media. To prepare APC for counting, 10 μ L of cells were diluted 1:10 in Trypan blue (0.4%; Thermo Fisher Scientific, USA). Cells were counted using a haemocytometer. Cells were plated on UV-sterilised, Geltrex-coated (1X; 2h; 37°C) coverslips at a density of 25,000-35,000 cells per coverslip for immunocytochemistry (ICC) staining. Cells were plated on 24-well, Geltrex-coated plates at 100,000 cells/well density for enzyme-linked immunosorbent assay (ELISA) and quantitative polymerase chain reaction (qPCR) analysis. For Extracellular Flux (Seahorse) assay, cells were plated on a 96-well Seahorse (XFe96), Geltrex-coated cell culture plate at a density of 40,000 per well. Cells were incubated at 37°C for 1 hour to adhere before the wells were topped up with AMM-EL (500 μ L per well for 24-well plates; 180 μ L per well for 96-well plates). Once cells reached confluency, AMM-EL media was changed to terminal differentiation media containing Serio supplemented with bone morphogenic protein 4 (BMP4; 20 ng/mL; R&D Systems, UK) and hLIF (20 ng/mL), referred to as AMM-BL. BMP4 promotes astrocyte development and inhibits the oligodendrocyte differentiation (Gomes, Mehler and Kessler, 2003). Cells were matured in AMM-BL for 7d, after which it was replaced with plain AMM with no supplementation for 72h prior to experimentation (Figure 2.2 above). APC and mature astrocytes may be expanded and used in experiments up to D120, after which they reach the end of their lifespan in culture. Cell treatments and stimulation has been described in the methods sections of each results chapter (Ch. 3-5) due to differences in stimulation and treatment regime depending on the study.

2.6. Gene Expression Analysis via qPCR

Post-treatment, cells were lysed, their RNA isolated, and cDNA manufactured. mRNA expression of genes of interest was determined in cell lysates by quantitative polymerase chain reaction (qPCR) analysis. All procedures, including cell lysis, RNA

isolation, cDNA manufacture and qPCR setup, were carried out in a designated molecular biology fume hood (Premier, Scotland) which was cleaned with 70% ethanol and RNase-away® (VWR International, UK) prior to use, to sterilise the area, and remove RNase and DNase, thus preventing the breakdown down of RNA and DNA, as well as reducing the chance of sample contamination.

2.6.1. Cell Lysis

Cells were lysed in lysis buffer consisting of RA1 buffer (350 µL per sample) from the NucleoSpin® total RNA isolation kit (Macherey-Nagel, Germany) and β-mercaptoethanol (3.5 µL per sample). Briefly, after treatment removal, each well was washed with PBS (500 µL per well). PBS was then removed and replaced with 350 µL of the lysis buffer per well. The cells were then scraped off the bottom of each well with a P1000 tip, and the lysates were collected and stored at -70°C prior to RNA isolation.

2.6.2. RNA Isolation

RNA was isolated from cell lysates using a NucleoSpin® total RNA isolation kit, as per the manufacturer's instructions. Before each isolation, reconstituted rDNase (10 µL per sample) and reaction buffer (90 µL per sample) were combined to prepare the DNase reaction mixture. A 70% ethanol dilution was also prepared by adding molecular biology grade ethanol (ethyl alcohol, 200 proof; Sigma-Aldrich, UK) to RNase-free H₂O from the NucleoSpin® total RNA isolation kit. To filter the lysate, thawed cell lysates were added to the violet NucleoSpin® filter units in collection tubes and centrifuged (11,000 x g; 1 min). The violet filter was then discarded and 350 µL of 70% ethanol was added to each of the collecting tubes and triturated 8-10 times to adjust the RNA-binding conditions. To bind the RNA, freshly adjusted lysates were loaded into the light blue NucleoSpin® RNA II columns in collection tubes and centrifuged (11,000 x g; 30s). The columns were placed into new collection tubes and 350 µL of the membrane desalting buffer (MDB) was added to each collection tube and centrifuged (11,000 x g; 1 min) to desalt the membrane. To digest the DNA, 95 µL of the DNase reaction mixture was added to the centre of each column and incubated (15 min; RT). After incubation, rDNase

was inactivated by the addition of 200 μ L of the RAW2 buffer to each column and centrifuged (11,000 $\times$ g; 30s). To wash the filter, 600 μ L of the RA3 buffer was added to each column and centrifuged (11,000 $\times$ g; 30s). The filter was further washed by the addition of another 250 μ L of the RA3 buffer to each column and centrifugation (11,000 $\times$ g; 2 min) to dry the membrane. The columns were placed into nuclease-free microcentrifuge tubes (1.5 mL). To elute the RNA, 40 μ L of the RNase-free H₂O was added to each column and centrifuged (11,000 $\times$ g; 1 min). The yield of RNA isolated from each sample was quantified by the use of the NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA). The RNA was equalised to 40 ng/mL by making the appropriate dilution with RNase-free H₂O in each sample. Equalised RNA was returned to the -70°C ultra-low temperature freezer for storage until cDNA manufacture.

2.6.3. cDNA Manufacture

The cDNA was reverse transcribed from the isolated RNA using the manufacturer's instructions that came with the High-Capacity cDNA manufacture kit (Applied Biosystems, USA). To prepare the 2X RT reaction mix, 2 μ L per reaction of 10X RT buffer, 2 μ L per reaction of 10X RT random primers, 1 μ L per reaction of MultiScribe RT, 0.8 μ L per reaction of 25X dNTP mix (100nM), and 4.2 μ L per reaction of nuclease-free H₂O were used. 10 μ L of the 2X RT reaction mix was added to 10 μ L of equalised RNA. The tubes were centrifuged (1,200 $\times$ g; 1 min), and the thermal cycler (PTC-200 Peltier Thermal Cycler, MJ Research, USA) was used to incubate the samples on the AB-cDNA program set to 37°C for 60 min, 95°C for 5 min and 4°C for ∞ . cDNA was then diluted 1:4 in nuclease-free H₂O and stored at -20°C prior to qPCR analysis.

2.6.4. qPCR

Multi-target qPCR was used to amplify the manufactured cDNA. To prepare the master mix, 0.5 μ L per sample of the target primer and 0.5 μ L per sample of the endogenous primer were added to 5 μ L per sample of the TaqMan master mix. 4 μ L of cDNA per sample was loaded on a 96-well PCR fast plate (ThermoFisher Scientific, USA), which was placed in an ice block. For each of the loaded

samples, 6 μ L of the PCR master mix was added, and the plate was centrifuged at 1,200 rpm for 1 min. The plate was then run using the StepOne™ Real-Time PCR System using the Comparative Ct program (Applied Biosystems, ThermoFisher Scientific, USA). For the endogenous control, GAPDH (Hs02786624_g1; VIC) was used. The following stages were used on the Comparative Ct program: Holding Stage 1 (50°C; 120s), Holding Stage 2 (95°C; 20s) and the Cycling Stage (95°C for 1s and 60°C for 20s) for 40 cycles. The Livak $\Delta\Delta$ Ct method was then used to produce a relative quantity (RQ) value of the target gene expression that is relative to GAPDH expression as a way of determining the gene expression of the gene of interest. A table containing each gene used can be found in the methods sections of results chapters (Ch. 3-5).

2.7. Cytokine Secretion Analysis via Enzyme-linked Immunosorbent Assay (ELISA)

To assess the potential release of cytokines and chemokines post-stimulation and/or treatment, enzyme-linked immunosorbent assay (ELISA; R&D Systems, USA) was used to quantify the protein secretion of CXCL10, IL-6, GM-CSF and RANTES. An adapted manufacturer's protocol was used for each of the ELISA kits used. Firstly, 50 μ L of the capture antibody (diluted 1:120 in PBS) was added to each well of the 96-well ELISA plate (Nunc by Thermo Fisher Scientific, USA). An adhesive strip (Thermo Fisher Scientific, USA) was used to seal the plate, which was then incubated overnight at RT. The next day, the capture antibody was removed and wash buffer (0.05% Tween-20 in PBS; Sigma-Aldrich, UK) was used to wash the plate three times. Reagent diluent (RD; 1% BSA in PBS) was then used at 50 μ L per well to block the plate for 1h at RT. The plate was then washed as described above. The standards (diluted in RD; ratio dependent on the specific kit), and samples (diluted 1:4 in RD) were loaded at 50 μ L per well and incubated for 2h at RT. Samples and standards were then removed, and the plate was washed three times with the wash buffer again. The detection antibody (1:60 in RD) was then loaded at 50 μ L per well and incubated for 2h at RT. The detection antibody was then removed, and the plate was washed three times again with the wash buffer.

Streptavidin conjugated to horseradish peroxidase (Streptavidin-HRP; 1:40 in RD) was loaded onto the plate using 50 μ L per well, and the plate was incubated at RT for 20 min in the dark. Wash buffer was used to wash the plate three times again, after which tetramethylbenzidine (TMB; Sigma-Aldrich, UK), which is the substrate solution, was loaded on the plate at 50 μ L per well and incubated in the dark at RT (length of incubation dependent on the kit; usually 10-20 min). The stop solution (H_2SO_4) was then loaded at 25 μ L per well to stop the reaction. A microplate reader (Synergy HT, BioTek Instruments, USA) and Gen5 software were used to read the plate at 450 nm absorbance, with a 540 nm absorbance set as the wavelength correction to maximise the accuracy of the reading. The standard curve produced was then used to quantify cytokine concentrations in each sample.

2.8. Cell Viability Assays

2.8.1. alamarBlue™

alamarBlue™ Cell Viability Reagent (Thermo Fisher Scientific, USA) was used to determine the viability of cells post-treatment via measurements of metabolic activity. alamarBlue™ was diluted 1:10 in AMM and used on the cells (400 μ L per well) right after treatment removal. The assay was then incubated for 1h at 37°C. Each sample was then loaded in triplicate onto a black-walled clear-bottom 96-well plate (Thermo Fisher Scientific, USA) at 100 μ L per well. Colorimetric analysis was conducted by using a microplate reader (Synergy HT, BioTek Instruments, USA) with the Gen5 software. The intensity of staining was read at 560 nm excitation and 590 nm emission after performing autogain. Cells treated with 10% DMSO in media served as a positive control for this assay. The viability of cells in each well was then quantified as the percentage of the mean of all controls for that experiment.

2.8.2. LDH Assay

CyQUANT™ LDH Cytotoxicity Assay kit (Invitrogen, USA) was used to quantify the cytotoxicity levels of cortical iPSC-derived astrocytes post-stimulation with pro-inflammatory cytokines and treatment with CBD. LDH positive control was

prepared by adding 1.5 μL of the stock solution of LDH positive control into 1 mL of 1% BSA in PBS. Supernatants collected after treatment were loaded in triplicate at 30 μL per well onto a clear-bottomed 96-well plate (Nunc by Thermo Fisher Scientific, USA). Negative control (diH_2O) and previously made-up positive control were loaded in triplicate at 30 μL per well. The reaction mixture from the kit was then loaded at 30 μL per well. The plate was sealed and incubated on an orbital shaker (200 rpm; Orbital Shaker SO3; Stuart Scientific, UK) for 30 min in the dark at RT. The stop solution included in the kit was then added at 25 μL per well to stop the reaction. Colorimetric analysis was conducted by using a microplate reader (Synergy HT, BioTek Instruments, USA) with the Gen5 software. The plate was read at 480 absorbance, with a 680 absorbance set as the wavelength correction to maximise the accuracy of the reading. Cells treated with 10% DMSO in media served as a positive control for this assay. The cytotoxicity of cells in each well was then quantified as the percentage of the mean of all positive controls for that experiment.

2.9. Immunocytochemistry (ICC)

2.9.1. Immunocytochemistry Staining

Cells on glass coverslips were fixed with 4% paraformaldehyde (PFA) for 25 minutes at RT. PFA was then removed, and the cells were washed three times with PBS. A blocking buffer was then made up, containing 10% normal goat serum (NGS) diluted in 0.3% Triton X-100 in PBS (PTx; Sigma-Aldrich, UK). The cells were blocked with the blocking buffer for 2h at RT. Primary antibodies were made up in 2% NGS and 0.06% PTx. Blocking buffer was removed from coverslips, and primary antibodies were added at 500 μL per coverslip and incubated overnight at 4°C. The next day, cells were washed three times for 5 min with the wash buffer (0.02% PTx). Species-specific secondary antibodies were diluted in 0.02% PTx. Secondary antibodies were then added at 500 μL per coverslip and incubated for 2h in the dark at RT. Cells were washed three times for 5 minutes with the wash buffer again. Hoechst (Cell Signalling Technology, USA), which is a nuclear stain, was then diluted 1:1000 in PBS and added to coverslips at 500 μL per well and incubated for 30 min in the dark.

at RT. Cells were then washed once with PBS and stored in 500 μ L of PBS until mounting. To mount the coverslips, 5 μ L of ProLong™ Diamond Antifade Mountant (Invitrogen by Thermofisher Scientific, USA) per coverslip was added onto a slide. The coverslips were then removed from the well using tweezers and placed upside down on the ProLong™ droplet. The slides were then left overnight in the dark at RT to dry. Specific primary and secondary antibodies and dilution factors used for each study can be found in the methods sections of the results chapters (Ch. 3-5).

2.9.2. Image Acquisition

Cells were imaged using a fluorescent microscope (Carl Zeiss Axio Imager Z1 microscope) using the x20 objectives, with an attached microscope camera (AxioCam HRm; Carl Zeiss, Germany). Camera exposure times were kept constant while collecting images. Approximately 15 images from 1-2 coverslips were taken per N. Multiple TIFF files were created for each image, with each colour channel kept separately for further analysis.

2.9.3. CellProfiler Analysis

All analysis was performed on unmodified images using CellProfiler (The Broad Institute <https://cellprofiler.org/home>). TIFF files were imported into CellProfiler and analysed using a modified fruit fly pipeline developed in the lab, which allows for quantification of intensity of staining as well as various morphology analysis. The NamesAndTypes module was used to assign different colour channels based on a consistent label prefix within the file name. IdentifyPrimaryObjects module was used to identify the nuclei using the Hoechst channel. The object diameter in pixel units as well as the threshold correction factor were adjusted for a set of sample images to ensure the nuclei were correctly identified. IdentifySecondaryObjects module was then used to identify the cells, again allowing for adjustments to be made for the threshold correction factor until the cells were correctly identified. Once optimised, the same pipeline parameters were used for all conditions. All images were analysed and data containing staining intensity as well as area, perimeter, circularity, eccentricity and Feret diameters (minimal and maximal) was produced for each nucleus and cell. Cell area measures the number of pixels in the

region, i.e. the cell, which allows for quantification of the size of each cell. Cell perimeter quantifies the total number of pixels around the boundary of each cell in the image. Measures of cell circularity, eccentricity and min and max Feret diameters provide an additional insight into astrocyte geometry and process extension. Cell circularity is a measure of how close the shape of the cell is to a circle. The more circular, i.e. the less stellate-shaped, an astrocyte is, the more likely it is that the cell is reactive. In CellProfiler, circularity is known as Form Factor and it is calculated using the following formula: $\text{Form Factor} = \frac{4 \times \pi \times \text{Area}}{\text{Perimeter}^2}$ (Janssen, Breusegem and Larrieu, 2022), which allows for the quantification of the overall cell compactness compared to a circle. A circularity value of 1 describes the perfectly circular object; the closer the value is to 1, the more circular the astrocyte is, and therefore potentially more reactive. Cell eccentricity is a measure of “the eccentricity of the ellipse with the same second-moments” as the cell. It is a ratio of the distance between the centre of the ellipse and its major axis length (Janssen, Breusegem and Larrieu, 2022). In simpler terms, cell eccentricity measures how elongated or non-circular the cell is or how much it deviates from the perfect circle. A perfect circle would have an eccentricity of 0, while eccentricity of 1 is considered a line. Therefore, the closer the value is to a 1, the more elongated and less circular the cell is. Feret diameter is defined as the distance between two parallel lines tangent to opposite sides of a cell’s outline (Janssen, Breusegem and Larrieu, 2022). Max Feret diameter represents the longest span across the cell, often reflecting the extent of process outgrowth, while Min Feret diameter corresponds to the narrowest span. Larger differences between Max and Min Feret diameters indicate a more elongated morphology, whereas similar Min and Max values indicate more circular cells.

2.10. Extracellular Flux (Seahorse) Assay

The Agilent Seahorse Glycolytic Stress Test kit and the Seahorse XFe96 Analyser were used to assess glycolysis, using the manufacturer’s protocol (Agilent, USA). The assay measures extracellular acidification rate (ECAR), an indirect indicator of glycolytic activity that primarily reflects lactate-associated proton release into the

extracellular environment, alongside oxygen consumption rate (OCR), a measure of mitochondrial respiration. Cells were seeded at 40,000 per well on a XFe96-well Seahorse cell culture plates and matured as described in Section 2.5.4. above. The cells were then stimulated for 24 h using a different combination of pro-inflammatory cytokines. Details of this for each specific study can be found in individual methodology sections within the results chapter (Ch. 3 and 4).

The day before the assay, the probe calibrant plate was immersed in the Seahorse XF calibrant media and hydrated overnight in a CO₂-free incubator (37°C). The next day, AMM media was replaced with Seahorse XF DMEM assay medium (Agilent, USA) and the cells were incubated in a CO₂-free incubator (37°C, 1h). Seahorse XF DMEM assay medium was used because it is a phenol red-free medium containing no glucose and low glutamine levels (2mM) and is formulated to maintain pH 7.4 without CO₂ buffering, thereby minimising interference with ECAR measurements and glycolytic flux.

The calibrant plate was loaded into the XFe96 Seahorse Analyser to allow for the equipment and sensor cartridge calibration. The cell culture plate was then loaded into the analyser and baseline ECAR and OCR measurements were recorded under glucose-starved conditions. Three ECAR and OCR readouts were then recorded post-glucose (10 mM), oligomycin (1 µM) and 2-deoxyglucose (2-DG; 50 mM) injections, with the run time of 3 x 6 min rest periods between readouts per injection. Glucose addition initiated glycolysis, oligomycin inhibited mitochondrial ATP synthase (Complex V) to shift cellular energy production toward maximal glycolytic activity, and 2-DG, a non-metabolisable glucose analogue, inhibited glycolysis to determine non-glycolytic acidification. The data were automatically recorded and generated by XFe96 Analyser software. This allowed for quantification of basal glycolysis, representing glycolytic activity under baseline conditions; glycolytic capacity, defined as the maximum glycolytic response following mitochondrial ATP synthase inhibition by oligomycin; glycolytic reserve, reflecting the ability of cells to increase glycolytic flux in response to energetic stress; and non-glycolytic acidification, representing extracellular acidification independent of glycolysis.

2.11. Statistical Analysis

Data were initially prepared in Microsoft Excel for statistical analysis. Data and graphs are expressed as mean $\pm$ standard error of the mean (SEM). The 'n' values refer to the number of cultures per group, while the 'r' value refers to the number of replicate samples within each experimental group. Normality of data distribution was assessed using the Shapiro-Wilk test prior to statistical analysis. Unpaired Student's t-Test, one-way, two-way and three way analysis of variance (ANOVA) was used for statistical analysis, followed by Tukey's post hoc test, where significance was inferred. A p-value of less than 0.05 ($p < 0.05$) was deemed significant. Statistical analysis was performed using GraphPad Prism version 10.6.1 for MacOS (GraphPad Software, USA). Details of statistical analysis for each data set can be found in the figure legends within each results chapter (Ch. 3-5).

Chapter 3. Cortical and Ventral Midbrain Astros Exhibit Regional Differences in Morphology, Response to Pro-Inflammatory Stimuli, and Function

3.1. Introduction

Astrocytes are crucial regulators of the central nervous system (CNS) homeostasis, with functions including supporting synaptic transmission, metabolic coupling, ion and neurotransmitter balance, blood-brain barrier (BBB) regulation and innate immune signalling, all of which aid neuronal survival (Yang and Jackson, 2019; Westergard and Rothstein, 2020; Endo *et al.*, 2022; Jiwaji and Hardingham, 2022; Zhang *et al.*, 2023). Although astrocytes are historically viewed as a uniform glial population, in recent years they have been recognised as highly diverse at molecular, morphological and functional levels. Transcriptomic and proteomic studies revealed pronounced astrocyte heterogeneity across brain regions, suggesting that this heterogeneity is shaped by developmental patterning cues, intra- and extracellular signals and neuronal interactions (Clarke *et al.*, 2021). Regional identity emerges early during CNS development via morphogen gradients, including WNT, fibroblast growth factors (FGFs) and sonic hedgehog (SHH), which help establish anterior-posterior and dorsal-ventral axes (Hochstim *et al.*, 2008; Goldman, 2016). These cues then help specify progenitor domains, which can be characterised by transcription factors such as FOXG1 (cortex) and LMX1A/FOXA2 (ventral midbrain), which in turn influence astrocyte lineage commitment to a specific brain region and imprint stable regional features that will persist throughout the cell lifespan (Huang *et al.*, 2020; Lozzi *et al.*, 2020).

Astrocyte reactivity is a central response to CNS injury or inflammation, involving cytokine and chemokine release, transcriptional activation, metabolic changes and structural remodelling (Zamanian *et al.*, 2012). However, it is becoming more evident that astrocyte reactivity is context-dependent and regionally specialised, rather than a uniform response across all cells (Verkhatsky *et al.*, 2023; Santos *et al.*, 2025). Astrocytes from distinct brain regions exhibit differences in inflammatory receptor expression, NFκB activation and signalling pathways involved, thus leading to diverse responses to identical stimuli (Bradley *et al.*, 2019; Batiuk *et al.*, 2020; Hasel *et al.*, 2021; Endo *et al.*, 2022). These intrinsic differences may possibly contribute to selective regional vulnerability in neurodegenerative

diseases (Zimmer, Orr and Orr, 2024), with cortical neurons degenerating in AD, while dopaminergic neurons of the ventral midbrain are targeted in PD.

iPSC-derived astrocytes provide an excellent system for studying region-specific astrocytes. When guided by appropriate patterning cues, iPSC can be differentiated into neural progenitors with forebrain, midbrain, hindbrain or spinal identities, which then can be further differentiated into regionally distinct astrocyte progenitors (Crompton *et al.*, 2013; Serio *et al.*, 2013; Tcw *et al.*, 2017; Bradley *et al.*, 2019; MacMahon Copas *et al.*, 2024; O'Toole *et al.*, 2024). These astrocytes retain key transcriptomic features reflective of their developmental origins and exhibit functional differences in glutamate uptake, synaptogenic capacity, cell metabolism and inflammatory responses (Sloan *et al.*, 2017; Batiuk *et al.*, 2020; Clarke *et al.*, 2020). However, the extent to which iPSC-derived cortical (CTX) and ventral midbrain (VM) astrocytes (Astros) maintain their regional identities after long-term differentiation, and how these identities influence functional responses such as inflammatory activation and metabolic adaptation, remains widely understudied, despite extensive genomic studies on astrocyte regional identity.

Regional identity is especially relevant to neurodegeneration. In AD, the main affected area is the cortex where astrocyte dysfunction contributes to chronic neuroinflammation and synaptic loss (Heneka *et al.*, 2015; K. Li *et al.*, 2019; Verkhratsky *et al.*, 2023). In contrast, the ventral midbrain is mainly affected in PD, where astrocytes exhibit unique metabolic and dopaminergic-supportive roles, thus potentially shaping this region-specific vulnerability (Chung *et al.*, 2013; Booth, Hirst and Wade-Martins, 2017). Therefore, comparing CTX and VM-Astros offers a potential answer to a crucial question; do intrinsic regional properties shape inflammatory responsiveness in astrocytes and does this influence disease progression and therapeutic outcomes?

The present study used well-established pro-inflammatory paradigms to model astrocyte reactivity. The TNF α , IL-1 α and C1q cocktail (TIC) is a combination of canonical cytokines released by microglia; several studies have shown that stimulating astrocytes with these pro-inflammatory factors will induce a reactive phenotype associated with neurotoxicity (Liddelow *et al.*, 2017; McComish *et al.*,

2024). TNF α and IL-17A have recently been shown to synergise together in order to enhance astrocyte activation in chronic inflammation (MacMahon Copas *et al.*, 2024). The stimulation with IL-1 α alone was also utilised to provide a more nuanced astrocyte response to a less potent pro-inflammatory stimulus. Together, this allowed for an investigation on how CTX and VM-Astros respond to distinct pro-inflammatory challenges, providing insight into region-specific astrocyte activation patterns.

Aims and Objectives

Research Question: **Do iPSC-derived astrocytes retain regional identity, and does this identity shape their baseline properties, inflammatory responses, and metabolism?**

Astrocyte heterogeneity is increasingly recognised as a key factor shaping regional brain function and vulnerability to disease. However, the extent to which iPSC-derived astrocytes retain their developmental identity, and how this identity influences their behaviour under baseline and inflammatory conditions, remains unclear. To address this gap, this chapter set out the following aims:

1. To confirm the regional identity of cortical (CTX) and ventral midbrain (VM) neural progenitors and their derived astrocytes at both protein and gene expression levels.
2. To characterise baseline differences in morphology, protein secretion and gene expression between CTX and VM-Astros using ICC, qPCR and ELISA.
3. To evaluate region-specific responses of CTX and VM-Astros to pro-inflammatory stimulation using ICC, qPCR and ELISA.
4. To compare the metabolic activity at baseline and after stimulation using Seahorse Extracellular Flux Analysis.

By integrating these complementary approaches, this chapter provides new insight into how developmental origin imprints regional identity on human astrocytes, shaping their morphology, molecular signatures, inflammatory reactivity and metabolic flexibility. Furthermore, it highlights the utility of iPSC-derived astrocytes as a powerful platform for modelling region-specific astrocyte behaviours in the context of health and neurodegeneration.

3.2. Methods

3.2.1. Cell Lines Utilised

The following cell lines were used in the study described in this chapter:

- NAS2 iPSC-derived CTX-Astros
- NAS2 iPSC-derived VM-Astros generated by Dr Adina MacMahon Copas

Both astrocyte lines were utilised to investigate the regional differences in astrocytes, at baseline and post-stimulation with pro-inflammatory insults.

3.2.2. Differentiation of VM-NPC

VM-NPC were differentiated using the in-lab protocol (MacMahon Copas *et al.*, 2024) adapted from Nolbrant *et al.* (2017). Briefly, iPSCs were detached from culture plates using EDTA (7 min, RT). The cells were counted and seeded on a VTN-coated plates at a density of 10,000 cells/cm² in NMM media supplemented with SB431542 (10 µM), LDN-193189 (100 ng/mL), SHH (300 ng/mL) and CHIR99021 (0.8 µM). Y-27632 (10 µM) was also added to aid cell survival. A complete media change was carried out after 48h to remove the Y-27632. The cells were cultured in the aforementioned media until D9. On D9, the media was changed to NMM media containing FGF8b (100 ng/mL), but other factors and small molecules were removed. On D11, the cells were dissociated using Accutase™ (10 min, 37°C) and seeded onto poly-L-ornithine (1 µg/cm²; 1 h, RT) and laminin (1 µg/cm²; 2h, 37°C)-coated plates as previously described in Chapter 2, Sections 2.4.2.-2.4.3. From this point onward, the cells were treated as neural progenitor cells and were cultured in NMM media supplemented with FGF8b (100 ng/mL), BDNF (20 ng/mL) and ascorbic acid (0.2 mM). On D16, FGF8b was replaced with GDNF (20 ng/mL), alongside BDNF and ascorbic acid. From D16 to D25 VM-NPC were taken for characterisation via ICC for TUJ1, LMX1A, FOXA2 and SOX6. The timeline of VM-NPC differentiation can be found in Figure 3.2.1. below.

CTX-NPC were differentiated as described in Chapter 2, Section 2.4 and characterised via ICC for TUJ1 and TBR1. Characterised VM-NPC and CTX-NPC

were further differentiated into APC and mature Astros following the protocols described in Chapter 2, Sections 2.5.1.-2.5.4.

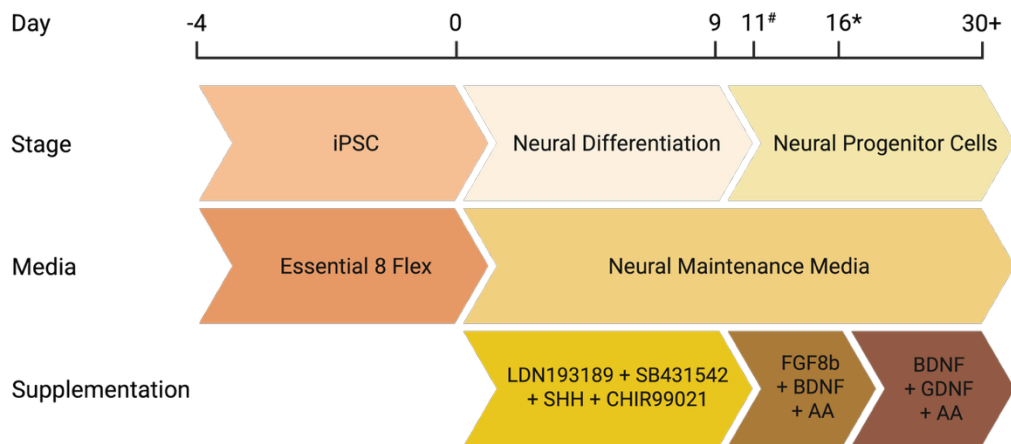


Figure 3.2.1 Ventral midbrain neuralisation timeline.

*iPSC can be used to differentiate into VM-NPC in a 16-day protocol using NMM media supplemented with SB, LDN, SHH and CHIR for the first 9 days. The media is then changed to NMM supplemented with FGF8b, BDNF and AA on D9. The cells are first passaged on D11 (marked as #). From D16 onward, cells can be seeded for characterisation (marked as *), and the cells are cultured in NMM media supplemented with BDNF, GDNF and AA for long-term maintenance. Figure created in BioRender (<https://www.biorender.com>).*

3.2.3. Stimulation with Pro-Inflammatory Insults

Mature CTX- and VM-Astros were subjected to stimulation with TNF α (30 ng/mL) + IL-1 α (3 ng/mL) and C1q (400 ng/mL; TIC) or TNF α (0.3 ng/mL) + IL-17A (10 ng/mL) or IL-1 α (0.3 ng/mL) for 24h to induce a reactive profile in astrocytes and assess the potential regional differences between CTX- and VM-Astros in terms of cytokine release, gene expression and metabolic activity. Concentrations and incubation time were selected based on previous studies demonstrating reliable induction of reactive astrocyte phenotypes in human iPSC-derived astrocytes (Liddelow *et al.*, 2017; Barbar *et al.*, 2020; MacMahon Copas *et al.*, 2024), together with preliminary optimisation experiments performed in-house to identify conditions that produced robust inflammatory responses without excessive cytotoxicity. Briefly, media was aspirated from wells, and the cells were gently washed with PBS to remove any remaining cell debris. Experimental wells were administered the stimulation

cocktails, TIC or TNF α +IL-17A or IL-1 α , while the control wells were administered AMM media alone. The cells were then incubated for 24 h (37°C; 5% CO₂) before supernatant collection and cell lysis.

3.2.4. Immunocytochemistry

ICC and subsequent analysis were performed on CTX- and VM- NPC, APC and Astros as described in Chapter 2, Section 2.9. The primary and secondary antibodies used are listed in Table 3.2.1. and Table 3.2.2. below.

Table 3.2.1 Primary Antibodies Used for Immunocytochemistry.

Primary Antibody	Manufacturer	Dilution
Rat anti-CD49f	BioLegend; GoH3	1:500
Rabbit anti- α Connexin-43	Sigma-Aldrich; C6219	1:1000
Rabbit anti-EAAT1	Bio-Techne; NBPI-2	1:1000
Rabbit anti-FOXA2	ThermoFisher Scientific; 9H5L7	1:1000
Chicken anti-GFAP	Merck; AB5541	1:1000
Hoechst 33342	Cell Signalling Technology; 4082	1:500
Rabbit anti-LMX1A	Merck; AB10533	1:1000
Rabbit anti-TBR1	Abcam; AB31940	1:300
Mouse anti-TUJ1	BioLegend; BL-801202	1:1000
Mouse anti-S100 β	Sigma-Aldrich; S2532	1:1000

Table 3.2.2 Secondary Antibodies Used for Immunocytochemistry.

Secondary Antibody	Manufacturer	Dilution
Goat Anti-chicken 488	Abcam; ab150169	1:500
Donkey Anti-mouse 488	ThermoFisher Scientific	1:500
Donkey anti-mouse 594	ThermoFisher Scientific	1:500
Donkey Anti-mouse 647	ThermoFisher Scientific	1:500
Donkey Anti-rabbit 594	ThermoFisher Scientific	1:500
Donkey Anti-rabbit 647	ThermoFisher Scientific	1:500

3.2.5. qPCR

qPCR analysis was performed on the control and stimulated CTX- and VM-Astros as described in detail in Chapter 2, Section 2.6. The relative quantification (RQ) of each target gene for CTX- and VM-Astros was calculated relative to iPSC to avoid bias toward either region. All primers were manufactured by ThermoFisher Scientific,

USA. VIC fluorescent reporter dye was used for GAPDH and FAM fluorescent reporter dye was used for all target primers. The endogenous and target primers used in this study are listed in Table 3.2.3. below.

Table 3.2.3 qPCR Primers Used in the Study.

Primers	Gene Name	Assay ID
<i>GAPDH</i>	glyceraldehyde-3-phosphate dehydrogenase	Hs02786624_g1
<i>TBR1</i>	T-box, brain 1	Hs00232429_m1
<i>FOXP1</i>	forkhead box G1	Hs01850784_s1
<i>EN1</i>	engrailed homeobox 1	Hs00154977_m1
<i>OTX2</i>	orthodenticle homeobox 2	Hs00222238_m1
<i>LMX1A</i>	LIM homeobox transcription factor 1 alpha	Hs00898455_m1
<i>GFAP</i>	glial fibrillary acidic protein	Hs00909233_m1
<i>C3</i>	complement component 3	Hs00163811_m1
<i>LCN2</i>	lipocalin 2	Hs01008571_m1
<i>SERPINA3</i>	serpin family A member 3	Hs00153674_m1
<i>TIMP1</i>	TIMP metalloproteinase inhibitor 1	Hs01092512_g1
<i>VIM</i>	vimentin	Hs00958111_m1
<i>IL6</i>	interleukin 6	Hs00174131_m1
<i>IL17RA</i>	interleukin 17 receptor A	Hs01056316_m
<i>CXCL10</i>	C-X-C motif chemokine ligand 10	Hs00171042_m1
<i>CCL5</i>	C-C motif chemokine ligand 5	Hs00982282_m1
<i>ICAM1</i>	intercellular adhesion molecule 1	Hs00164932_m1
<i>PTX3</i>	pentraxin 3	Hs00173615_m1
<i>TLR4</i>	toll like receptor 4	Hs00152939_m1
<i>IL1B</i>	interleukin 1 beta	Hs01555410_m1
<i>CSF2</i>	colony stimulating factor 2	Hs00929873_m1
<i>ITGAM</i>	integrin subunit alpha M	Hs00167304_m1
<i>IFNA2</i>	interferon alpha 2	Hs00265051_s1
<i>IFNB1</i>	interferon beta 1	Hs01077958_s1
<i>ITGA6</i>	integrin subunit alpha	Hs01041011_m1
<i>SLC1A2</i>	solute carrier family 1 member 2	Hs01102423_m1
<i>SLC1A3</i>	solute carrier family 1 member 3	Hs00904823_g1
<i>GJA1</i>	gap junction protein alpha 1	Hs00748445_s1
<i>GLUL</i>	glutamate-ammonia ligase	Hs00365928_g1
<i>DRD2</i>	dopamine receptor D2	Hs00241436_m1
<i>LDHA</i>	lactate dehydrogenase A	Hs01378790_g1
<i>HK2</i>	hexokinase 2	Hs00606086_m1
<i>PFKFB3</i>	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	Hs00998698_m1
<i>SLC2A1</i>	solute carrier family 2 member 1	Hs00892681_m1
<i>SLC6A3</i>	solute carrier family 6 member 3	Hs00997374_m1
<i>PPARG</i>	peroxisome proliferator activated receptor gamma	Hs01115513_m1

3.3. Results

To address the aims outlined above, a series of experiments were conducted to determine whether iPSC-derived cortical (CTX) and ventral midbrain (VM) astrocytes retain their regional identity and whether this influences their properties at baseline and under pro-inflammatory conditions. We first confirmed regional identity in neural progenitors and astrocytes at the protein and gene expression levels. We then examined baseline morphological, molecular and functional differences between CTX- and VM-Astros, followed by their responses to pro-inflammatory stimulation. Finally, we compared metabolic activity across regions using Seahorse extracellular flux analysis. The results are presented below in the order of these experimental aims.

3.3.1. Cortical and ventral midbrain NPC express regional markers at the protein and gene levels

In order to study the differences in astrocytes from different brain regions, CTX and VM-NPC from these were assessed for their expression of regional markers at the protein and gene levels. As seen in **Figure 3.3.1.A-B** below, CTX-NPC stained positive for TUJ1 (β Tubulin III), a classical neuronal marker, and TBR1, a cortical marker. Positive TUJ1, as well as LMX1A and FOXA2 (ventral midbrain markers) staining was observed in VM-NPC. Both CTX-NPC and VM-NPC were also counterstained for the markers from the other region to further confirm the region specificity of these cells. Both CTX- and VM-NPC stain positive for TUJ1 (see **Fig. 3.3.1.C** below); however, the intensity of staining was higher in CTX-NPC by approximately 1,000 A.U. (**** $p < 0.0001$) compared to VM-NPC. As expected, TBR1 intensity (see **Fig. 3.3.1.D** below) was nearly 10-fold higher in CTX-NPC compared to VM (**** $p < 0.0001$). On the other hand, VM-NPC expressed 3-fold higher intensity of LMX1A (**** $p < 0.0001$) and 2-fold higher intensity of FOXA2 (**** $p < 0.0001$), compared to CTX (see **Fig. 3.3.1.E-F** below).

As expected, CTX-NPC differentiated in the lab expressed the cortical region genes *TBR1* and *FOXP1*, while VM-NPC express *EN1* and *OTX2*, which are primarily expressed in the ventral midbrain region. Additionally, both CTX and VM-NPC were

then used to compare their expression of *TBR1* and *EN1* mRNA to further confirm their correct regionality. As seen in **Fig. 3.3.1.G** below, CTX-NPC expressed significantly more *TBR1* mRNA compared to VM-NPC (**** $p < 0.0001$), with the expression in VM-NPC being virtually non-existent at ~0.0005 RQ compared to 1 RQ in the CTX. On the other hand, the *EN1* mRNA expression (**Fig. 3.3.1.H** below) was 10-fold higher in VM-NPC compared to CTX (**** $p < 0.0001$).

Overall, the expression of correct regional markers at both protein and gene levels confirmed the distinction between CTX and VM-NPC. This allowed for further differentiation of these cells into astrocyte progenitors and functionally mature CTX- and VM-Astros.

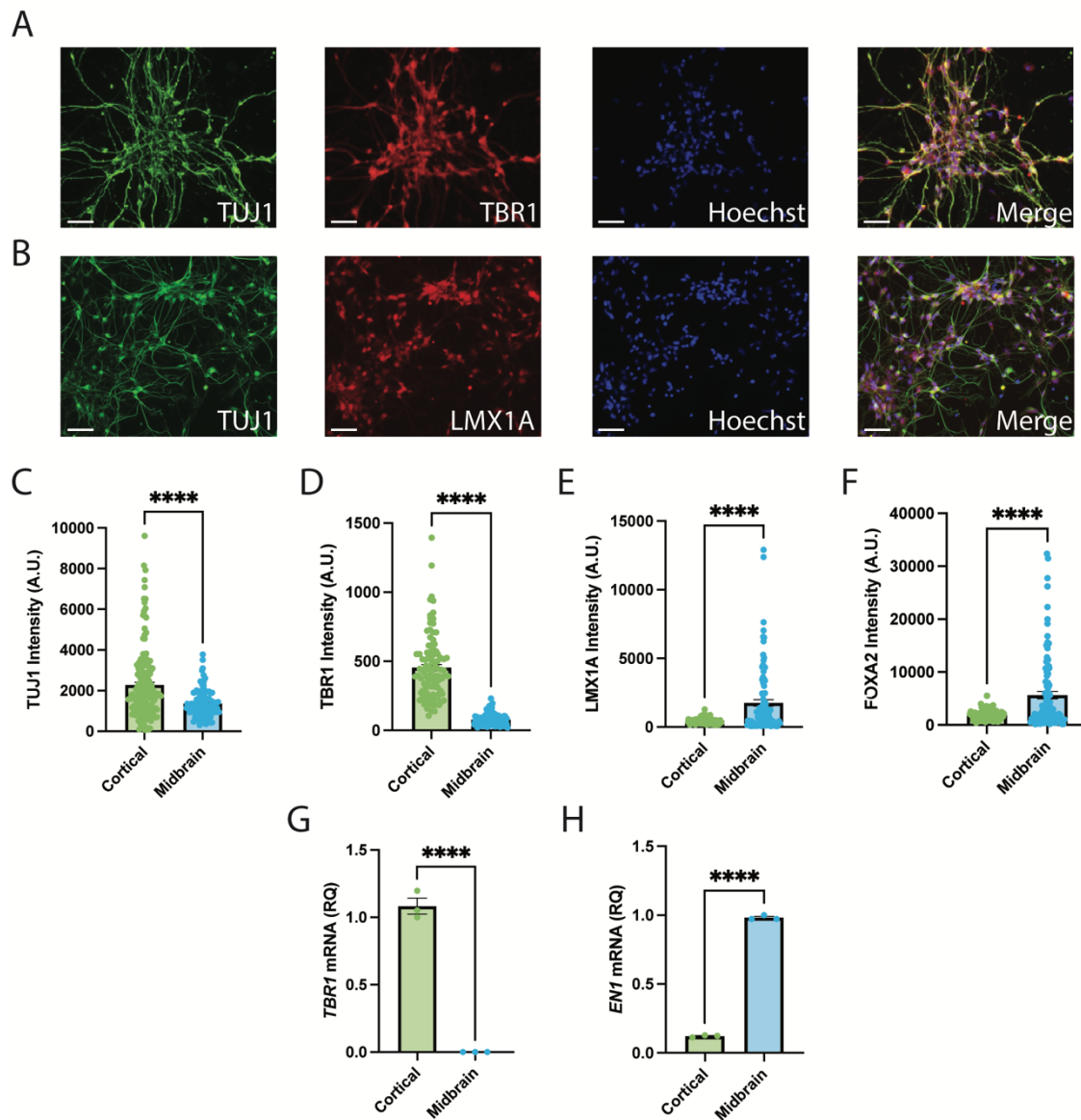


Figure 3.3.1 Cortical and ventral midbrain NPC express regional markers at the protein and gene level.

CTX and VM-NPC were analysed for their expression of CTX and VM markers. **(A)** Representative images of CTX-NPC stained positive for the TUJ1 (green) neural marker and TBR1 (red), a classical cortical marker. **(B)** Representative images of VM-NPC also stained positive for TUJ1 (green) as well as LMX1A (red). **(C)** TUJ1 intensity is higher in CTX-NPC compared to VM-NPC. **(D)** CTX-NPC also express 5-fold higher intensity of TBR1 compared to VM-NPC. On the other hand, VM-NPC express significantly higher intensity of **(E)** LMX1A and **(F)** FOXA2. **(G)** CTX-NPC express significantly more TBR1 mRNA compared to VM-NPC. VM-NPC express higher levels of EN1 mRNA **(H)** compared to CTX-NPC. Scale bars: 50 μ m. Data expressed as mean $\pm$ SEM (N=3, r=3; 100 cells per N for ICC). **** p <0.0001 vs cortical NPC. Two-tailed unpaired Student's t-Test.

3.3.2. Cortical and ventral midbrain Astros express astrocyte markers at different staining intensities

Although the ventral midbrain neural differentiation protocol differs slightly from the cortical neuralisation by adding the patterning factors for the midbrain development (SHH and CHIR), the differentiation process from neurons to astrocytes is the same regardless of the origin (see Chapter 2, Section 2.5 for the astrocyte differentiation protocol). As seen in **Fig. 3.3.2.A-B** below, both cortical and midbrain astrocytes stained positively for astrocytic markers (S100 β , GFAP, EAAT1), as well as astrocyte-associated proteins (Cx43, CD49f). Positive staining was observed both at baseline and following stimulation with pro-inflammatory factors.

The intensity of S100 β was 5-fold higher in CTX-Astros at baseline, compared to VM (++++p<0.0001), as seen in **Figure 3.3.3.A** below. Regardless of stimulation, CTX-Astros expressed 4-fold more S100 β compared to VM (++++p<0.0001 for all stimulation groups). Although there were no differences in S100 β intensity between different stimulation groups, compared to the control, S100 β intensity significantly decreased when CTX-Astros were stimulated with IL-1 α (****p<0.0001) or TIC (*p=0.0358). No significant differences in S100 β intensity were observed in VM-Astros between control and stimulation groups.

Both CTX and VM-Astros GFAP intensity (**Fig. 3.3.3.B** below) is high at baseline, with the intensity being higher by approximately 1,000 A.U. in VM-Astros compared to CTX (++++p<0.0001). GFAP intensity remained significantly higher in VM-Astros post-IL-1 α (++++p<0.0001) and TNF α +IL-17A stimulation (++++p<0.0001), but not TIC, compared to CTX. TNF α +IL-17A stimulation of VM-Astros resulted in a higher GFAP intensity of these cells compared to the control (****p<0.0001) and IL-1 α stimulation (####p<0.0001). GFAP intensity was significantly lower in VM-Astros stimulated with TIC compared to the control (****p<0.0001), IL-1 α (####p<0.0001) or TNF α +IL-17A stimulation (^^^p<0.0001). No significant differences in GFAP intensity were observed in CTX-Astros.

On the other hand, EAAT1 intensity (**Fig 3.3.3.C** below) was 3-fold higher in CTX-Astros control and 2-fold higher across all stimulation groups, compared to VM (++++p<0.0001). In CTX-Astros, stimulation with IL-1 α (****p<0.0001), TNF α +IL-17A (*p=0.0482) and TIC (****p<0.0001) resulted in a significantly higher EAAT1 intensity compared to the control. Although there were no significant differences between IL-1 α and TNF α +IL-17A stimulations in CTX-Astros, stimulation with TIC resulted in a significantly lower EAAT1 intensity, compared to TNF α +IL-17A (^^^p<0.0001). There were no significant differences observed in EAAT1 intensity between control and stimulation groups in VM-Astros.

As seen in **Fig. 3.3.3.D** below, CD49f intensity also differed between the regions and stimulation groups. At baseline, CTX-Astros expressed approximately 13 times more CD49f compared to VM (++++p<0.0001). This significantly higher intensity of CD49f in CTX-Astros stayed true for all stimulation groups compared to VM (++++p<0.0001). In CTX-Astros, stimulation with IL-1 α and TNF α +IL-17A resulted in a significantly lower expression of CD49f compared to the control (****p<0.0001), with CD49f levels being significantly higher post-TIC stimulation compared to IL-1 α (####p<0.0001) and TNF α +IL-17A (^^^p<0.0001), but not different from the control. No significant differences were observed between any of the stimulation groups in CD49f intensity in VM-Astros.

Just like in the case of GFAP intensity, Cx43 intensity (**Fig. 3.3.3.E** below) was significantly higher in VM-Astros compared to CTX at baseline (++++p<0.0001), as well as IL-1 α (++++p<0.0001) or TNF α +IL-17A (++++p<0.0001) stimulation groups, but not the TIC-stimulated group. In CTX-Astros, stimulation with IL-1 α (*p=0.0191) or TNF α +IL-17A (*p=0.0185) resulted in significantly lower Cx43 intensity compared to the control, but no significant differences were observed between different stimulation groups. In VM-Astros, stimulation with IL-1 α (****p<0.0001) and TIC (****p<0.0001) resulted in significantly lower Cx43 intensity compared to the control, with the TIC stimulation being lowest across the groups (####p<0.0001). When VM-Astros were stimulated with TNF α +IL-17A, Cx43 intensity was significantly higher compared to IL-1 α (####p<0.0001) or TIC (^^^p<0.0001) stimulations, but not the control.

Overall, CTX-Astros stained more intensely for S100 β , EAAT1 and CD49f, while GFAP and Cx43 intensity was higher in VM-Astros. Stimulation with pro-inflammatory factors generally resulted in lower staining intensity, except for TNF α +IL-17A stimulation, which resulted in higher GFAP, CD49f and Cx43 intensity in VM-Astros.

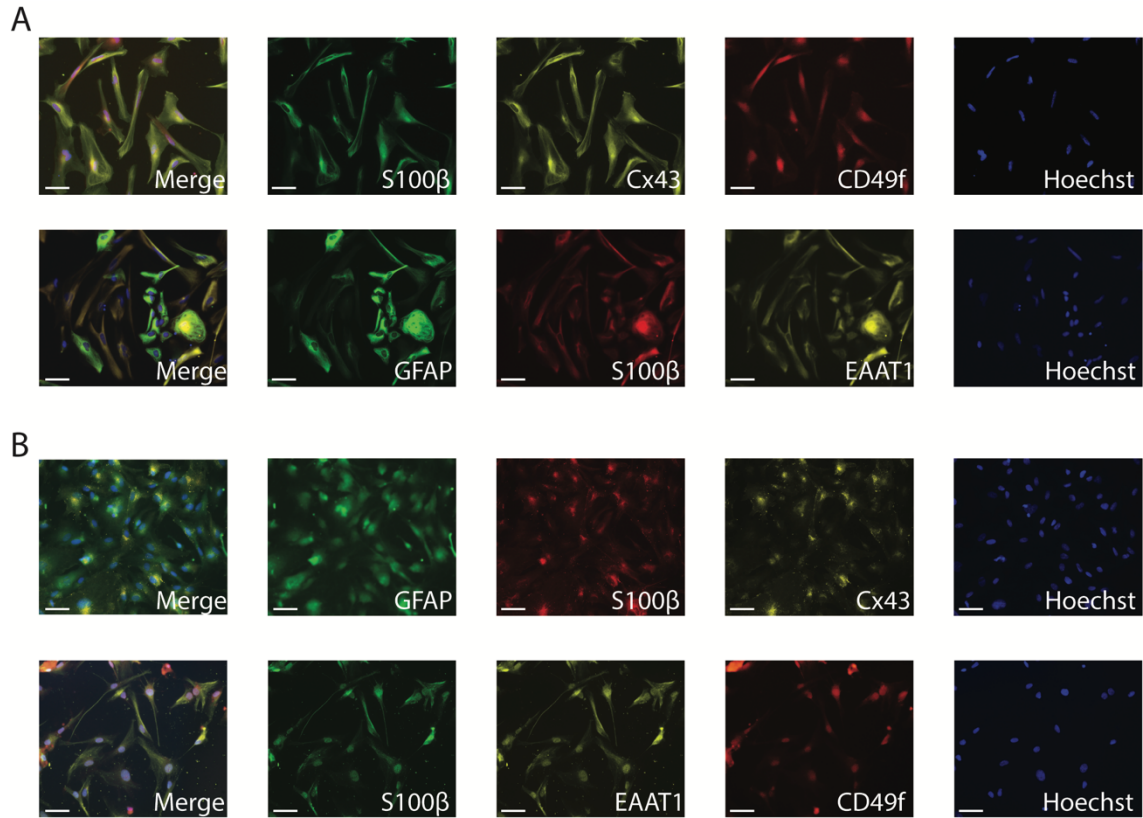


Figure 3.3.2 Cortical and ventral midbrain Astros stain positive for astrocyte markers and astrocyte-associated proteins.

CTX-Astros and VM-Astros were matured on coverslips and fixed for ICC analysis. Staining for anti-S100 β , GFAP, α Connexin-43, CD49f and EAAT1 was performed on both sets of cells. (A) Representative images of CTX-Astro controls staining positive for S100 β , Cx49, CD49f, GFAP and EAAT1. (B) Representative images of VM-Astro controls staining positive for S100 β , GFAP, Cx43, EAAT1 and CD49f. Imaged at x20 magnification. Scale bars: 50 μ m.

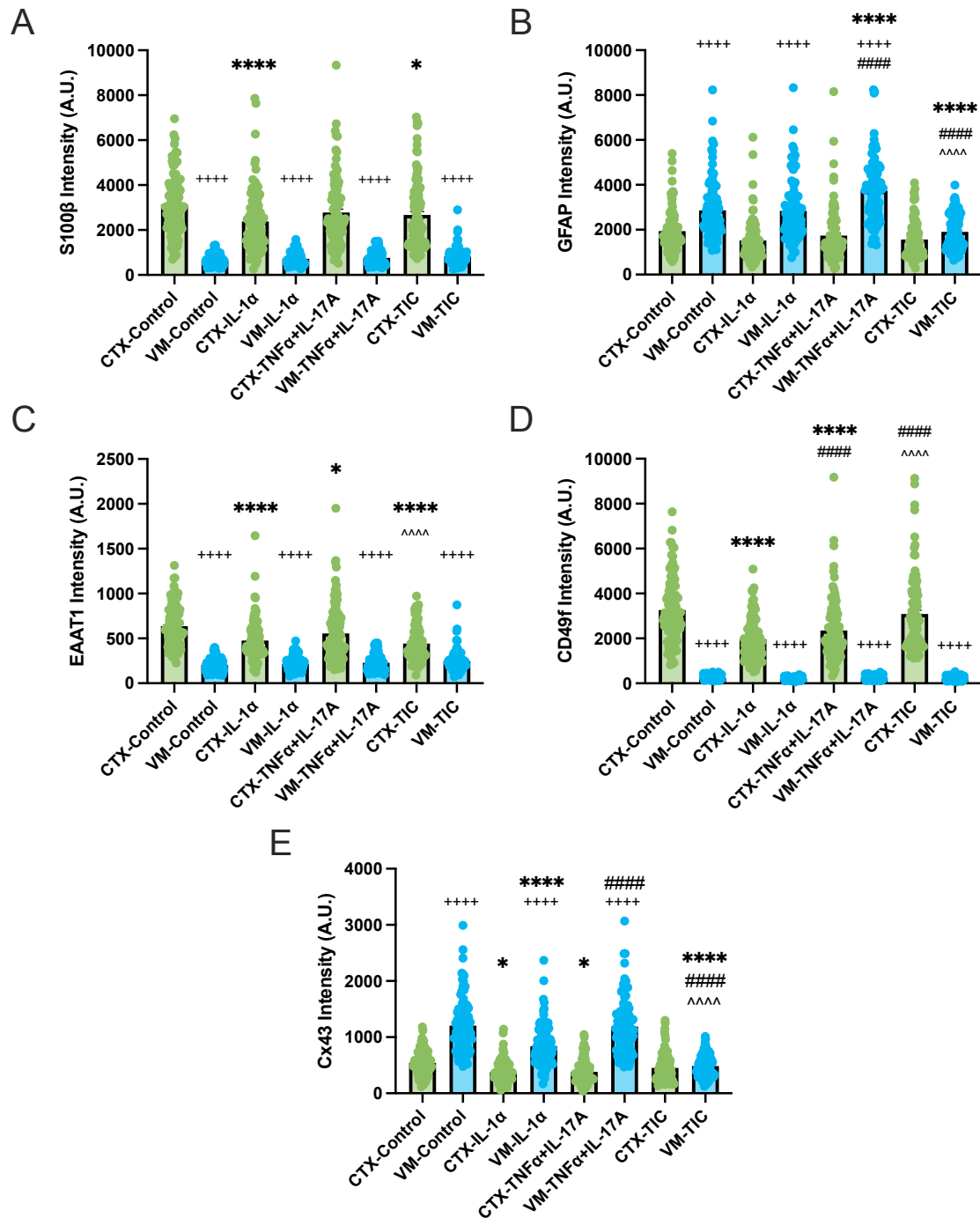


Figure 3.3.3 The intensity of staining for astrocyte markers and astrocyte-associated proteins differs between CTX- and VM-Astros.

(A) S100 β was highly expressed in CTX-Astros and, to a lesser extent, in VM-Astros. Stimulating CTX-Astros with IL-1 α or TIC resulted in a higher intensity of S100 β compared to the control. In contrast, **(B)** GFAP was more highly expressed in VM-Astros compared to CTX across control, IL-1 α and TNF α +IL-17A stimulation. Stimulation with TNF α +IL-17A significantly increases GFAP in VM-Astros compared to the control or IL-1 α stimulation. However, stimulation with TIC reduced GFAP intensity in the same cells, compared to the control, IL-1 α or TNF α +IL-17A stimulation. **(C)** Both CTX-

and VM-Astros expressed EAAT1, with the staining intensity being significantly higher in CTX-Astros compared to VM, regardless of stimulation. Compared to the control, stimulated CTX-Astros expressed significantly less EAAT1, with the intensity post-TIC stimulation being significantly lower compared to TNF α +IL-17A. Similarly, CD49f (**D**) was also expressed more highly in CTX-Astros compared to VM. Once stimulated with IL-1 α or TNF α +IL-17A, CD49f expression was significantly lower in CTX-Astros compared to the control. CD49f expression was much higher in CTX-Astros stimulated with TNF α +IL-17A or TIC compared to IL-1 α . In VM-Astros, CD49f intensity stayed the same regardless of stimulation. (**E**) Cx43 was expressed more highly in VM-Astros compared to CTX, except for TIC stimulation. Once stimulated with IL-1 α or TNF α +IL-17A, Cx43 intensity decreased significantly compared to the control. Similarly, in VM-Astros, stimulation with IL-1 α or TIC resulted in a significant decrease in Cx43 intensity compared to the control, with the expression being lowest post-TIC stimulation. Stimulation with TNF α +IL-17A did not change the Cx43 expression compared to the control but was significantly higher compared to the IL-1 α or TIC stimulation. Data expressed as mean $\pm$ SEM (N=3, 100 cells per N). *p<0.05, ****p<0.0001 vs control. ++++p<0.0001 vs CTX-Astros. ####p<0.0001 vs IL-1 α stimulation. ^^^p<0.0001 vs TNF α +IL-17A stimulation. Two-way ANOVA with Tukey's post hoc test.

3.3.3. Quiescent and reactive astrocytes from cortical and midbrain ventral regions exhibit morphological differences

The S100 β and Hoechst channels were used to quantify the differences between quiescent and reactive CTX- and VM-Astros. Namely, changes in cell area, perimeter, circularity and eccentricity, as well as Max and Min Feret diameter were taken into consideration. S100 β was selected for morphological analysis because it provides robust labelling of the astrocytic soma and cytoplasm in human iPSC-derived astrocytes, including populations with low basal GFAP expression. In contrast, GFAP expression in iPSC-derived astrocytes is heterogeneous and highly state-dependent, particularly under quiescent conditions, which limit its suitability for unbiased whole-cell morphological quantification (Zhang *et al.*, 2016; Barbar *et al.*, 2020; Escartin *et al.*, 2021). Representative S100 β immunocytochemistry images demonstrating morphological appearance of CTX- and VM-Astros are shown in **Fig. 3.3.2.A-B**.

As shown in **Fig. 3.3.4.A** below, at baseline, the cell area was approximately 5-fold larger in CTX-Astros compared to VM (++++p<0.0001). Cell area in CTX-Astros

stimulated with IL-1 α or TNF α +IL-17A was significantly lower compared to the control (**** $p<0.0001$ for both). However, stimulation with TIC resulted in a significantly higher cell area in CTX-Astros compared to IL-1 α (#### $p<0.0001$) and TNF α +IL-17A (^^^ $p<0.0001$) stimulation, but not the control. No significant differences were observed between the control and stimulation groups in VM-Astros.

Cell circularity (**Fig. 3.3.4.B** below) was significantly lower in VM-Astros at baseline compared to CTX (+ $p=0.0163$). It was also lower in VM-Astros stimulated with TNF α +IL-17A compared to CTX-Astros stimulated with the same cytokines(++ $p=0.0051$). In CTX-Astros, cell circularity was significantly lower post-TIC stimulation compared to IL-1 α (# $p=0.0230$), but not the control or TNF α +IL-17A stimulation. In VM-Astros, cell circularity significantly increased post-IL-1 α stimulation compared to the control (* $p=0.0287$). No other changes were observed in terms of cell circularity between the regions.

Similarly to the cell area, cell perimeter (**Fig. 3.3.4.C** below) was also significantly higher in CTX-Astros compared to VM (++++ $p<0.0001$), both at baseline and across all stimulation groups. In CTX-Astros stimulated with IL-1 α , the cell perimeter was significantly lower compared to the control (**** $p<0.0001$), TNF α +IL-17A (#### $p<0.0001$) and TIC (#### $p<0.0001$) stimulations. No changes in cell perimeter were observed between the control and stimulation groups in VM-Astros.

Very few changes were observed in terms of cell eccentricity (see **Fig. 3.3.4.D** below) in both CTX and VM-Astros. Namely, the singular regional difference between CTX and VM-Astros was observed post-stimulation with TIC where VM-Astros' cell eccentricity was significantly lower than CTX (+ $p=0.0144$). In CTX-Astros, stimulation with TNF α +IL-17A resulted in a significantly lower cell eccentricity compared to the control (* $p=0.0218$). No other changes in cell eccentricity were observed between the regions or post-stimulation with pro-inflammatory factors.

Max Feret diameter (**Fig. 3.3.4.E** below) was also significantly lower in VM-Astros compared to CTX (++++ $p < 0.0001$), both at baseline and across all stimulation groups. In CTX-Astros, max Feret diameter significantly decreased post-stimulation with IL-1 α (**** $p < 0.0001$), TNF α +IL-17A (**** $p < 0.0001$), and TIC (** $p = 0.0040$). Stimulation with TIC in CTX-Astros resulted in a higher max Feret diameter compared to IL-1 α (#### $p < 0.0001$) and TNF α +IL-17A ($^{\wedge}p = 0.0403$) stimulations, whereas stimulation with TNF α +IL-17A yielded a significantly higher max Feret diameter compared to IL-1 α stimulation ($\#p = 0.0292$). No significant changes in max Feret diameter were observed in any of the VM-Astro groups.

Similarly, the min Feret diameter (as shown in **Fig. 3.3.4.F** below) was also significantly lower in VM-Astros compared to CTX across all groups (++++ $p < 0.0001$). In CTX-Astros, stimulation with IL-1 α resulted in a significantly lower min Feret diameter compared to the control (**** $p < 0.0001$), TNF α +IL-17A ($\#p = 0.0017$) and TIC (#### $p < 0.0001$) stimulation. No significant changes were observed in min Feret diameter across any of the VM-Astro groups.

To summarise, CTX-Astros were larger and more morphologically diverse than VM-Astros, with the cells becoming smaller in terms of cell area, perimeter and both min and max Feret diameter post-stimulation. VM-Astros, although smaller in size than CTX-Astros, were less prone to morphological changes post-stimulation.

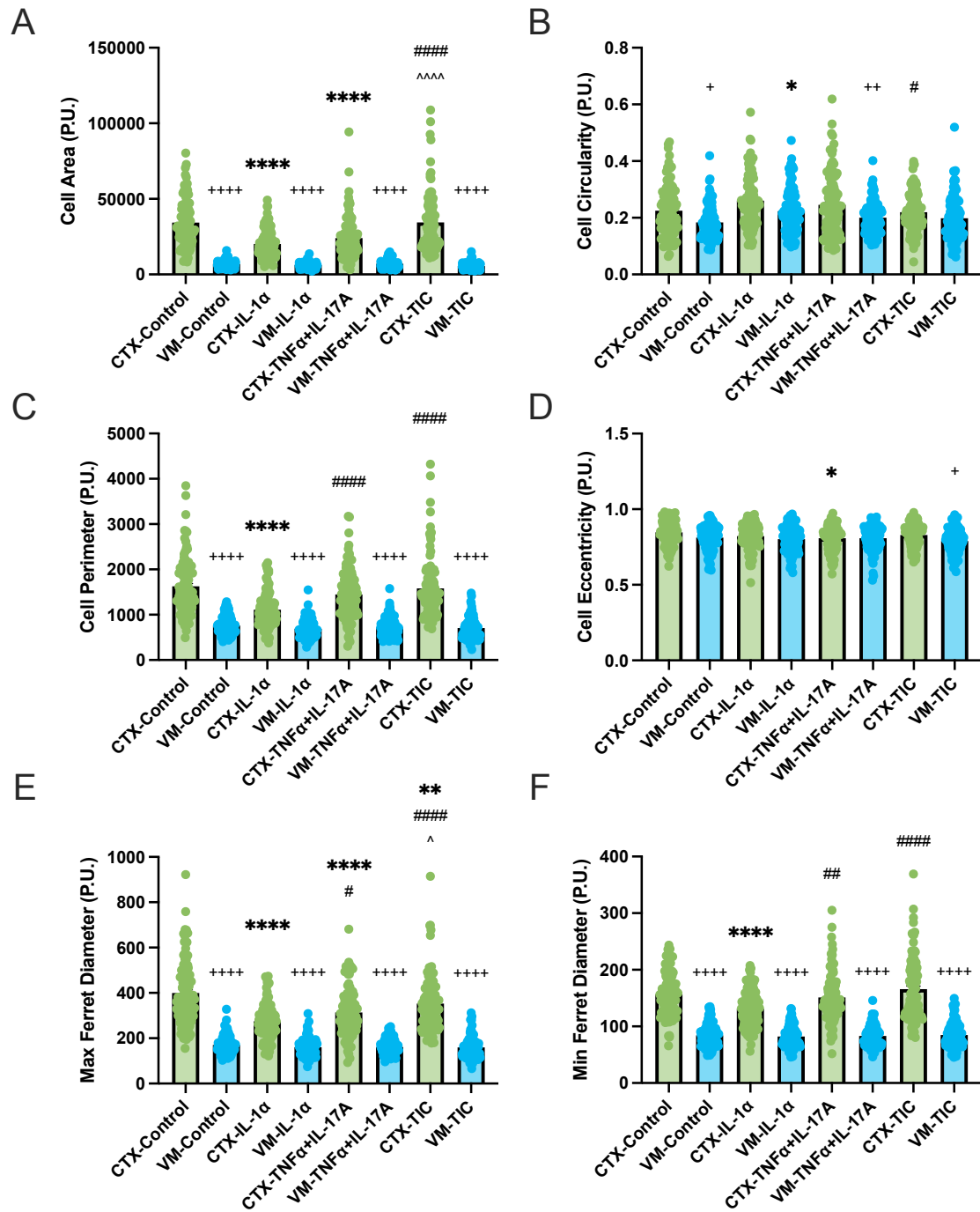


Figure 3.3.4 Astrocyte morphology differs in cortical and ventral regions.

(A) VM-Astros cell area was smaller at baseline and across all stimulation groups compared to CTX. Cell area significantly decreased in CTX-Astros stimulated with IL-1α or TNFα+IL-17A compared to control. Stimulation of CTX-Astros with TIC resulted in a significantly higher cell area compared to IL-1α or TNFα+IL-17A stimulations. **(B)** Cell circularity was significantly lower in VM-Astros at baseline and post-TNFα+IL-17A stimulation compared to CTX. However, stimulation with IL-1α resulted in a

significantly lower cell circularity in VM-Astros compared to the control. Stimulation of CTX-Astros with TIC resulted in significantly lower cell circularity compared to IL-1 α stimulation. **(C)** In VM-Astros, the cell perimeter was significantly lower at baseline and post-stimulation compared to CTX. Stimulation with IL-1 α significantly lowered cell perimeter in CTX-Astros, compared to the control, while both TNF α +IL-17A and TIC stimulations resulted in a higher cell perimeter compared to IL-1 α stimulation. **(D)** Cell eccentricity only differed between CTX and VM-Astros post-TIC stimulation. CTX-Astros stimulated with TNF α +IL-17A had a reduced cell eccentricity compared to the control. **(E)** Max Feret Diameter was significantly lower in VM-Astros at baseline and across all stimulation groups, compared to CTX. In CTX-Astros, Max Feret diameter significantly reduced post-stimulation with IL-1 α , TNF α +IL-17A and TIC compared to the control. Stimulation of CTX-Astros with TIC resulted in the highest Max Feret diameter across stimulation groups, while TNF α +IL-17A stimulation yielded a higher Max Feret diameter compared to IL-1 α . **(F)** Min Feret diameter was also lower in VM-Astros compared to CTX, regardless of stimulation. In CTX-Astros, IL-1 α stimulation resulted in a significantly lower Min Feret diameter compared to the control, whereas both TNF α +IL-17A and TIC stimulations resulted in a higher Min Feret diameter compared to IL-1 α . Data expressed as mean $\pm$ SEM (N=3, 100 cells per N). * p <0.05, ** p <0.01, **** p <0.0001 vs control. + p <0.05, ++ p <0.01, +++ p <0.0001 vs CTX-Astros. # p <0.05, ## p <0.01, #### p <0.0001 vs IL-1 α stimulation. ^ p <0.05, ^^^ p <0.0001 vs TNF α +IL-17A stimulation. Two-way ANOVA with Tukey's post hoc test.

3.3.4. Confirmation of regional gene expression in CTX- and VM-Astros

CTX- and VM-Astros, including the stimulated groups, were assessed for the expression of the regional marker genes, namely, *FOXP1* for the cortical region, and *LMX1A*, *EN1* and *OTX2* for the ventral midbrain region. CTX-Astros expressed *FOXP1* mRNA (see **Fig. 3.3.5.A** below) both at baseline and post-stimulation. *FOXP1* mRNA expression significantly increased post-IL-1 α (** p =0.0077) and TIC (**** p <0.0001) stimulation compared to the control, with a 4-fold and 7-fold increase, respectively. Expression of *FOXP1* mRNA differed between stimulation groups in CTX-Astros, with the TNF α +IL-17A group having similar expression to the control and being significantly lower than the IL-1 α stimulation group (# p =0.0216). On the other hand, stimulation with TIC resulted in the highest expression of *FOXP1* mRNA compared to the control (**** p <0.0001), IL-1 α (## p =0.0037) and TNF α +IL-17A (^^^ p <0.0001) stimulation groups.

VM-Astros expressed mRNA for their regional markers *LMX1A*, *EN1* and *OTX2* (see **Fig. 3.3.5.B-D** below). *LMX1A* mRNA (**Fig. 3.3.5.B** below) expression increased from ~5 RQ at baseline to ~27 RQ post-stimulation with TNF α +IL-17A (****p<0.0001). Stimulation with TNF α +IL17A resulted in a significantly higher *LMX1A* mRNA expression compared to IL-1 α stimulation (####p<0.0001). However, stimulation with TIC significantly reduced *LMX1A* mRNA expression almost to zero, compared to the control (*p=0.0469) or TNF α +IL-17A stimulation (^^^p<0.0001). Similarly, stimulation with IL-1 α also severely reduces *LMX1A* mRNA expression compared to the control, but this did not reach significance (p=0.0580).

VM-Astros expressed high levels of *EN1* mRNA (**Fig. 3.3.5.C** below) at 8,000 RQ at baseline. This expression was significantly decreased post-IL1 α (**p=0.0042) and TIC (****p<0.0001) stimulation compared to the control, to ~6,000 and ~4,500 RQ, respectively. TNF α +IL-17A stimulation yields the highest *EN1* mRNA expression at ~11,800 RQ compared to the control (****p<0.0001) and IL-1 α stimulation (####p<0.0001). Among all stimulation groups, *EN1* mRNA levels are lowest post-TIC stimulation compared to the control (****p<0.0001), IL-1 α (###p=0.0006) and TNF α +IL-17A (^^^p<0.0001) stimulation.

As seen in **Fig. 3.3.5.D** below, *OTX2* mRNA expression levels in VM-Astros were quite low at approximately 2.5-3 RQ for the control, IL-1 α and TNF α +IL-17A stimulation groups. *OTX2* mRNA expression further decreased post-TIC stimulation compared to the control (****p<0.0001), IL-1 α (###p=0.0007) and TNF α +IL-17A stimulation (^^^p=0.0001).

Overall, both CTX and VM-Astros expressed their respective regional markers at the gene level, thus confirming their regionality post-astrocyte differentiation protocol, with the *FOXP1* mRNA levels increasing in CTX-Astros stimulated with IL-1 α and TIC, while *LMX1A* and *EN1* mRNA increased in VM-Astros post-TNF α +IL-17A stimulation but decreased post-IL-1 α and TIC stimulation. *OTX2* mRNA expression in VM-Astros was only affected post-TIC stimulation, with the expression decreasing.

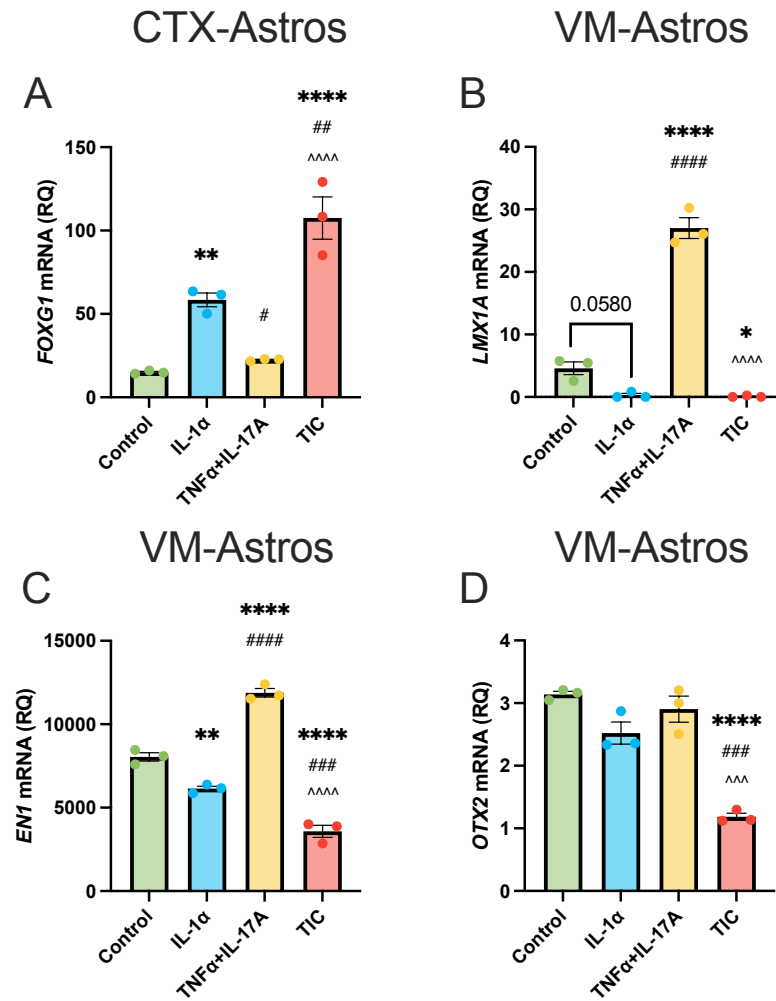


Figure 3.3.5 CTX- and VM-Astros Express the Region-Specific Genes.

CTX- **(A)** and VM-Astros **(B-D)** expressed the mRNA for their regional genes. **(A)** CTX-Astros expressed the FOXG1 mRNA at baseline, as well as post-stimulation, with significantly higher expression post-IL-1 α and TIC stimulation. VM-Astros expressed LMX1A **(B)**, EN1 **(C)** and OTX2 **(D)** mRNA at baseline and post-stimulation. Higher expression of LMX1A **(B)** was observed post TNF α +IL-17A stimulation in VM-Astros, while TIC-stimulation reduced the mRNA expression. EN1 **(C)** mRNA in VM-Astros was reduced by IL-1 α and TIC stimulation, while TNF α +IL-17A stimulation resulted in a higher expression. OTX2 **(D)** mRNA expression in VM-Astros was reduced with TIC stimulation. Data expressed as mean $\pm$ SEM (N=3, r=3). * p <0.05, ** p <0.01, *** p <0.0001 vs control. # p <0.05, ## p <0.01, ### p <0.001, #### p <0.0001 vs IL-1 α stimulation. ^^^ p <0.001, ^^^^ p <0.0001 vs TNF α +IL-17A stimulation. One-way ANOVA with Tukey's post hoc test.

3.3.5. Differential expression of reactive markers in CTX- and VM-Astros stimulated with pro-inflammatory factors

One of the functions of astrocytes is their ability to respond to pro-inflammatory stimuli and become reactive. There are many reactive genes expressed by astrocytes, including *GFAP* and *VIM*, intermediate filament proteins associated with astrocyte structural remodelling; *C3*, a complement component linked to inflammatory and neurotoxic astrocyte states; *LCN2*, an acute phase inflammatory mediator involved in iron homeostasis and immune signalling; *SERPINA3*, a reactive protease inhibitor associated with neuroinflammation; and *TIMP1*, a regulator of extracellular matrix remodelling and inflammatory responses (Escartin *et al.*, 2021).

Unlike at the protein level, at baseline, *GFAP* mRNA expression (see **Fig. 3.3.6.A** below) was lower in VM-Astros compared to CTX (++++ $p < 0.0001$). Similar levels of *GFAP* mRNA were observed post-stimulation with TNF α +IL-17A, where the expression was lower in VM-Astros compared to CTX (+++ $p = 0.0001$). In CTX-Astros, *GFAP* mRNA expression was reduced in cells stimulated with IL-1 α (**** $p < 0.0001$) or TIC (**** $p < 0.0001$), compared to the control. Stimulation with TNF α +IL-17A resulted in significantly higher *GFAP* mRNA levels compared to IL-1 α (### $p = 0.0006$), while *GFAP* expression in CTX-Astros stimulated with TIC was considerably lower compared to TNF α +IL-17A (^^^ $p < 0.0001$). No significant changes in *GFAP* gene expression were observed between VM-Astros, regardless of stimulation.

The *C3* mRNA expression (**Fig. 3.3.6.B** below) significantly increased in both regions across all stimulation groups compared to the control (**** $p < 0.0001$). Although at baseline, both CTX- and VM-Astros expressed the same levels of *C3* mRNA, VM-Astrocytes expressed significantly higher levels of *C3*, compared to CTX, once stimulated with IL-1 α (+++ $p = 0.0001$), TNF α +IL-17A (+++ $p = 0.0003$) or TIC (++++ $p < 0.0001$). In both CTX- and VM-Astros, *C3* mRNA levels were significantly lower in TNF α +IL-17A-stimulated groups compared to IL-1 α (#### $p < 0.0001$). However, cells stimulated with TIC expressed significantly more *C3* mRNA

compared to IL-1 α (###p=0.0001 for CTX, ####p<0.0001 for VM-Astros) or TNF α +IL-17A stimulation (^^^p<0.0001 for both) across both CTX and VM regions.

At baseline, CTX and VM-Astros expressed low levels of *LCN2* mRNA (**Fig. 3.3.6.C** below), and although CTX-Astros expressed more *LCN2* than VM, this result was not significant. However, regional differences were observed in *LCN2* mRNA levels post-stimulation with IL-1 α where VM-Astros expressed 3-fold less *LCN2* compared to CTX (++++p<0.0001). Overall, stimulation with pro-inflammatory factors resulted in a higher expression of *LCN2* in CTX-Astros (****p<0.0001 for IL-1 α , *p=0.0398 for TNF α +IL-17A and **p=0.0070 for TIC) and to an extent in VM-Astros, but this was only significant post-stimulation with TNF α +IL-17A (**p=0.0073). In CTX-Astros, *LCN2* expression was reduced post-TNF α +IL-17A (####p<0.0001) and TIC (###p=0.0005) stimulation compared to IL-1 α . No significant differences in *LCN2* mRNA expression were observed between stimulation groups in VM-Astros.

The expression of *SERPINA3* mRNA (**Fig. 3.3.6.D** below) significantly differed between VM- and CTX-Astros, with a 4-fold higher expression in VM-Astros compared to CTX, both at baseline and post-stimulation (++++p<0.0001 for all groups). Stimulation of VM-Astros with IL-1 α , TNF α +IL-17A or TIC resulted in a significantly higher *SERPINA3* mRNA expression compared to the control (****p<0.0001 for all), with the TNF α +IL-17A (#p=0.0310) and TIC (##p=0.0013) stimulations inducing higher *SERPINA3* levels compared to IL-1 α . CTX-Astros also expressed more *SERPINA3* mRNA post-stimulation (****p<0.0001 for all three groups), but there were no significant differences observed between the stimulation groups.

Similarly to *LCN2*, *TIMP1* (**Fig. 3.3.6.E**) mRNA were expressed at low levels in CTX and VM-Astros at baseline. In both VM and CTX-Astros, *TIMP1* gene expression increased post stimulation with TIC (****p<0.0001), with the expression being significantly lower in VM-Astros compared to CTX (+++p=0.0008). No other regional differences were observed in *TIMP1* expression between these cells. Stimulation with TIC in CTX-Astros resulted in a significant increase of *TIMP1* mRNA expression compared to IL-1 α (####p<0.0001) and TNF α +IL-17A (^^^p<0.0001) stimulations. Stimulation of CTX-Astros with IL-1 α also resulted in a *TIMP1* mRNA level increase

compared to the control and TNF α +IL-17A stimulation, but these results were non-significant ($p=0.0516$ and $p=0.0555$, respectively). In contrast, stimulation of VM-Astros with TIC resulted in a significant increase in *TIMP1* mRNA expression compared to the control (**** $p<0.0001$), IL-1 α (# $p=0.0175$) and TNF α +IL-17A stimulation (^^^ $p<0.0001$).

VIM mRNA expression (**Fig. 3.3.6.F** below) in CTX and VM-Astros followed a similar pattern to that of *GFAP*, although at a much lower level, with the expression decreasing post-stimulation compared to the control. Although the mRNA expression levels were higher in VM-Astros compared to CTX across all groups, this was only significant post-stimulation with TIC (+ $p=0.0344$). *VIM* mRNA was significantly lower in CTX-Astros post-stimulation with IL-1 α (** $p=0.0002$), TNF α +IL-17A (* $p=0.0334$) and TIC stimulation (**** $p<0.0001$) compared to the control, with the mRNA levels being lowest in the TIC-stimulated group compared to TNF α +IL-17A stimulation (^ $p=0.0015$). However, in VM-Astros, *VIM* mRNA levels were only significantly decreased post-stimulation with IL-1 α (** $p=0.0013$) and TIC (** $p=0.0009$) compared to control, with TIC levels being lowest compared to the TNF α +IL-17A stimulation group (^ $p=0.0462$). No other significant changes were detected in *VIM* mRNA expression between the two regions or stimulation groups.

To summarise, most of the reactive genes looked at resulted in higher mRNA expression post-stimulation, with the exception of *GFAP* and *VIM*. Some of the genes were more highly expressed in CTX-Astros (*GFAP*, *LCN2* and *TIMP*), while others had a higher mRNA expression in VM-Astros (*C3*, *SERPINA3* and *VIM*), although this differed depending on the stimulation. However, overall, VM- and CTX-Astros expressed reactive genes, which were altered in both regions with the introduction of pro-inflammatory stimuli.

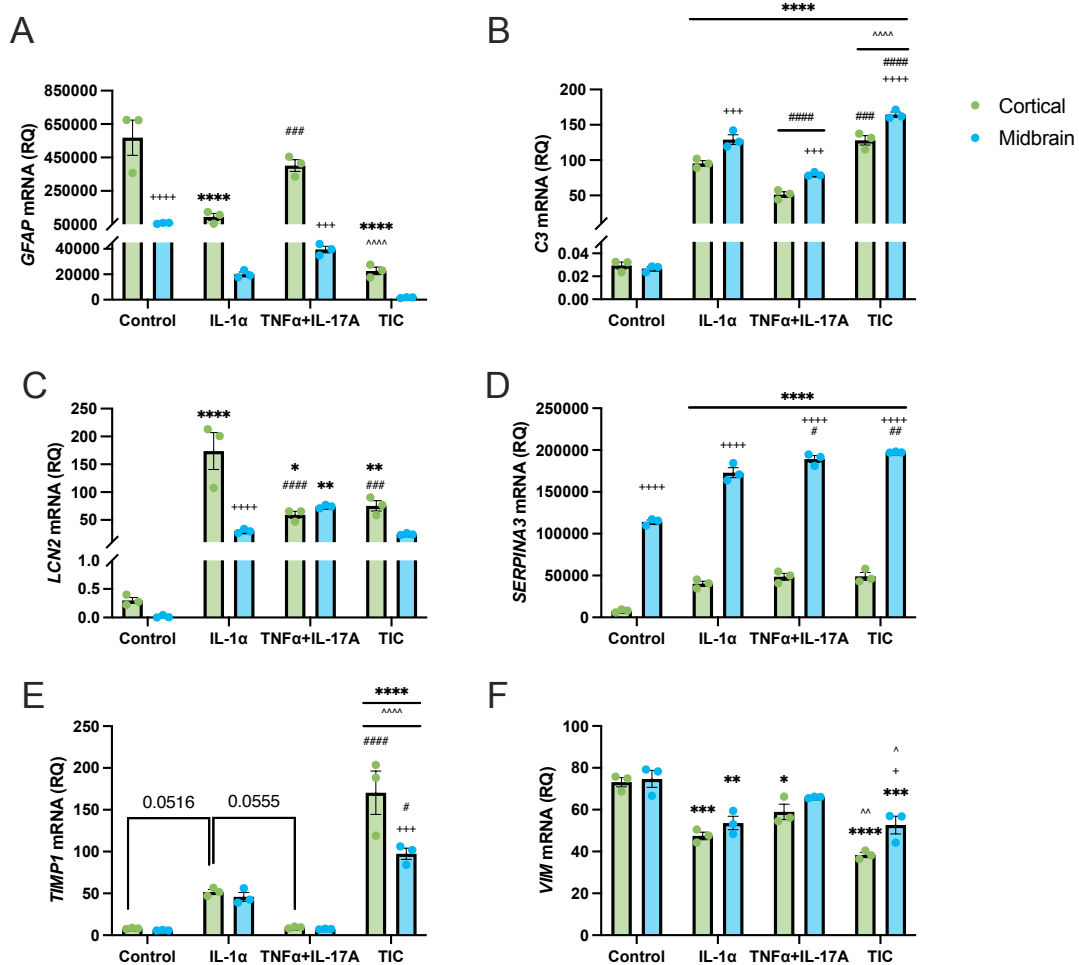


Figure 3.3.6 CTX- and VM-Astros differentially express reactive markers mRNA at baseline and post-stimulation.

Stimulation with pro-inflammatory cytokines resulted in a downregulation of GFAP (A) and VIM (F) mRNA, while upregulating (B) C3, (C) LCN2, (D) SERPINA3 and (E) TIMP1 mRNA. At baseline, (A) GFAP mRNA expression was higher and SERPINA3 (D) was lower in CTX-Astros compared to VM-Astros. (A) GFAP mRNA expression was higher in CTX-Astros post-stimulation with TNF α +IL-17A. VM-Astros expressed more C3 (B) and SERPINA3 (D) mRNA across all stimulation groups. CTX-Astros expressed more LCN2 (C) post-stimulation with IL-1 α and more TIMP1 (E) post-stimulation with TIC factors. There was a higher expression of VIM (F) in VM-Astros post-stimulation with TIC factors. Data expressed as mean $\pm$ SEM (N=3, r=3). * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001 vs control. + p <0.05, +++ p <0.001, ++++ p <0.0001 vs CTX-Astros. # p <0.05, ## p <0.01, ### p <0.001, #### p <0.0001 vs IL-1 α stimulation. ^ p <0.05, ^^ p <0.01, ^^ p <0.0001 vs TNF α +IL-17A stimulation. Two-way ANOVA with Holm-Šidák post hoc test.

3.3.6. CTX- and VM-Astros differ in their response to pro-inflammatory stimuli

One of the most critical aspects of culturing iPSC-derived astrocytes is to ensure that they are functional, and therefore capable of releasing cytokines/chemokines such as IL-6 or CXCL10 in response to pro-inflammatory stimuli, just like astrocytes in the brain would. CTX and VM-Astros supernatants were used to assess their ability to release IL-6, CXCL10, GM-CSF, and RANTES, which are all signs of astrocyte reactivity.

Both CTX and VM-Astros released low levels of IL-6 at baseline (see **Fig. 3.3.7.A** below), with no regional differences between the two. However, once stimulated with IL-1 α (+p=) or TNF α +IL-17A (++++p<0.0001), VM-Astros released significantly more IL-6 compared to CTX. VM-Astros released potent amounts of IL-6 regardless of the stimulant compared to the control (****p<0.0001 for all three), ranging between 2,000 pg/mL in TNF α +IL-17A stimulation and ~7,000 pg/mL post-IL-1 α or TIC stimulation. IL-6 release post-TNF α +IL-17A stimulation in VM-Astros was significantly lower compared to IL-1 α (####p<0.0001) or TIC stimulation (^^^p<0.0001). CTX-Astros also released significantly higher levels of IL-6 post-stimulation with IL-1 α (****p<0.0001) or TIC (****p<0.0001), but not TNF α +IL-17A, compared to the control. Stimulation of CTX-Astros with TIC resulted in the most potent IL-6 release of ~7,200 pg/mL compared to IL-1 α (~6,500 pg/mL, ###p=0.0004) and TNF α +IL-17A (~500 pg/mL, ^^^p<0.0001).

CXCL10 (**Fig. 3.3.7.B** below), a pro-inflammatory chemokine, was also significantly increased post-stimulation in both CTX- and VM-Astros. At baseline, Astros from both regions released only small amounts of CXCL10, with no significant difference between the two regions. However, once stimulated with pro-inflammatory factors, both CTX- and VM-Astros released enormous amounts of CXCL10 of ~8,000 pg/mL, with the exception of TNF α +IL-17A stimulation in CTX-Astros, where CXCL10 release reached approximately 3,000 pg/mL, compared to the control (****p<0.0001 for all stimulated groups in CTX and VM-Astros). Regional differences were only found in TNF α +IL-17A-stimulated Astros, where VM-

Astrocytes released ~2.5-fold more CXCL10 compared to CTX (++++p<0.0001). Overall, stimulation of CTX-Astros with TNF α +IL-17A resulted in a significantly lower CXCL10 release compared to IL-1 α (####p<0.0001) or TIC (^^^p<0.0001). There were no significant differences observed in CXCL10 release post-stimulation in VM-Astros.

In contrast, GM-CSF release (**Fig. 3.3.7.C** below) was much lower in both CTX and VM-Astros. There were no regional differences observed in terms of GM-CSF at baseline, but stimulation of CTX-Astros with TIC yielded a ~1.5-fold higher GM-CSF release compared to VM (++++p<0.0001). The other two stimulation groups, IL-1 α and TNF α +IL-17A, displayed no regional differences. CTX and VM-Astros released high levels of GM-CSF post-stimulation with IL-1 α (**p=0.0007 for CTX and ***p=0.0004 for VM) and TIC (****p<0.0001) compared to the control. Stimulation with TIC yielded significantly higher release of GM-CSF compared to the control (****p<0.0001), IL-1 α (####p<0.0001) and TNF α +IL-17A stimulation (^^^p<0.0001). In contrast, stimulation with TNF α +IL-17A did not increase the release of GM-CSF compared to the control and was significantly less effective than IL-1 α stimulation (####p<0.0001), in both CTX and VM-Astros.

As seen in **Fig. 3.3.7.D** below, there were no regional differences in RANTES release at baseline. However, once stimulated with pro-inflammatory factors, RANTES release in VM-Astros was significantly higher compared to CTX (****p<0.0001 for IL-1 α , *p=0.0246 for TNF α +IL-17A and **p=0.0031 for TIC). CTX-Astros stimulated with TIC released the highest levels of RANTES compared to the control (****p<0.0001), IL-1 α (####p<0.0001) and TNF α +IL-17A (^^^p<0.0001) stimulation. This was followed by IL-1 α stimulation which resulted in a significant release of RANTES compared to the control (****p<0.0001) and IL-1 α (####p<0.0001). RANTES release in CTX-Astros post-TNF α +IL-17A stimulation was similar to that of the control group. In contrast, TNF α +IL-17A stimulation in VM-Astrocytes resulted in a significant release of RANTES compared to the control (*p=0.0120), but it was significantly lower compared to the IL-1 α (####p<0.0001) and TIC stimulation (^^^p<0.0001). Similarly to the TIC stimulation in CTX, VM-Astros yielded the highest release of RANTES post-TIC

stimulation compared to the control (**** $p < 0.0001$), IL-1 α (#### $p < 0.0001$) and TNF α +IL-17A stimulation (^^^ $p < 0.0001$).

Overall, stimulation of CTX and VM-Astros with pro-inflammatory factors resulted in a further release of pro-inflammatory cytokines and chemokines by Astros, especially when stimulated with IL-1 α and TIC. However, stimulation with TNF α +IL-17A was mainly effective in VM-Astros only in terms of immune response, except for CXCL10, which was also released by CTX-Astros post-TNF α +IL-17A stimulation.

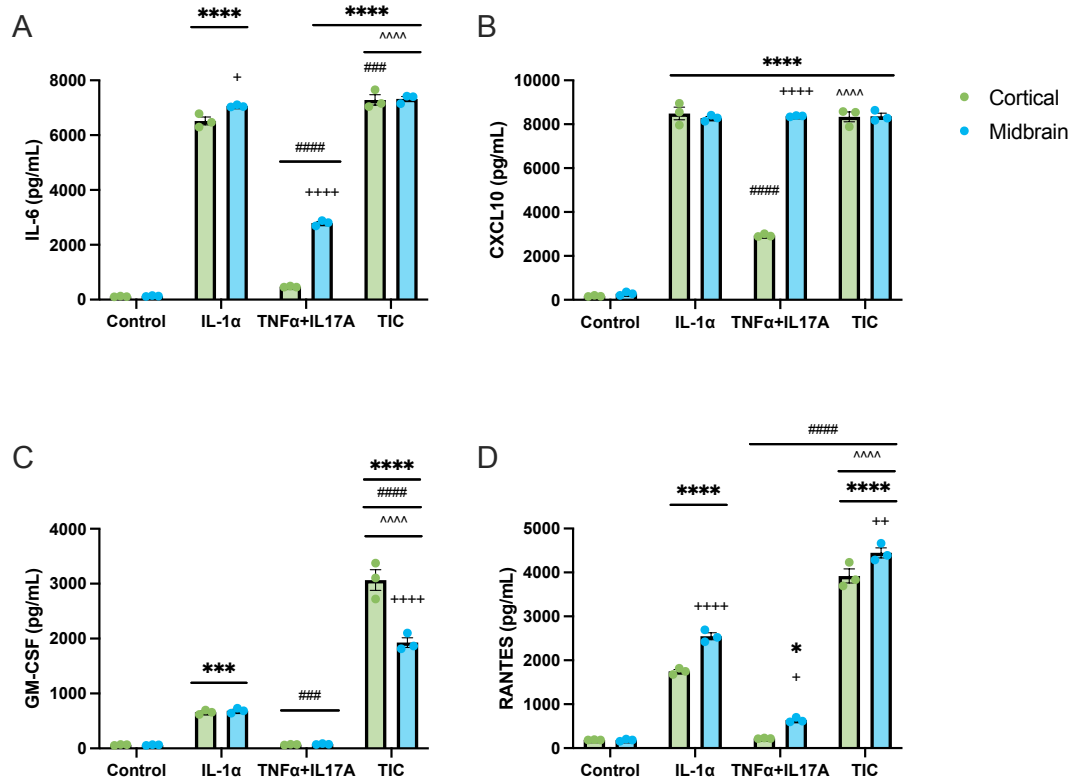


Figure 3.3.7 Cytokine and chemokine release by astrocytes is dependent on the region and stimuli.

CTX- and VM-Astros released pro-inflammatory cytokines and chemokines (A-D) upon stimulation with IL-1α and TIC factors, but stimulation with TNFα+IL-17A was only effective in VM Astros. (A) Both CTX- and VM-Astros released IL-6 when reactive, but VM-Astros released significantly more IL-6 when stimulated with IL-1α or TNFα+IL-17A. Both regions released significantly less IL-6 post TNFα+IL-17A stimulation compared to IL-1α or TIC stimulation. (B) The same trend was observed in terms of CXCL10 release; however, the regional differences were only observed in TNFα+IL-17A stimulation, with VM-Astros releasing significantly more CXCL10. Stimulation with TNFα+IL-17A was also significantly less effective in CTX-Astros compared to stimulation with IL-1α or TIC factors. (C) GM-CSF was released by both CTX and VM-Astros when stimulated with IL-1α and TIC, with TIC stimulation resulting in the highest GM-CSF release. CTX-Astros stimulated with TIC released much higher levels of GM-CSF compared to VM. (D) All stimulation groups resulted in a significant increase in RANTES release except for TNFα+IL-17A stimulation of CTX Astros. VM-Astros released significantly higher levels of RANTES across all stimulation compared to CTX, with IL-1α and TIC stimulation resulting in a much higher RANTES release compared to TNFα+IL-17A stimulation. Data expressed as mean ± SEM (N=3, r=3). *p<0.05, ***p<0.001, ****p<0.0001 vs control. +p<0.05, +++p<0.0001 vs CTX-Astros. ###p<0.001, ####p<0.0001 vs IL-1α stimulation. ^^^p<0.0001 vs TNFα+IL-17A stimulation. Two-way ANOVA with Holm-Šidák post hoc test.

3.3.7. Inflammatory response in CTX and VM-Astros also differs at gene level

Apart from comparing the responses of CTX and VM-Astros to pro-inflammatory stimuli at protein level, we were also interested in looking at it at the gene level. Therefore, several genes associated with the inflammatory response in astrocytes were assessed between the two regions, including *IL6*, *IL17RA*, *CXCL10*, *CCL5*, *ICAM1*, *PTX3*, *TLR4*, *IL1B*, *CSF2*, *ITGAM*, *IFNA2* and *IFNB1*, as seen in **Figure 3.3.8.A-L** below.

Similarly to the protein expression, *IL6* mRNA (**Fig. 3.3.8.A**) levels were relatively similar between the two regions. Regional differences were only displayed post-stimulation with TIC, where *IL6* mRNA was significantly higher in VM-Astros compared to CTX (++++ $p < 0.0001$). In CTX-Astros, stimulation with IL-1 α and TIC effectively induced *IL6* mRNA expression compared to the control (**** $p < 0.0001$). However, stimulation with TNF α +IL-17A did not increase *IL6* mRNA expression in CXT-Astros. In this case, *IL6* mRNA levels were similar to the control, and were significantly lower compared to the IL-1 α and TIC stimulation groups (#### $p < 0.0001$, ^^^^ $p < 0.0001$, respectively). On the other hand, stimulation of CTX-Astros with TIC, resulted in very potent *IL6* gene levels, which were significantly higher than the IL-1 α stimulation group (#### $p < 0.0001$). Similarly to CTX, VM-Astros also expressed high levels of *IL6* mRNA post-stimulation with IL-1 α (**** $p < 0.0001$), TNF α +IL-17A (* $p = 0.0441$) and TIC (**** $p < 0.0001$), compared to the control. Although *IL6* gene levels in VM-Astros post-stimulation with TNF α +IL-17A were still significantly lower than post-IL-1 α stimulation (#### $p < 0.0001$), these levels were much higher than in CTX-Astros. VM-Astros stimulated with TIC express significantly more *IL6* mRNA than the cells stimulated with IL-1 α (#### $p < 0.0001$) or TNF α +IL-17A (^^^ $p < 0.0001$).

Unlike *IL6*, the *IL17RA* (**Fig. 3.3.8.B** below) mRNA expression was much lower in both CTX and VM-Astros at baseline and post-stimulation. VM-Astros expressed significantly less *IL17RA* mRNA compared to CTX at baseline (+ $p = 0.0112$) as well as post stimulation with IL-1 α (++ $p = 0.0017$), TNF α +IL-17A (+ $p = 0.0324$) or

TIC (+p=0.0313). Although stimulation of CTX-Astros with IL-1 α (*p=0.0493) and TNF α +IL-17A (*p=0.0176) resulted in a higher *IL17RA* mRNA expression compared to the control, this increase was still relatively low. In VM-Astros, a similar increase was observed post-stimulation with TNF α +IL-17A (**p=0.0055) and TIC (*p=0.0493) compared to the control. There were no significant differences observed between different stimulation groups in CTX or VM-Astros.

CXCL10 mRNA expression (**Fig. 3.3.8.C**) also differed between the two regions. Although at baseline *CXCL10* gene levels were similar, once stimulated, VM-Astros expressed significantly more *CXCL10* mRNA compared to CTX (++++p<0.0001 for IL-1 α and TIC; ++p=0.0068). Unlike at protein level, *CXCL10* mRNA had a significantly higher expression in CTX-Astros stimulated with TIC only compared to the control (****p<0.0001), but not IL-1 α or TNF α +IL-17A. This *CXCL10* mRNA expression post-TIC stimulation in CTX-Astros was significantly higher compared to IL-1 α (###p=0.0009) and TNF α +IL-17A (^^^p<0.0001). However, in VM-Astros, stimulation with IL-1 α (****p<0.0001), TNF α +IL-17A (*p=) and TIC (****p<0.0001) resulted in a higher *CXCL10* mRNA expression compared to the control. At a staggering 50,000-fold increase from the control, VM-Astros stimulated with TNF α +IL-17A resulted in a lower *CXCL10* mRNA expression compared to IL-1 α (####p<0.0001) stimulation, which is 3-fold higher. *CXCL10* mRNA expression post-stimulation of VM-Astros with TIC was approximately 2-fold higher compared to IL-1 α (####p<0.0001) and ~5-fold higher compared to TNF α +IL-17A-stimulated group (^^^p<0.0001).

The expression of *CCL5* mRNA (**Fig. 3.3.8.D** below) followed a similar trend to that of *IL6* mRNA. Although there were no regional differences observed at baseline or post-stimulation with IL-1 α or TNF α +IL-17A, stimulation with TIC yielded a higher *CCL5* mRNA expression in VM-Astros compared to CTX (+++p=0.0002). Stimulation of CTX-Astros with IL-1 α (**p=0.0016) or TIC (****p<0.0001) resulted in a higher *CCL5* mRNA expression compared to the control, with the *CCL5* gene levels post-TIC stimulation being significantly higher compared to IL-1 α (####p<0.0001) or TNF α +IL-17A (^^^p<0.0001) stimulation. On the other hand, stimulation with TNF α +IL-17A resulted in a significantly lower *CCL5* mRNA expression compared to

IL-1 α (##p=0.0025). Similar was observed in VM-Astros where stimulation with IL-1 α (****p<0.0001) or TIC (****p<0.0001) resulted in a higher *CCL5* mRNA expression compared to the control, and where TIC stimulation was significantly higher compared to IL-1 α (####p<0.0001) and TNF α +IL-17A (^^^p<0.0001) stimulation. Just like in CTX, VM-Astros stimulated with TNF α +IL-17A resulted in a significantly lower *CCL5* mRNA expression compared to IL-1 α stimulation (^^p=0.0002).

This trend was further followed in terms of *ICAM1* mRNA expression (see **Fig. 3.3.8.E** below), except that there were no regional differences observed between CTX and VM-Astros, regardless of stimulation. Stimulation with IL-1 α resulted in a significantly higher *ICAM1* mRNA expression by CTX (**p=0.0025) and VM-Astros (***p=0.0001) compared to the control. Although stimulation with TNF α +IL-17A did not yield significantly higher *ICAM1* mRNA expression compared to the control, in VM-Astros, this expression was significantly lower compared to IL-1 α stimulation (##p=0.0040). On the other hand, stimulation with TIC resulted in a very potent expression of *ICAM1* mRNA in both CTX and VM-Astros, which was significantly higher compared to the control (****p<0.0001), IL-1 α (####p<0.0001) and TNF α +IL-17A stimulation (^^^p<0.0001).

PTX3 mRNA (**Fig. 3.3.8.F**) was one of the examples where CTX-Astros expressed more of the gene than VM-Astros, with the *PTX3* gene levels being significantly higher post-IL-1 α (+p=0.0140) and TIC (+p=0.0464) stimulation compared to VM. Stimulation with IL-1 α and TIC yielded a significantly higher *PTX3* mRNA expression in CTX-Astros compared to the control, with TIC stimulation levels being higher compared to TNF α +IL-17A stimulation (^^^p<0.0001). On the other hand, *PTX3* mRNA expression post-TNF α +IL-17A stimulation in CTX-Astros was significantly lower compared to IL-1 α stimulation (##p=0.0033). Stimulation with IL-1 α (*p=0.0464) or TIC (***p=0.0003) also resulted in a significantly higher expression of *PTX3* mRNA compared to the control, but there were no significant differences observed between stimulation groups in these cells.

In contrast, *TLR4* mRNA (**Fig. 3.3.8.G** below) expression was significantly higher in VM-Astros compared to CTX regardless of stimulation (++++p<0.0001 across all

groups, including baseline). Higher expression of *TLR4* mRNA was observed in VM-Astros stimulated with IL-1 α (**** $p < 0.0001$) and TIC (**** $p < 0.0001$) compared to the control. However, stimulation with TNF α +IL-17A did not further increase *TLR4* mRNA expression in VM-Astrocytes and was therefore significantly lower compared to IL-1 α (#### $p < 0.0001$) or TIC stimulation (^^^ $p < 0.0001$). Similar was observed in the CTX-Astros, although at a much lower level. Once stimulated with IL-1 α (** $p = 0.0005$) or TIC (**** $p < 0.0001$), *TLR4* mRNA expression was significantly increased compared to the control. Both TNF α +IL-17A (## $p = 0.0016$) and TIC (# $p = 0.0422$) stimulated groups expressed less *TLR4* mRNA compared to IL-1 α , but the gene levels post-TIC stimulation were still significantly higher compared to TNF α +IL-17A (^^^ $p < 0.0001$).

IL1B mRNA (**Fig. 3.3.8.H**) was only expressed at high levels in CTX-Astros post-stimulation with IL-1 α (**** $p < 0.0001$) or TIC (**** $p < 0.0001$) compared to the control. However, TNF α +IL-17A stimulation did not increase *IL1B* mRNA compared to the control and was significantly lower compared to IL-1 α (#### $p < 0.0001$) and TIC (^^^ $p < 0.0001$) stimulation. Stimulation of CTX-Astros with TIC (#### $p < 0.0001$) resulted in a significantly higher *IL1B* mRNA expression compared to IL-1 α stimulation. Due to the fact that VM-Astros did not significantly increase *IL1B* mRNA expression post-stimulation, *IL1B* mRNA levels post-IL-1 α (+++ $p = 0.0001$) and TIC (++++ $p < 0.0001$) stimulation in VM-Astros were significantly lower compared to CTX.

Similar was observed in *CSF2* mRNA expression (**Fig. 3.3.8.I** below) in both CTX and VM-Astros, where stimulation with IL-1 α or TNF α +IL-17A did not increase *CSF2* mRNA expression. However, stimulation with TIC did significantly increase *CSF2* gene levels in both CTX and VM-Astros compared to the control (**** $p < 0.0001$), IL-1 α (#### $p < 0.0001$ for CTX and ### $p = 0.0001$ for VM) or TNF α +IL-17A stimulation (^^^ $p < 0.0001$). Regional differences in *CSF2* mRNA expression were observed post-TIC stimulation only, with the gene levels being significantly lower in VM-Astros compared to CTX (++++ $p < 0.0001$).

Although not generally associated with astrocytes, the mRNA expression of *ITGAM* (**Fig. 3.3.8.J** below) was also assessed between the two regions. At baseline,

both CTX and VM-Astros expressed similar levels of *ITGAM*. However, this changed once the cells were stimulated. CTX-Astros stimulated with IL-1 α , TNF α +IL-17A and TIC yielded *ITGAM* mRNA expression that was significantly increased compared to the control (**** p <0.0001) and VM-Astros (++++ p <0.0001). *ITGAM* mRNA levels were also significantly increased in CTX-Astros post-TNF α +IL-17A (## p =0.0026) and TIC stimulation (## p =0.0026), compared to IL-1 α . Stimulation of VM-Astros with pro-inflammatory factors did not have an effect on *ITGAM* mRNA levels compared to the control.

The mRNA expression of *IFNA2* (**Fig. 3.3.8.K**) and *IFNB1* (**Fig. 3.3.8.L**) was also assessed between the two regions and post-stimulation. Although *IFNA2* mRNA was slightly higher in CTX-Astros compared to VM, this was not significant. However, regional differences were observed in *IFNB1* mRNA levels with the expression being higher in CTX-Astros post-IL-1 α (++ p =0.0035), TNF α +IL-17A (+ p =0.0368) and TIC stimulation (++++ p <0.0001). *IFNB1* mRNA expression in CTX-Astros was significantly higher post-IL-1 α (* p =0.0368) and TIC (**** p <0.0001) stimulation compared to the control, but not TNF α +IL-17A stimulation. TIC stimulation in CTX-Astros resulted in significantly higher *IFNB1* gene levels compared to IL-1 α (#### p <0.0001) and TNF α +IL-17A stimulation (^^^ p <0.0001). However, only VM-Astros stimulated with TIC expressed significantly more *IFNB1* mRNA compared to the control (** p =0.0010), IL-1 α (# p =0.0144) or TNF α +IL-17A (^ p =0.0047).

Overall, both CTX and VM-Astros expressed mRNA of pro-inflammatory markers at various levels. Some genes, like *CXCL10* or *TLR4*, were expressed more highly in VM-Astros, while others, e.g. *PTX3* and *IFNB1*, had a higher expression in the cortical region. Stimulation with TIC was the most effective at inducing higher expression of pro-inflammatory genes, but some genes like *CXCL10*, *CCL5* and *PTX3* were also highly expressed post IL-1 α and TNF α +IL-17A stimulation.

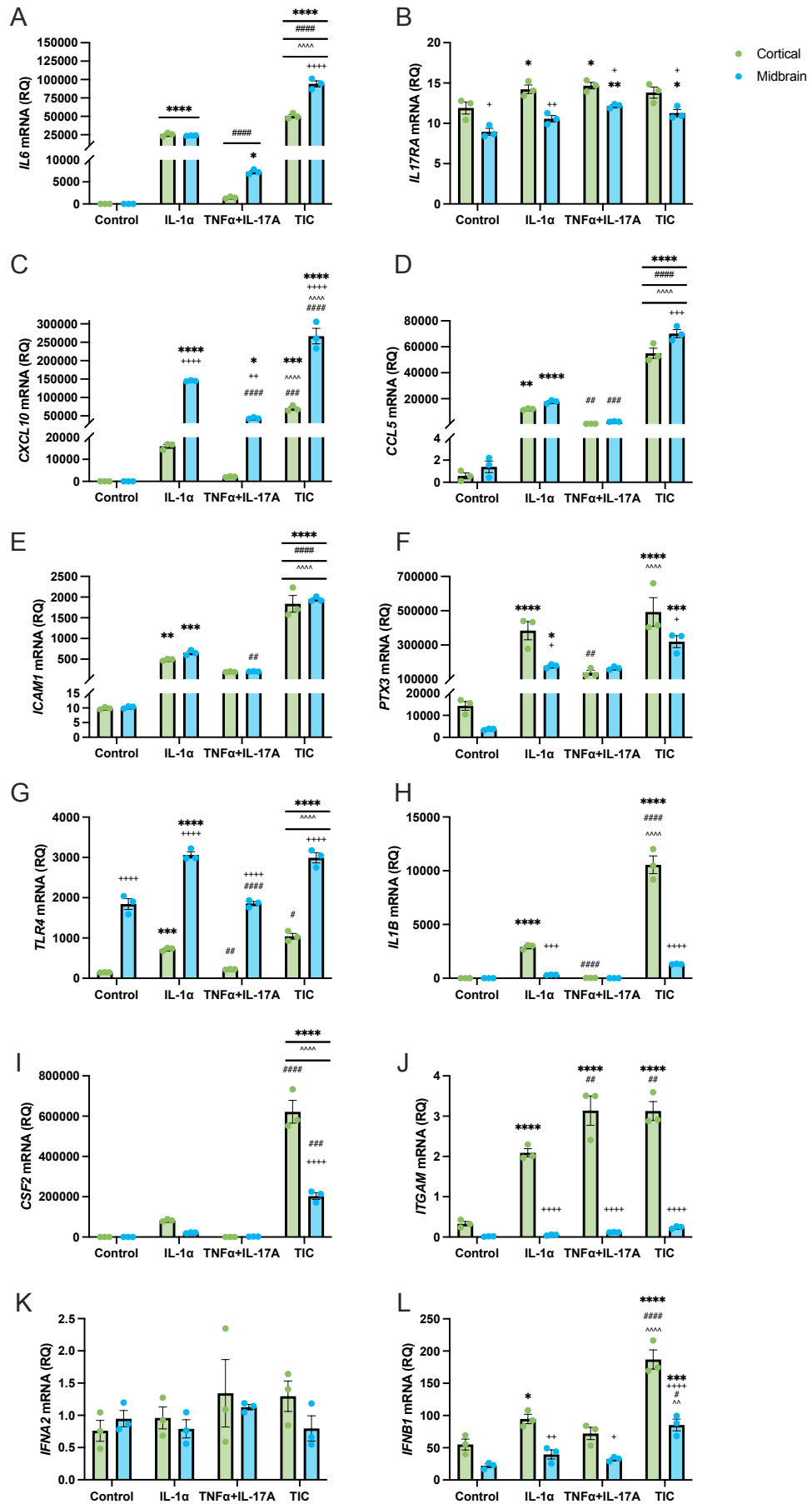


Figure 3.3.8 Inflammatory response gene expression differs between CTX and VM-Astros (previous page).

Stimulation with pro-inflammatory cytokines resulted in an upregulated mRNA expression of IL6 (**A**), IL17RA (**B**), CXCL10 (**C**), CCL5 (**D**), ICAM1 (**E**), PTX3 (**F**), TLR4 (**G**), IL1B (**H**), CSF2 (**I**), ITGAM (**J**) and (**L**) IFNB1. At baseline, CTX-Astros expressed more IL17RA (**B**) and less TLR4 (**G**) mRNA compared to VM-Astros. VM-Astros expressed more (**A**) IL6 and (**D**) CCL5 mRNA post-stimulation with TIC factors compared to CTX. More IL17RA (**B**) mRNA was expressed by CTX-Astros across all stimulation groups, while VM-Astros expressed more CXCL10 (**C**) mRNA. CTX-Astros also expressed more (**F**) PTX3 and (**H**) IL1B mRNA when stimulated with IL-1 α or TIC factors. (**G**) TLR4 mRNA expression was higher in VM-Astros across all stimulation groups compared to CTX. Stimulation with TIC factors resulted in higher (**I**) CSF2 mRNA expression by CTX-Astros, while (**J**) ITGAM and (**L**) IFNB1 mRNA expression was higher in CTX-Astros across all stimulation compared to VM. Data expressed as mean $\pm$ SEM (N=3, r=3). * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001 vs control. + p <0.05, ++ p <0.01, +++ p <0.001, ++++ p <0.0001 vs CTX-Astros. # p <0.05, ## p <0.01, ### p <0.001, #### p <0.0001 vs IL-1 α stimulation. ^^ p <0.01, ^^ p <0.001, ^^ p <0.0001 vs TNF α +IL-17A stimulation. Two-way ANOVA with Holm-Šidák post hoc test.

3.3.8. CTX- and VM-Astros display differential expression of functional genes

Astrocytes, like any other cell in the body, express a variety of genes that aid their function, with some of these genes including *ITGA6*, *SLC1A2*, *SLC1A3*, *GJA6*, *GLUL* and *DRD2*. Unlike at the protein level, *ITGA6* (also known as CD49f) mRNA was expressed more highly by VM-Astros, compared to CTX (++++ p <0.0001 for all groups), regardless of stimulation (see **Fig. 3.3.9.A** below). *ITGA6* mRNA expression by VM-Astros decreased by approximately 30% post-stimulation with IL-1 α , TNF α +IL-17A or TIC (**** p <0.0001 for all three), with no significant differences between the stimulation groups. In contrast, *ITGA6* mRNA expression in CTX-Astros was only increased post-stimulation with TIC, compared to the control (**** p <0.0001), IL-1 α (#### p <0.0001) and TNF α +IL-17A stimulation (^^ p <0.0001). No other significant changes were observed in *ITGA6* gene expression.

SLC1A2 mRNA (also known as EAAT2), did not differ from the control in CTX- or VM-Astros, and any small changes such as the decrease of gene expression post IL-1 α stimulation were non-significant (**Fig. 3.3.9.B**). Stimulation with TNF α +IL-17A in VM-Astros resulted in higher *SLC1A2* mRNA expression compared to IL-1 α (##p=0.0067), but not the control. Similarly, stimulation of CTX-Astros with TIC caused a significantly higher *SLC1A2* mRNA expression compared to IL-1 α stimulation (#p=0.0129), but not the control. There was a potential regional difference in *SLC1A2* gene expression post-TIC stimulation, with the expression in CTX-Astros being higher than in VM, but this did not reach significance (p=0.0520).

The opposite was true, however, for *SLC1A3* (EAAT1) mRNA expression (see **Fig. 3.3.9.C** below). CTX-Astros expressed significantly more *SLC1A3* mRNA compared to VM (++++p<0.0001 for all groups), at baseline and post-stimulation. *SLC1A3* mRNA expression was significantly increased in CTX-Astros stimulated with IL-1 α (****p<0.0001) and TNF α +IL-17A (*p=0.0371), compared to the control. Stimulation with TNF α +IL-17A (###p=0.0002) or TIC (####p<0.0001) in CTX-Astros significantly reduced *SLC1A3* mRNA levels compared to IL-1 α stimulation. No significant differences were observed in *SLC1A3* mRNA expression post-stimulation of VM-Astros, compared to the control or each other.

Similarly to *SLC1A3*, the expression of *GJA1* mRNA (**Fig. 3.3.9.D**), also known as Cx43, was significantly higher in CTX-Astros compared to VM, at baseline (++++p<0.0001) and post-stimulation with IL-1 α , TNF α +IL-17A (++++p<0.0001 for both) or TIC (++p=0.0012). Stimulation of CTX-Astros with IL-1 α (**p=0.0061) or TIC (****p<0.0001) significantly reduced *GJA1* mRNA expression, while stimulation with TNF α +IL-17A increased it (**p=0.0034) compared to the control. TNF α +IL-17A-stimulated group in CTX-Astros yielded a significantly higher expression of *GJA1* mRNA compared to IL-1 α stimulation (####p<0.0001). However, the *GJA1* gene expression was significantly reduced by TIC stimulation compared to IL-1 α (####p<0.0001) or TNF α +IL-17A (^^^p<0.0001) stimulation. In VM-Astros, *GJA1* expression levels were increased by TNF α +IL-17A stimulation compared to IL-1 α (##p=0.0038) or TIC (^p=0.0038), but not the control.

Unlike some other genes, the mRNA expression of *GLUL* (**Fig. 3.3.9.E** below) increased post-stimulation with pro-inflammatory factors. This was especially apparent in CTX-Astros where stimulation with IL-1 α (**** $p < 0.0001$), TNF α +IL-17A (* $p = 0.0447$) or TIC (**** $p < 0.0001$) resulted in a significantly higher expression of *GLUL* mRNA. Stimulation of CTX-Astros with TNF α +IL-17A yielded significantly lower *GLUL* mRNA levels compared to IL-1 α (## $p = 0.0059$) and TIC (^ $p = 0.0126$). In the case of VM-Astros, *GLUL* mRNA levels remained mostly unaffected by stimulation, except for TNF α +IL-17A stimulation (* $p = 0.0290$) compared to the control. Overall, CTX-Astros expressed significantly more *GLUL* mRNA compared to VM at baseline (+ $p = 0.0108$) as well as post-stimulation with IL-1 α (++++ $p < 0.0001$), TNF α +IL-17A (+ $p = 0.0157$) or TIC (++++ $p < 0.0001$).

The opposite was true for *DRD2* mRNA expression (see **Fig. 3.3.9.F**). Regional differences in *DRD2* mRNA expression were only observed at baseline (+++ $p = 0.0004$) and post IL-1 α stimulation (+++ $p = 0.0008$), with CTX-Astros expressing significantly more *DRD2* mRNA compared to VM. The expression of *DRD2* mRNA significantly decreased in CTX-Astros post stimulation with IL-1 α (** $p = 0.0005$), TNF α +IL-17A (**** $p < 0.0001$) and TIC (**** $p < 0.0001$) compared to the control. The same was observed in VM-Astros with *DRD2* gene expression decreasing significantly post-stimulation with IL-1 α (** $p = 0.0011$), TNF α +IL-17A (* $p = 0.0266$) and TIC (** $p = 0.0004$) compared to the control. No significant differences in *DRD2* mRNA expression were observed between any of the stimulation groups.

To summarise, most of the genes supporting normal astrocyte function were more highly expressed by CTX-Astros compared to VM, with the exception of *ITGA6*. The expression of half the genes increased with pro-inflammatory stimulation (e.g. *SLC1A3*), while the other half, like *DRD2*, were significantly decreased by stimulation.

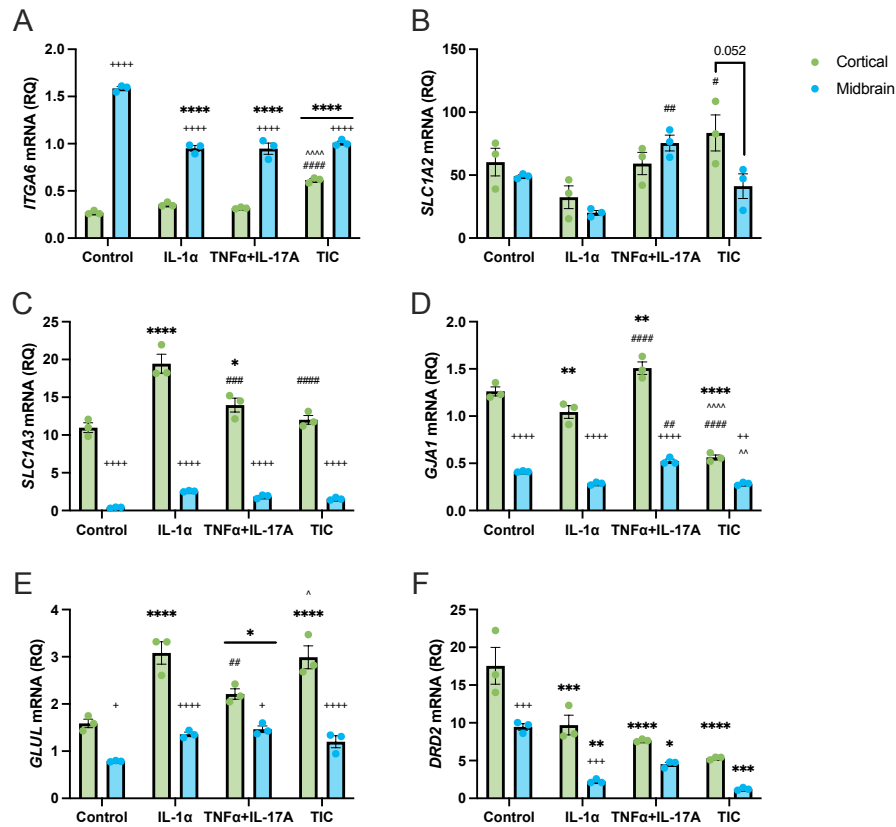


Figure 3.3.9 CTX and VM-Astros display differential expression of functional genes.

At baseline, there was a higher expression of **(A)** ITGA6 mRNA by VM-Astros, while CTX-Astros expressed more **(C)** SLC1A3, **(D)** GJA1, **(E)** GLUL and **(F)** DRD2. Stimulation with pro-inflammatory cytokines resulted in a decreased expression of **(A)** ITGA6 mRNA by VM-Astros, while stimulation with TIC factors resulted in an increase of ITGA6 mRNA in CTX-Astros, with the mRNA levels being higher in VM-Astros than CTX. Although there was no regional difference in **(B)** SLC1A2 expression, in VM-Astros, the mRNA expression post-TNFα+IL-17A stimulation was significantly higher compared to IL-1α stimulation. **(C)** SLC1A3 mRNA was increased by stimulation with IL-1α and TNFα+IL-17A in CTX-Astros and was overall higher in CTX-Astros compared to VM. Expression of GJA1 **(D)** mRNA was higher in CTX-Astros across all stimulation compared to VM. Stimulation with IL-1α and TIC factors resulted in a lower GJA1 expression in CTX-Astros compared to control, while TNFα+IL-17A stimulation increased GJA1 mRNA. **(E)** GLUL mRNA expression was higher across all stimulations in CTX-Astros and TNFα+IL-17A stimulation only in VM-Astros compared to the control, with the expression being higher in CTX-Astros post-stimulation compared to VM. **(F)** DRD2 expression was lower across all stimulation compared to control in both CTX and VM-Astros. Regional difference was only observed post-stimulation with IL-1α, resulting in a higher DRD2 mRNA expression in CTX-Astros compared to VM. Data expressed as mean ± SEM (N=3, r=3). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs control. +p<0.05, ++p<0.01, +++p<0.001, ++++p<0.0001 vs CTX-Astros. #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001 vs IL-1α stimulation. ^p<0.05, ^^p<0.01, ^^p<0.0001 vs TNFα+IL-17A stimulation. Two-way ANOVA with Holm-Šidák post hoc test.

3.3.9. The differential expression of metabolic function and stress response genes in CTX and VM-Astros

Before further assessing astrocyte functionality via Seahorse, several metabolic function and stress response genes were analysed. Namely, the expression of *LDHA*, *HK2*, *PFKFB3*, *SLC2A1*, *SLC6A3* and *PPARG* mRNA was assessed in CTX and VM-Astros, as seen in **Fig. 3.3.10.A-F** below.

LDHA mRNA expression (**Fig. 3.3.10.A**), although relatively low overall, varied significantly between CTX and VM-Astros, both at baseline and post-stimulation, with the VM-Astros expressing significantly less *LDHA* mRNA compared to CTX (++++ $p < 0.0001$ for all groups). VM-Astros also expressed significantly more *LDHA* mRNA post-stimulation with IL-1 α (**** $p < 0.0001$), TNFA+IL-17A (* $p = 0.0257$) and TIC (**** $p < 0.0001$) compared to the control. Highest levels of *LDHA* mRNA expression were observed in the TIC-stimulated group of VM-Astros, which was significantly higher compared to IL-1 α (# $p = 0.0257$) and TNFA+IL-17A stimulation (^^^ $p < 0.0001$). *LDHA* mRNA post-stimulation with TNFA+IL-17A was significantly lower compared to both IL-1 α (#### $p < 0.0001$) and TNFA+IL-17A (^^^ $p < 0.0001$). Similar was observed in CTX-Astros, with the stimulation groups yielding a significant increase in *LDHA* mRNA compared to the control (**** $p < 0.0001$ for IL-1 α , * $p = 0.0199$ for TNFA+IL-17A and **** $p < 0.0001$ for TIC). Stimulation of CTX-Astros with TNFA+IL-17A resulted in a significantly lower *LDHA* gene expression compared to IL-1 α (#### $p < 0.0001$). In contrast, stimulation with TIC caused a significantly higher *LDHA* mRNA expression in CTX-Astros compared to IL-1 α (#### $p < 0.0001$) and TNFA+IL-17A (^^^ $p < 0.0001$).

Unlike *LDHA*, the mRNA expression of *HK2* (**Fig. 3.3.10.B** below) did not differ between CTX and VM-Astros at baseline, or post-TNFA+IL-17A stimulation. Significance was reached in the IL-1 α (+++ $p = 0.0004$) and TIC-stimulated (+++ $p = 0.0001$) groups due to the fact that stimulation did not have an effect on *HK2* mRNA expression in VM-Astros, but it did in CTX-Astros. Stimulation of CTX-Astros with IL-1 α (** $p = 0.0005$) and TIC (**** $p < 0.0001$) resulted in a significantly higher *HK2* mRNA expression compared to the control, or VM-

Astros. Because this effect was not seen in CTX-Astros stimulated with TNFA+IL-17A, these cells yielded a significantly lower level *HK2* mRNA compared to IL-1 α (##p=0.0047) and TIC (^^^p<0.0001). No significant changes were observed in VM-Astros, regardless of stimulation.

As seen in **Fig. 3.3.10.C** below, a similar trend was observed in terms of *PFKFB3* mRNA expression, but this time at higher levels. Although at baseline CTX and VM-Astros expressed the same levels of *PFKFB3* mRNA, stimulation with IL-1 α and TIC significantly increased this expression in CTX-Astros compared to VM (++++p<0.0001 for both). CTX-Astros stimulated with IL-1 α (****p<0.0001) or TIC (****p<0.0001), but not TNFA+IL-17A, expressed higher levels of *PFKFB3* mRNA compared to the control. The same was observed in VM-Astros where IL-1 α and TIC stimulation increased the *PFKFB3* mRNA expression (****p<0.0001 for both) compared to the control. In CTX-Astros, stimulation with TNFA+IL-17A resulted in significantly lower *PFKFB3* gene levels compared to IL-1 α (####p<0.0001), while TIC-stimulation caused a significant increase compared to IL-1 α (####p<0.0001) and TNFA+IL-17A stimulation (^^^p<0.0001). *PFKFB3* mRNA expression post-TNFA+IL-17A stimulation was also significantly lower compared to IL-1 α (####p<0.0001) in VM-Astros, whereas post-TIC stimulation, the expression levels were significantly higher compared to IL-1 α (#p=0.0229) and IL-1 α (^^^p<0.0001).

In contrast, *SLC2A1* mRNA (**Fig. 3.3.10.D**) was significantly higher in VM-Astros at baseline compared to CTX (+p=0.0101), but post-stimulation with IL-1 α (++p=0.0036) and TIC (+++p=0.0007), the expression was higher in CTX-Astros compared to VM. *SLC2A1* mRNA expression was significantly decreased in both VM and CTX-Astros post-stimulation compared to the control (****p<0.0001 for all). Although no significant differences in *SLC2A1* mRNA expression were observed between stimulation groups in CTX-Astros, in VM-Astros, stimulation with TNFA+IL-17A significantly increased *SLC2A1* gene levels compared to IL-1 α (####p<0.0001) while TIC-stimulation caused a significant *SLC2A1* decrease compared to TNFA+IL-17A stimulation (^^^p<0.0001).

Unlike the rest of the genes, *SLC6A4* mRNA levels (**Fig. 3.3.10.E** below) are near zero at baseline and post stimulation, with the exception of TIC stimulation in CTX-Astros which significantly increases the *SLC6A4* mRNA levels to 0.3 RQ, compared to the control (**** $p < 0.0001$), IL-1 α (#### $p < 0.0001$) and TNFA+IL-17A (^^^ $p < 0.0001$) within the region, and TIC stimulation in VM-Astros (++++ $p < 0.0001$). No other significant differences were observed in terms of *SLC6A3* mRNA expression.

Similar to *SLC2A1*, the *PPARG* mRNA expression (see **Fig. 3.3.10.F**) is significantly higher in VM-Astros compared to CTX, at baseline and post-stimulation (++++ $p < 0.0001$ for all groups). Stimulation with pro-inflammatory cytokines resulted in a lower expression of *PPARG* compared to the controls in both regions (**** $p < 0.0001$). *PPARG* mRNA expression varied between the stimulation groups, with TNFA+IL-17A-stimulation resulting in a higher expression of *PPARG* compared to IL-1 α (### $p = 0.003$ for CTX and #### $p < 0.0001$ for VM). In contrast, stimulation with TIC yielded a significantly lower *PPARG* mRNA expression compared to IL-1 α (# $p = 0.0292$ for both CTX and VM) and TNFA+IL-17A (^^^ $p < 0.0001$ for both regions).

Overall, the expression of certain metabolic genes such as *PFKFB3* or *HK2* tended to be higher in CTX-Astros than VM, while other genes such as *PPARG* were more highly expressed in VM-Astros. Stimulation with pro-inflammatory factors could result in a higher gene expression, e.g. *PFKFB3*, while other genes, such as *SLC2A1*, would be downregulated. These responses were highly dependent on gene, region, and pro-inflammatory stimuli.

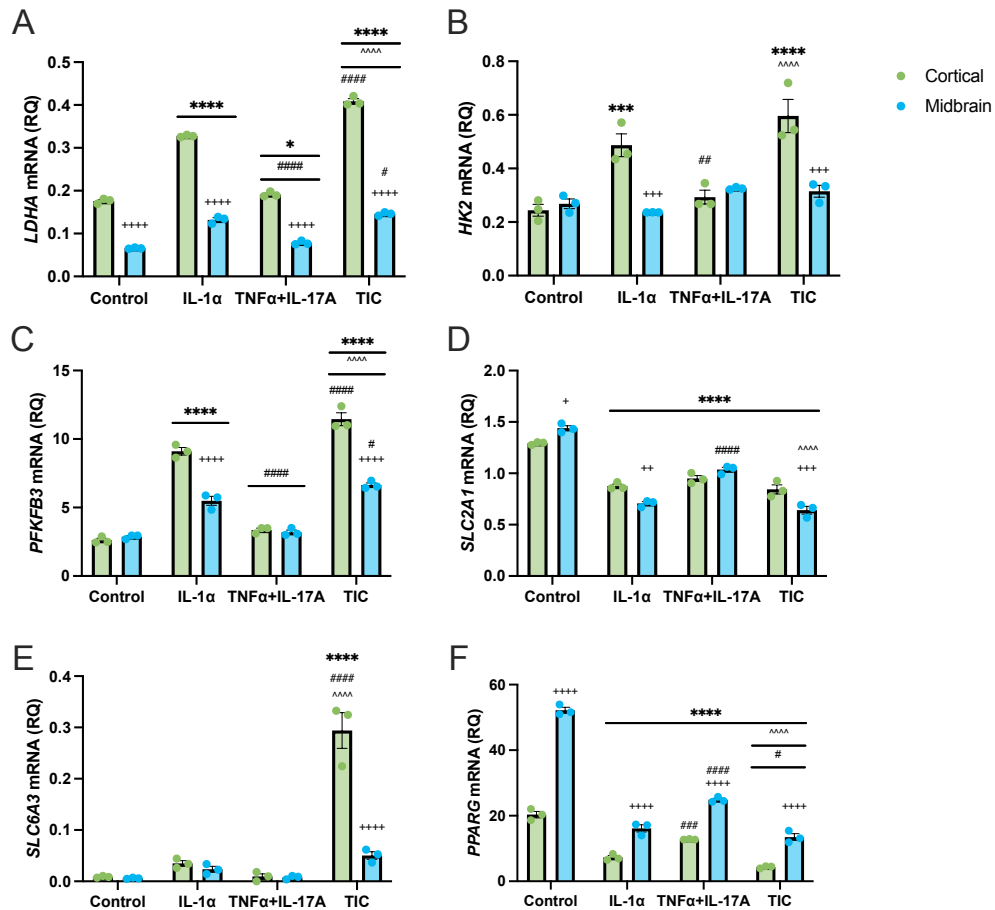


Figure 3.3.10 The expression of metabolic function and stress response genes differs between CTX and VM-Astros.

LDHA (A) mRNA expression was higher in CTX-Astros at baseline compared to VM, while the opposite was true for (D) SLC2A1 and (F) PPARG mRNA expression. Stimulation with pro-inflammatory cytokines resulted in a higher expression of LDHA (A), HK2 (B), and PFKFB3 (C) across all stimulation groups compared to control, while higher SLC6A3 (E) mRNA expression was only observed post-stimulation with TIC factors. SLC2A1 (D) and PPARG (G) mRNA levels were reduced across all stimulation compared to control. (A) LDHA mRNA was more highly expressed by CTX-Astros across all stimulation, while a higher HK2 mRNA (B) expression was only observed post IL-1 α and TIC stimulation compared to VM-Astros. A similar effect was observed in PFKFB3 (C) mRNA, with the expression being higher in CTX-Astros stimulated with IL-1 α and TIC factors compared to VM. Stimulation with IL-1 α and TIC factors also resulted in a significantly higher SLC2A1 mRNA expression in CTX-Astros compared to VM. In terms of SLC6A3 (E) mRNA expression, this higher expression by CTX-Astros was only observed post-TIC stimulation. In contrast, PPARG (F) mRNA expression was significantly higher in VM Astros compared to CTX, across all stimulation groups. Data expressed as mean $\pm$ SEM (N=3, r=3). * p <0.05, ** p <0.001, **** p <0.0001 vs control. + p <0.05, ++ p <0.01, +++ p <0.001, ++++ p <0.0001 vs CTX-Astros. # p <0.05, ## p <0.01, #### p <0.0001 vs IL-1 α stimulation. ^^^ p <0.0001 vs TNF α +IL-17A stimulation. Two-way ANOVA with Holm-Šídák post hoc test.

3.3.10. Stimulation with pro-inflammatory factors increases glycolysis in CTX-Astros to a greater extent than in VM-Astros

To assess the real-time changes in the metabolism of CTX and VM-Astros, cells were deprived of glucose for 1 hour before subjecting them to the Glycolytic Stress Test in the Seahorse Analyser, involving injections of glucose, oligomycin and 2-DG (as depicted in **Figure 3.3.11.A** below).

At baseline, stimulated CTX-Astros (**Fig. 3.3.11.B** below) had a significantly higher ECAR rate compared to the control ($***p=0.0002$, $****p<0.0001$ for IL-1 α ; $^^^p=0.0003$, $^^^^p<0.0001$ for TNFA+IL-17A; $++++p<0.0001$ for TIC). Once glucose was injected, the ECAR rate increased for all groups, with the highest rate observed in TIC-stimulated group ($++++p<0.0001$), followed by IL-1 α ($****p<0.0001$) and TNFA+IL-17A ($^^^^p<0.0001$) compared to the control. The addition of Oligomycin allowed the CTX-Astros to reach their maximal glycolysis, where the ECAR rate was still highest post-stimulation with TIC ($++++p<0.0001$), then IL-1 α ($****p<0.0001$) and TNFA+IL-17A ($^^p=0.0022$) compared to the control, albeit the ECAR post-TNFA+IL-17A stimulation was only significant during the very first measurement post-Oligomycin injection. Most of the significance between different groups was lost once 2-DG was injected with the exception of TIC-stimulated cells, as the ECAR of these cells remained significantly higher compared to the control ($+p=0.0288$), IL-1 α ($\pounds p=0.0118$) and TNFA+IL-17A stimulation ($\&\&\&p<0.0001$). Throughout the assay, up to the 2-DG injection, the ECAR rate of TIC-stimulated group remained significantly higher compared to IL-1 α ($\pounds\pounds\pounds p<0.0001$) and TNFA+IL-17A ($\&\&\&p<0.0001$). Although at baseline there was no significant difference in ECAR between IL-1 α and TNFA+IL-17A-stimulated group, this changed with the injection of glucose where the ECAR in IL-1 α -stimulated group became significantly higher compared to TNFA+IL-17A, and remained that way until the addition of 2-DG ($###p=0.0004$ at 21 min, $##p=0.0047$ at 27 min, $####p<0.0001$ 34-53 min).

Stimulation of VM-Astros with pro-inflammatory factors also increased the ECAR rate, but at a much lower level than in CTX-Astros (see **Fig. 3.3.11.C** below). At

baseline, there was no significant difference between the control and any of the stimulation groups. Once glucose was injected, the ECAR in the TIC-stimulated group was significantly increased compared to the control (++p=0.0045 at 21 min, ++p=0.0051 at 27 min, ++p=0.0042 at 34 min). Post-oligomycin injection, the ECAR rates of IL-1 α (*p=0.0391 at 40 min, *p=0.0498 at 47 min, *p=0.0465 at 53 min) and TIC-stimulated groups (++++p<0.0001 at 40-53 min) were significantly higher compared to the control. ECAR rate post-TIC stimulation was also significantly higher compared to IL-1 α (££p=0.0054 at 40 min, ££p=0.0025 at 47 min, ££p=0.0027 at 53 min) and TNFA+IL-17A-stimulation groups (***p=0.0005 at 40-53 min).

Although there were no regional differences observed before the addition of glucose (**Fig. 3.3.11.D**), once it has been injected, the ECAR rates of the control (**p=0.0061 at 21 min, *p=0.0202 at 27 min, **p=0.0016 at 34 min, ****p<0.0001 at 40-53 min), IL-1 α (^^^p=0.0004 at 21 min, ^^p=0.0024 at 27 min, ^^^p<0.0001 at 34-53 min), TNFA+IL-17A (+p=0.0337 at 21 min, +p=0.0151 at 34 min, ++++p<0.0001 at 40-53 min) and TIC-stimulated (####p<0.0001 at 21-53 min) groups were significantly higher in CTX-Astros compared to VM. Injection of 2-DG towards the end of the assay stopped all glycolytic activity in these cells and therefore all regional differences were no longer observed.

As previously mentioned, raw ECAR data obtained from the Seahorse Analyser were exported to Microsoft Excel and expressed as milli-pH units per minute (mpH/min). Basal glycolysis was calculated from the ECAR value following glucose injection, glycolytic capacity as the maximum ECAR value following oligomycin injection, glycolytic reserve as glycolytic capacity minus basal glycolysis, and non-glycolytic acidification from the residual ECAR value following 2-DG injection. These calculated values were used to compare basal glycolysis, glycolytic capacity, glycolytic reserve, and non-glycolytic acidification rate, as seen in **Fig. 3.3.12.A-D** below. Basal glycolysis (**Fig. 3.3.12.A**) was slightly higher in CTX-Astros across all stimulation groups, but only in the TIC-stimulated group was it significantly higher compared to VM (+++p=0.0007). CTX-Astros stimulated with TIC also had a significantly higher basal glycolysis compared to the control (***p=0.0004), IL-1 α (#p=0.0289) and TNFA+IL-17A-stimulated group (^^p=0.0013). Although basal

glycolysis was trending towards an increase in VM-Astros post-stimulation, these results were non-significant.

Glycolytic capacity (**Fig. 3.3.12.B**) was also higher in CTX-Astros compared to VM at baseline ($++p=0.0030$), as well as post-IL-1 α ($+++p=0.0004$) and TIC stimulation ($++++p<0.0001$). In both VM and CTX-Astros, the glycolytic capacity was significantly increased only in the TIC-stimulated group ($**p=0.0052$ for VM and $****p<0.0001$ for CTX) compared to the control. Stimulation with TIC in CTX-Astros also resulted in a much higher glycolytic capacity compared to IL-1 α ($##p=0.0033$) and TNFA+IL-17A ($^^^^p<0.0001$), while TNFA+IL-17A-stimulation was also significantly lower compared to IL-1 α ($\#p=0.0251$).

Similarly to the previous measures, glycolytic reserve (**Fig. 3.3.12.C** below) was approximately 3-4-fold higher in CTX-Astros at baseline ($++++p<0.0001$), post-IL-1 α ($++++p<0.0001$), TNFA+IL-17A ($++p=0.0026$) and TIC stimulation ($++++p<0.0001$). In CTX-Astros, the glycolytic reserve was significantly increased with IL-1 α ($*p=0.0243$) and TIC stimulation ($****p<0.0001$), while TNFA+IL-17A stimulation caused a slight decrease, albeit insignificant compared to the control. In VM-Astros, stimulation with TIC also caused a significant increase in the glycolytic reserve compared to the control ($****p<0.0001$). Stimulation with TIC significantly increased the glycolytic reserve in both CTX and VM-Astros compared to IL-1 α ($###p=0.0006$ for both) and TNFA+IL-17A ($^^^^p<0.0001$ for CTX, $^^^p=0.0001$ for VM).

The non-glycolytic acidification (**Fig. 3.3.12.D**) was also higher in CTX-Astros compared to VM, regardless of stimulation ($++++p<0.0001$ for all). No significant changes in non-glycolytic acidification rate were observed between stimulation and control groups. However, in CTX-Astros, the TIC-stimulated cells had a higher non-glycolytic acidification rate compared to TNFA+IL-17A ($^^p=0.0099$).

Overall, CTX-Astros were more glycolytic than the VM-Astros, especially when stimulated. Stimulation with pro-inflammatory cytokines, such as TIC, increased the rate of glycolysis at baseline, as well as the glycolytic capacity and reserve. However, astrocyte reactivity did not affect the non-glycolytic acidification rate.

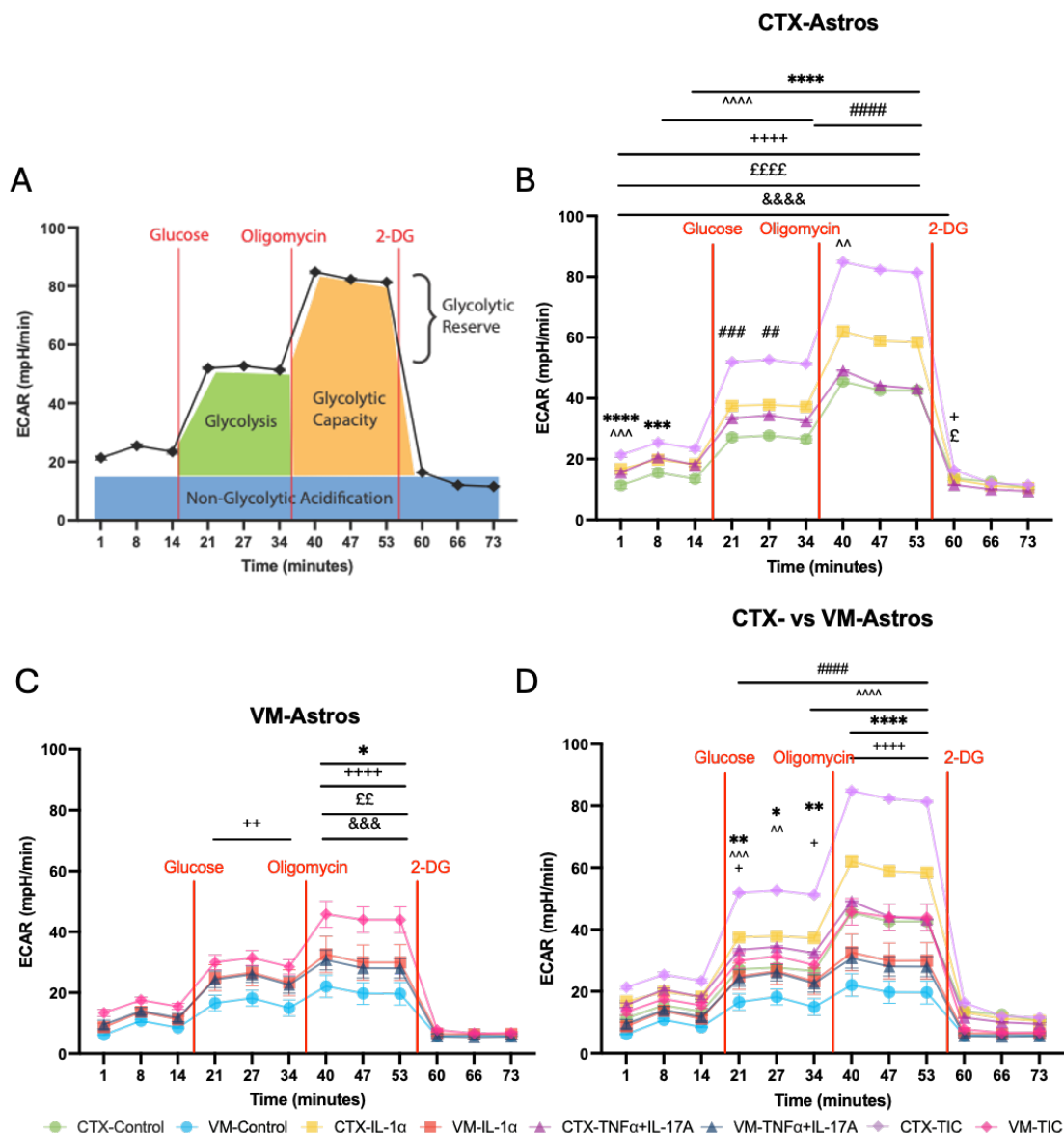


Figure 3.3.11 Stimulation with pro-inflammatory factors increases glycolysis in CTX-Astros to a greater extent than in VM-Astros.

Glycolysis was assessed in CTX and VM-Astros at baseline and post-stimulation with pro-inflammatory cytokines via Seahorse. **(A)** A schematic depicting the typical trajectory over time during the Glycolytic Stress Test in Seahorse Analyser; created in Adobe Illustrator by the author. **(B)** Stimulation with TIC and IL-1α increased ECAR rate in CTX-Astros across all stages of the assay, compared to the control, while ECAR post-TNFA+IL-17A stimulation was only increased as far as the addition of oligomycin. Overall, in CTX-Astros, ECAR rate was highest in TIC, followed by IL-1α stimulation, compared to the control and TNFA+IL-17A-stimulation. **(C)** ECAR in VM-Astros did not differ before the addition of glucose, regardless of stimulation. Post-glucose injection, TIC-stimulated VM-Astros had increased ECAR compared to the control. This again changed with the addition of oligomycin, where the TIC-stimulated group had the highest ECAR rate, followed by IL-1α and no difference in ECAR rate between the control and TNFA+IL-17A-stimulation. **(D)** Over the time

course of the assay, regional differences were observed between CTX and VM-Astros at baseline and post-stimulation with IL-1 α , TNFA+IL-17A, and TIC. Data expressed as mean $\pm$ SEM (N=3, r=1-2). For **(B-C)** * p <0.05, *** p <0.001, **** p <0.0001 control vs IL-1 α . ^^ p <0.01, ^^ p <0.001, ^^ p <0.0001 control vs TNFA+IL-17A. + p <0.05, ++ p <0.01, +++ p <0.0001 control vs TIC. ## p <0.01, ### p <0.001, #### p <0.0001 IL-1 α vs TNFA+IL-17A. £ p <0.05, ££ p <0.01, ££££ p <0.0001 IL-1 α vs TIC. && p <0.001, &&& p <0.0001 TNFA+IL-17A vs TIC. For **(D)** * p <0.05, ** p <0.01, **** p <0.0001 control (CTX vs VM). ^^ p <0.01, ^^ p <0.001, ^^ p <0.0001 IL-1 α stimulation (CTX vs VM). + p <0.05, +++ p <0.0001 TNFA+IL-17A stimulation (CTX vs VM). #### p <0.0001 TIC stimulation (CTX vs VM). Two-way ANOVA with Tukey's post hoc test.

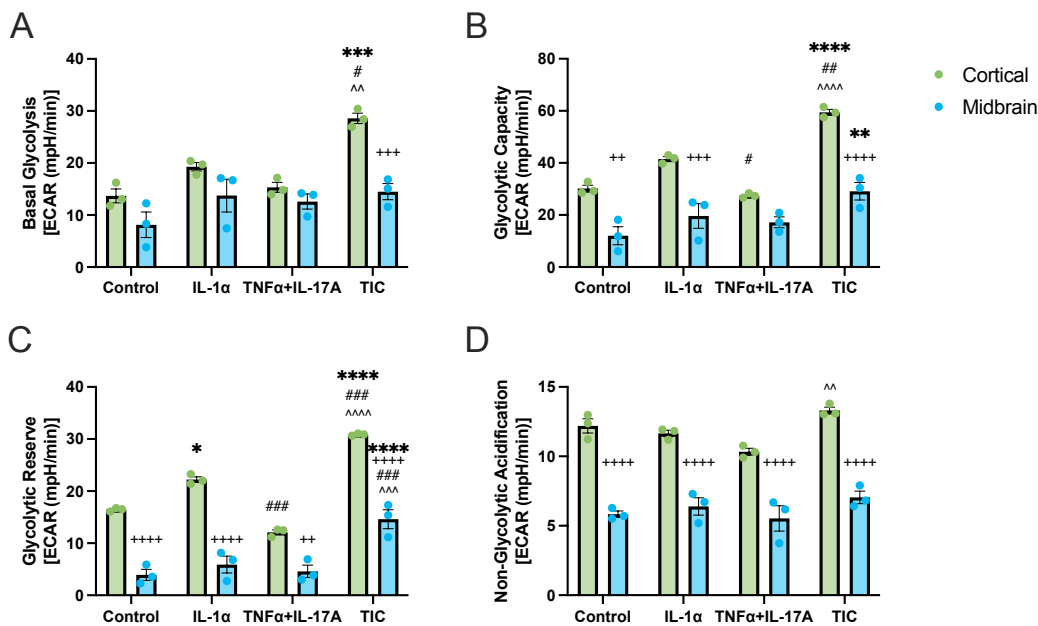


Figure 3.3.12 Basal Glycolysis, Glycolytic Capacity and Glycolytic Reserve are higher in CTX-Astros than in VM-Astros.

(A) CTX-Astros stimulated with TIC had a higher basal glycolytic rate compared to the control, IL-1 α and TNFA+IL-17A stimulation, while TIC-stimulated VM-Astros showed lower basal glycolysis than CTX. **(B)** Glycolytic capacity was lower in VM-Astros than CTX at baseline, and post IL-1 α and TIC stimulation. TIC stimulation increased glycolytic capacity compared to the control, IL-1 α and TNFA+IL-17A stimulation. **(C)** Glycolytic reserve was lower in VM-Astros than CTX, at baseline and post stimulation. IL-1 α and TIC increased the glycolytic reserve in CTX-Astros compared to control, with TIC producing greatest increase. TIC also increased glycolytic reserve in VM-Astros compared to control, IL-1 α and TNFA+IL-17A stimulation. **(D)** The non-glycolytic acidification rate was significantly higher in CTX-Astros compared to VM. Data expressed as mean $\pm$ SEM (N=3, r=1-2). * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001 vs control. ++ p <0.01, +++ p <0.001, ++++ p <0.0001 vs CTX-Astros. # p <0.05, ## p <0.01, ### p <0.001 vs IL-1 α stimulation. ^^ p <0.01, ^^ p <0.001, ^^ p <0.0001 vs TNFA+IL-17A stimulation. Two-way ANOVA with Tukey's post hoc test.

3.4. Discussion

Over the past decade, astrocyte reactivity has been widely accepted as a critical factor in the pathogenesis and progression of neurodegenerative diseases (Phatnani and Maniatis, 2015). Although one of the main functions of astrocytes is to support neuronal survival, in conditions such as AD, ALS or PD, astrocytes will undergo profound transcriptional and functional changes, which will indeed either support neuronal survival or exacerbate the pathology, thus contributing to neuronal damage and loss (Jiwaji and Hardingham, 2022). Thanks to several *in vitro* and *in vivo* studies, it is becoming increasingly apparent that astrocytes are not a homogeneous population. Instead, they will display region-specific differences in morphology, gene expression, cytokine release and other functional and physiological properties (Endo *et al.*, 2022). This heterogeneity is thought to arise from developmental patterning cues (Krencik *et al.*, 2011; Bradley *et al.*, 2019) and, therefore, possibly underlie the region-specific vulnerability observed in several neurological disorders, such as the cortex in AD, or the midbrain ventral region in PD. However, current research primarily focuses on the regional differences within neuronal populations only, often disregarding the fact that astrocytes, and most likely other types of brain cells, will also exhibit differences related directly to their regional identity.

In this study, we aimed to assess whether human iPSC-derived astrocytes retain their cortical and ventral midbrain identity and whether this would further influence their baseline characteristics, function, response to pro-inflammatory stimuli and metabolic activity. Our findings demonstrate that these cells indeed preserve the key regional signatures at the protein and gene level, as well as exhibit distinct reactivity profiles and metabolic changes, thus supporting the view that the regional diversity of astrocytes is not only linked to their developmental origin but will also influence their function *in vitro*. These regional signatures were already evident at the neural progenitor stage, where differences could be detected in protein and marker expression.

3.4.1. Retention of Regional Identity in NPCs and Astros

Both CTX and VM-NPC stained positive for TUJ1 (β Tubulin III), a classical neuronal marker, though the intensity was substantially higher in CTX-NPC. TUJ1 is widely used as a neuronal marker (Kriks *et al.*, 2011; Shi, Kirwan and Livesey, 2012; Tcw *et al.*, 2017), and its expression levels can vary between different brain regions during development, as well as depending on the age of the cells. Although no study directly compares the development of CTX and VM-NPC under identical conditions, studies investigating developmental neurobiology suggest that differentiation timing is region-dependent. A 2020 study (Brazdis *et al.*, 2021) compared CTX neurons to VM in terms of dopamine release. In this study, a lower proportion of midbrain dopaminergic neurons stained positive for TUJ1 compared to the cortical ones. Western Blot analysis of these cells also confirmed that midbrain dopaminergic neurons express less TUJ1 protein compared to cortical neurons. Therefore, our finding that TUJ1 intensity is higher in CTX-NPC than VM is consistent with the idea that cortical neural progenitors would commit to a neuronal fate earlier than midbrain NPC, which follow a dopaminergic lineage trajectory and may upregulate β Tubulin III more gradually. To further explore whether these regional differences extended beyond TUJ1, we also examined the expression of transcription factors associated with cortical (TBR1, FOXG1) and ventral midbrain (LMX1A, FOXA2) identity.

TBR1, an excitatory cortical neuron marker, and FOXG1, a more general forebrain transcription factor (Crompton *et al.*, 2013; Scuderi *et al.*, 2021), are both present in CTX-NPC at the protein and gene levels. qPCR analysis confirmed that CTX-NPC express both *TBR1* and *FOXG1* mRNA. Moreover, qPCR of VM-NPC for *TBR1* re-affirmed that this gene is only present at extremely low levels in the midbrain cells. This was further confirmed in ICC staining, where TBR1 intensity was at least 5-fold higher in CTX-NPC than in VM. Although TBR1 is normally considered primarily a nuclear transcription factor, we have observed its presence in the cytoplasm of CTX-NPC as well. However, cytoplasmic localisation of TBR1 was previously detected in postnatal and adult rats and mice. Hong and Hsueh (2007) observed that TBR1 was present in the cytoplasm and the dendrites of cortical neurons and that this

translocation away from the nucleus had a role in the enrichment of synaptic transmission in the neurons, thus increasing the neuronal communication. Büttner *et al.* (2010) noted that the overexpression of the *AF9* gene (also known as *MLLT3*) resulted in the TBR1 shift away from the nucleus into the cytoplasm, and even led to co-localisation with the mitochondrial marker Prohibitin. AF9, although not widely studied for its function within the CNS, has been shown to have a role in the forebrain and cerebellar development (Vogel and Gruss, 2009).

In contrast, VM-NPC expressed *EN1* and *OTX2* mRNA, and the comparison of *EN1* mRNA expression between the VM and CTX confirmed the 10-fold increased expression in VM-NPC. ICC staining confirmed a significant increase in LMX1A and FOXA2 intensity in VM-NPC compared to the CTX. Although both LMX1A and FOXA2 are predominantly expressed in the midbrain region, they are also present in the CTX NPC, albeit at much lower intensity. LMX1A and FOXA2 are well recognised for their role in the ventral neural tube patterning, where they act as key transcription factors of floor plate identity and dopaminergic progenitor specification (Norton *et al.*, 2005; Kittappa *et al.*, 2007; Bayly, Brown and Agarwala, 2012). However, regional patterning *in vitro* is gradual over time, with the presence of transitional progenitor states during which cells could potentially co-express factors generally associated with a different lineage before committing to a specific region. It is also important to note that recently, LMX1A has been shown to play a central role in cortical development by acting as a master regulator of the cortical hem as well as promoting the generation of Cajal-Retzius neurons (Chizhikov *et al.*, 2010; Fregoso, Dwyer and Franco, 2019; Iskusnykh *et al.*, 2023), which are crucial for the development of the hippocampus and the formation of cortical layers (Hevner *et al.*, 2003; Gil-Sanz *et al.*, 2013; Elorriaga, Pierani and Causeret, 2023). The involvement of LMX1A within the cortical hem occurs around embryonic day (E) 10.5-12.5 in mice, whereas its involvement in the differentiation of dopaminergic neurons within the substantia nigra occurs at E13-15 (Abeliovich and Hammond, 2007; Moore and Iulianella, 2021). This dual role may partly explain the LMX1A immunoreactivity in CTX-NPC; however, the significantly increased intensity in the VM-NPC region supports its primary association with midbrain identity.

Therefore, our interpretation focuses on the relative enrichment in the mRNA expression and staining intensity, rather than the absolute presence or absence of the transcription factors usually associated with a specific brain region.

To ensure that the CTX and VM-Astros retain the expression for their regional markers, CTX-Astros were assessed for *FOXG1* mRNA expression, while VM-Astros were assessed for the expression of *LMX1A*, *EN1* and *OTX2* mRNA. Indeed, regional identity was preserved throughout astrocyte differentiation. CTX-Astros expressed *FOXG1* mRNA while VM-Astros expressed *LMX1A*, *EN1* and *OTX2* mRNA at baseline. However, stimulation with pro-inflammatory factors had an effect on the gene expression by either upregulating or downregulating it. Namely, *FOXG1* mRNA expression was significantly increased in CTX-Astros by stimulation with IL-1 α and TIC, but not TNFA+IL-17A. In contrast, stimulation with IL-1 α and TIC downregulated the expression of *LMX1A*, *EN1* and *OTX2* mRNA in VM-Astros, while TNFA+IL-17A stimulation upregulated *LMX1A* and *EN1*. These results are supported in a study by Batiuk *et al.* (2020), where the authors observe multiple subtypes of astrocytes in adult mice. Although a lot of genes would be shared between all astrocytes, they have observed differences between subtypes that would suggest specialisations of astrocytes located within the specific brain region to distinct functions.

In 2011, Krencik *et al.* (2011) showed that once neural progenitors are patterned towards a specific regional fate, any of the universal astrocyte differentiation protocols, such as Serio *et al.* (2013), can be used to generate astrocyte progenitors that remain similar to the region of origin in terms of gene expression. Similar observations were made by Muroyama *et al.* (2005) and Hochstim *et al.* (2008) in embryonic mice, where transcriptional codes in forebrain and spinal cord neural progenitors were used to dictate the differentiation of region-specific astrocytes. Moreover, Zhao *et al.* (2014) have shown that the removal of crucial transcription factors during neural differentiation *in vitro* would lead to loss of astrocytes in that specific region. Therefore, this correlates with our observation that APC derived from cortical or midbrain ventral neural progenitors will continue to express the genes associated with that specific brain region. Taking all of this into account, one might assume that mRNA expression of all genes, such as regional markers,

functional, reactive, inflammatory response and those related to the metabolism, to name a few, would not only differ between astrocytes from different brain regions, but most likely would also change depending on the pro-inflammatory stimuli. This also implies that protein expression, cytokine release and morphology differences would also be present between different astrocytic phenotypes.

3.4.2. Baseline Astrocytic Characteristics Across Regions

Both CTX and VM-Astros stained positive for S100 β , GFAP, EAAT1, CD49f and Cx43, with varying degrees of intensity between the regions and different stimulation groups. S100 β is a calcium-binding protein that is highly expressed in astrocytes and has been found to play a crucial role in the regulation of intra- and extracellular activities within these cells (Brozzi *et al.*, 2009). It has long been established that in a healthy brain, S100 β is involved in neurite outgrowth, stimulating proliferation in astrocytes, enhancing neuronal survival and acting as a neurotrophic factor in general, especially after injury (Van Eldik and Wainwright, 2003). However, in neurodegeneration, the overexpression of S100 β is often stimulated by pro-inflammatory cytokines and, in the case of AD, β -amyloid, thus contributing to astrocyte reactivity and overall neuroinflammation (Griffin *et al.*, 1989; Hasel *et al.*, 2021; Clementi *et al.*, 2023). However, in our study, an overall low expression of S100 β in VM-Astros was observed across all groups, and the increase in S100 β intensity post-stimulation was not significant. A substantial decrease in S100 β intensity in CTX-Astros post-stimulation with IL-1 α and TIC was also observed. Although contrary to the accepted view about S100 β for the past few decades, indeed, other labs have also observed a significant decrease in S100 β intensity in recent years. For example, Jones *et al.* (2017) have documented a 10-fold reduction in S100 β fluorescence in Astros derived from FAD and SAD patient samples with PSEN1 or APOE4 phenotypes, respectively. MacMahon Copas *et al.* (2024), however, reported a minimal, albeit significant, increase in S100 β intensity in VM-Astros post-stimulation with TNF α , IL-17A or a combination of both, similar to what was observed in our study. Similarly, an overall low expression of S100 β in PD patients has been reported by Langeh and Singh (2020), who also suggest that S100 β may play a significant role not only in inflammation but also in

neurodegenerative diseases, activation of pro-inflammatory cytokine release and contribution to dopaminergic neuron damage. Therefore, any significant change to S100 β expression may potentially have a detrimental effect on astrogliosis and/or neuronal survival and death (Langeh and Singh, 2020).

GFAP is a hallmark intermediate filament protein expressed only in astrocytes, hence why it is also known as the “canonical astrocytic marker” (Jones *et al.*, 2017). Overall, intermediate filament proteins are involved in a wide range of cellular processes both during homeostasis and cellular stress (Kamphuis *et al.*, 2014). It has been widely accepted that in the case of GFAP, an increase is associated with astrogliosis and astrocyte damage and, in some cases, can be used as a prediction of the severity of neurodegeneration post-injury, such as ischemic stroke, as described by Amalia (2021). An increase in GFAP has been observed in AD mouse models by Kamphuis *et al.* (2012), both at the protein and gene levels. This same group then observed an increase in GFAP⁺ astrocytes in proximity to plaques in human AD tissue (Kamphuis *et al.*, 2014). However, Jones *et al.* (2017) did not observe any change in GFAP mean fluorescence in FAD or SAD compared to the control. In contrast, McComish *et al.* (2024) reported a significant decrease in GFAP intensity in CTX-Astros stimulated with TIC. Similarly, another study reported a decrease in GFAP⁺ astrocytes in their chronic stress model compared to the control (Fang *et al.*, 2022). This holds true for our study, where GFAP intensity does trend towards a decrease in CTX-Astros, but the results are non-significant. In VM-Astros, however, GFAP intensity increases when stimulated with TNF α +IL-17A and decreases post-stimulation with TIC, thus reaffirming the heterogeneity of astrocyte reactivity and the importance of stimulating astrocytes with factors and proteins relevant to the disease of interest. Overall, more studies recognise the fluidity of GFAP expression in the context of astrogliosis, which contradicts the widely accepted view of an increase in GFAP expression by reactive astrocytes (Lawrence *et al.*, 2023).

EAAT1, referred to as GLAST in rodent studies, is one of the glutamate transporters expressed in astrocytes. The primary role of EAAT1 is the termination of glutamate signalling within the cells and the maintenance of glutamate levels

extracellularly (Shigeri, Seal and Shimamoto, 2004). It has been shown in the past that inhibition of GLAST results in increased extracellular glutamate levels in rats, thus contributing to excitotoxic neuronal death (Rothstein *et al.*, 1996). In a study by Jones *et al.* (2017), a decrease in EAAT1 immunoreactivity has been observed in post-mortem frontal cortex tissue from FAD and SAD patients compared to controls, while Almad *et al.* (2022) did not observe any differences in protein expression of GLAST between the control and the ALS samples. In our study, a decrease in EAAT1 intensity was observed in CTX-Astros post-stimulation with pro-inflammatory factors with the largest decrease after TIC stimulation. In VM-Astros on the other hand, a trend towards an increase in EAAT1 intensity was observed, but this was not significant. Importantly, expression of *SLC1A3*, a gene encoding for EAAT1, was also detected by qPCR, which will be discussed below in the context of functional gene regulation.

CD49f, also known as Integrin alpha 6, is an integrin alpha chain protein. Integrins in general are involved in supporting cell adhesion and are involved in extracellular matrix interactions (Barbar *et al.*, 2020). Although CD49f is a very novel astrocyte marker, it has been observed that it is a reactivity-independent marker, meaning it would be expressed in control and reactive astrocytes, and therefore it could be used to purify iPSC-derived astrocytic cultures. Barbar *et al.* (2020) used transcriptomics and RNA-sequencing methods to analyse primary human and iPSC-derived astrocytes that were CD49f positive. They also confirmed that these astrocytes will indeed react to pro-inflammatory stimuli such as TIC and TNF α +IL-1 β to mimic neuroinflammation, with potent immune response (Barbar *et al.*, 2020). Moreover, they have shown that forebrain astrocytes are more positive for CD49f than spinal astrocytes. McComish *et al.* (2024) have also successfully used CD49f as one of the markers for characterising CTX-Astros. This is consistent in our study, where CD49f intensity is significantly decreased in CXT-Astros by stimulation with IL-1 α and TNF α +IL-17A, but not TIC. This intensity is at least 10-fold lower in VM-Astros and is not affected by any of the pro-inflammatory stimuli used. Importantly, qPCR analysis of the *ITGA6* mRNA was also assessed as part of this study and is discussed below in the context of functional gene regulation.

Cx43 is a major connexin, and a gap junction protein found in astrocytes and is therefore responsible for astrocytic intracellular communication. Cx43 is involved in synchronisation of astrocyte networks via calcium signalling, providing metabolic support to neurons as well as modulating synaptic activity and plasticity (Giaume and Liu, 2012). It has been reported in numerous studies that alterations in Cx43 expression are observed in neurodegenerative diseases such as ALS (Almad *et al.*, 2016, 2022), or PD (Fujita *et al.*, 2018). Just like in the case of GFAP, Cx43 intensity is significantly higher in our VM-Astros, rather than CTX in all groups, except for TIC-stimulated cells. The intensity is reduced post-IL-1 α and TIC stimulation in VM-Astros, but not TNFA+IL-17A, with the most substantial decrease in cells stimulated with TIC. In CTX-Astros instead, the intensity is significantly reduced in IL-1 α and TNFA+IL-17A-stimulated groups, and although a trend towards a decrease in TIC-stimulated group is observed, this is non-significant. This is consistent with McComish *et al.* (2024) who have observed a significant decrease in Cx43 intensity post-TIC stimulation. Fujita *et al.* (2018) however observed an increase in the number of Cx43 immunoreactive puncta in a mouse model of PD. qPCR analysis of the *GJA1* mRNA, the gene encoding for Cx43, was also assessed as part of this study and is discussed below in the context of functional gene regulation.

Although in rodent and murine studies GFAP is the most reliable astrocyte marker, in the case of iPSC-derived cells, not all cells stain for GFAP with the same intensity, which does not allow for a uniform morphology analysis. Some studies suggest that even as little as 15% of astrocytes stain positive for GFAP (Khakh and Sofroniew, 2015; Baldwin, Murai and Khakh, 2024), thus putting its reliability as an astrocyte marker in question. Therefore, S100 β immunoreactivity was used in this study to quantify cell area, circularity, perimeter, eccentricity, maximal (max) Feret diameter and minimal (min) Feret diameter.

In our study, CTX-Astros are approximately 5-times the size of VM-Astros at baseline. Cell area in CTX-Astros does, however, decrease post-stimulation with IL-1 α and TNFA+IL-17A, while in VM-Astros, the cell area does not seem to be affected by stimulation with pro-inflammatory factors. Other studies also report similar results. For example, Endo *et al.* (2022) found significant differences in cell

sizes of astrocytes from 13 different brain regions of adult mice CNS, including the cortical and midbrain regions. In their study, the cell area of an astrocyte from the motor cortex is approximately 500 μm^2 larger than that of a midbrain astrocyte. The astrocytes found in the visual and somatosensory cortex were also substantially larger than those found in the midbrain area. A mouse study by Chai *et al.* (Chai *et al.*, 2017) also found differences in astrocyte sizes between striatum and the hippocampus, with the striatal astrocytes being larger than the ones based in the hippocampal region of the brain. Although not part of the cortex or the midbrain regions, this study further proves that astrocyte size will differ between the brain regions.

The cell perimeter results are similar to the cell area, with the perimeter being significantly larger in CTX-Astros than VM, regardless of stimulation. This is consistent with previous observations that astrocyte territorial domains (the volume of tissue that is covered by their processes that ensure full parenchymal coverage) and process complexity vary by brain region (Zhou, Zuo and Jiang, 2019; Baldwin, Murai and Khakh, 2024; Bocchi *et al.*, 2025). In CTX-Astros, cell perimeter is significantly decreased post-stimulation with IL-1 α , but not other stimuli. There is a trend towards a decreased cell perimeter in VM-Astros stimulated with IL-1 α and TIC, but this result is not significant. Similar observations were observed by McComish *et al.* (2024) in CTX-Astros, where cell perimeter and nuclear perimeter were significantly reduced post-stimulation with TIC and the 10-fold dilution of TIC. In contrast, cell perimeter increased in reactive cortical astrocytes post-TBI injury in mice (Delgado-García *et al.*, 2024). MacMahon Copas *et al.* (2024) also observed a decrease in nuclear perimeter of VM-Astros post-72h and 7d stimulation with TNF α , but not at 24h of stimulation. Other perimeter-related changes in morphology have been observed in reactive astrocytes, such as reduced process extension and cell hypertrophy (Hyvärinen *et al.*, 2019). One limitation is that few studies include cell perimeter as one of the outputs for morphology analysis. Instead, most scientists report differences in astrocyte area or territory, from which perimeter changes can only be inferred. However, because changes in perimeter generally

follows the trends in cell area and process complexity, our findings are in line with prior reports of region- and stimulation-dependent morphological diversity.

In our study, cell circularity does not change a lot. There is a trend towards an increase in circularity post-stimulation with IL-1 α and TNFA+IL-17A in CTX-Astros. Similarly, in VM-Astros, IL-1 α and TNFA+IL-17A stimulation also increase cell circularity, with the increase being significant in IL-1 α -stimulated group only. Stimulation with TIC does not have an effect on cell circularity in either region. However, a regional difference is observed at baseline and post-TNFA+IL-17A stimulation with the VM-Astros being less circular than CTX-Astros. In their study, Endo *et al.* (2022) also observed that cortical astrocytes are more circular than the midbrain ones in adult mice. In fact, out of 13 different brain regions assessed, cortical astrocytes have the most circular morphology, while those located in the cerebellum are the least circular. In terms of circularity being affected by neuroinflammation, both MacMahon *et al.* (2024) and McComish *et al.* (2024) observed an increase in nuclear and cell circularity in VM- and CTX-Astros, respectively. Similarly, Hyvärinen *et al.* (2019) observed an increase in cell circularity in CTX-Astros post-stimulation with IL-1 β and TNF α , while Delgado-Garcia *et al.* (2024) report a decrease in circularity in various subsets of reactive cortical astrocytes in a TBI mice model.

Not many changes in cell eccentricity are observed in our study. The eccentricity does trend towards a decrease post-stimulation, but this is only significant in the case of CTX-Astros stimulated with TNFA+IL-17A. A significant difference is also observed between the regions post-stimulation with TIC, where the cell eccentricity is lower in VM-Astros. To our knowledge, there are no studies reporting eccentricity as a morphology metric in astrocytes. However, prior work on astrocyte heterogeneity frequently discusses differences in the process length, branch directionality and territorial domain complexity (Endo *et al.*, 2022; Delgado-García *et al.*, 2024), which are linked to shape anisotropy, that is, the extent to which cells deviate from an isotropic, round morphology. In some context, anisotropy may also reflect aspects of polarisation, where cells adopt a more elongated or directionally biased morphology in response to environmental cues, such as pro-inflammatory

stimulation. Our inclusion of eccentricity, along with Feret diameters discussed below, adds nuance to morphological analysis by quantifying the elongation and directional bias of astrocytes within two separate brain regions, which is beyond what area or perimeter alone can convey.

Consistent with their overall larger size, our CTX-Astros displayed a 2-fold larger Max Feret diameter compared to VM-Astros. However, once stimulated, the max Feret diameter decreases in CTX-Astros across all stimulation groups. Similarly, the max Feret diameter in VM-Astros also trends towards a decrease post-stimulation, but this is non-significant. Just like with the max Feret diameter, the min Feret diameter is higher in CTX-Astros compared to VM, across all stimulation groups. The min Feret diameter decreases once stimulated, but this is only significant in CTX-Astros stimulated with IL-1 α . Although a trend toward a decrease is observed in VM-Astros and the other stimulated groups in CTX, these results are non-significant. Similar is observed by Endo *et al.* (2022), with both max and min Feret diameters being higher in cortical astrocytes of adult mice compared to midbrain. In a study published by McComish *et al.* (2024), the stimulation of CTX-Astros with TIC and a 10-fold dilution also resulted in a significantly reduced min and max Feret diameters. Taken together, the Feret diameter analysis reinforces our findings in terms of cell area, perimeter circularity and eccentricity, thus demonstrating that CTX-Astros not only occupy a larger spatial domain than their VM counterparts, but also undergo a more pronounced reduction in cell span following stimulation with pro-inflammatory factors.

Overall, these morphological analyses show that CTX-Astros are substantially larger than VM-Astros and more prone to structural remodelling following pro-inflammatory stimulation. Even at baseline, significant regional differences are evident; however, upon stimulation, it is primarily the CTX-Astros that undergo substantial morphological changes. In contrast, VM-Astros showed only non-significant trends toward reduced perimeters and Feret diameters. These results suggest that intrinsic heterogeneity, which is potentially linked to differences in metabolic activity or gene expression, rather than external stimuli alone, underlies the distinct regional responses that were observed.

3.4.3. Astrocyte Reactivity and Inflammatory Response

Building on these morphological findings, we next investigated whether regional heterogeneity was also reflected in the transcriptional and secretory profiles associated with astrocyte reactivity and inflammatory signalling. Astrocyte reactivity is classically characterised by an upregulation of intermediate filaments such as *GFAP* and *VIM*, alongside the induction of genes linked to inflammatory and stress responses such as *LCN2*, *C3* or *SERPINA3*. These changes are often also accompanied by the induction of genes encoding for inflammatory mediators and receptors (e.g. *IL6*, *CXCL10*, *ICAM1* and *TLR4*), as well as altered cytokine and chemokine release, which can modulate neuronal survival and potentially immune cell recruitment. To assess these processes in CTX- and VM-Astros, we quantified the expression of canonical reactive markers together with a panel of cytokine, chemokine and adhesion-related genes, followed by complementing these with ELISAs for key secreted proteins, including IL-6, CXCL10, GM-CSF and RANTES.

GFAP, the classical astrocytic filament protein, was expressed at much higher levels in CTX-Astros at baseline, in contrast to immunostaining intensity, and decreased post-IL-1 α or TIC stimulation. Similar trend was observed in VM-Astros, but this was non-significant. *VIM*, also a filament protein upregulated during astrocyte reactivity, was robustly expressed at baseline, but consistently downregulated post-stimulation in both regions, particularly when stimulated with TIC, with mRNA levels slightly lower in CTX-Astros.

Complement component C3, a hallmark of “A1-like” reactive astrocytes, was barely detectable at baseline, but increased dramatically in both regions following stimulation, with consistently higher expression in VM-Astros. *LCN2* expression, a pro-inflammatory lipocalin, also increased significantly post-stimulation, with the strongest response in CTX-Astros post-IL-1 α stimulation and post-TNF α +IL-17A stimulation in VM-Astros. In contrast, *SERPINA3*, a serine protease inhibitor and human-specific reactivity marker, was expressed at high levels in VM-Astros at baseline and remained several-fold higher than CTX-Astros across all stimulation groups. *TIMP1*, which plays a role in extracellular matrix remodelling, was expressed at very low baseline levels in both regions, but showed a modest induction after TIC-

stimulation, particularly in CTX-Astros. Together, these results demonstrate that while some markers such as *GFAP*, *VIM* or *TIMP1* show modest, or even downregulated responses to stimuli, others (e.g. *C3*, *SERPINA3*, *LCN2*) exhibit substantial upregulation that varies between brain regions. This suggests that CTX and VM-Astros adopt distinct reactivity profiles, with cortical astrocytes showing higher expression of stress-responsive genes such as *LCN2*, whereas midbrain astrocytes exhibit preferences towards the classical astrocytic reactivity markers such as *C3* and maintain higher basal levels of *SERPINA3*. This aligns with transcriptomic and injury models *in vivo*, where distinct subsets of reactive astrocytes have been reported (Zamanian *et al.*, 2012; Liddelow *et al.*, 2017), and with more recent human iPSC-derived astrocyte studies showing context-dependent *GFAP*, *LCN2*, *C3* and *SERPINA3* regulation (Hyvärinen *et al.*, 2019; Crompton *et al.*, 2023; MacMahon Copas *et al.*, 2024; McComish *et al.*, 2024). Thus, our findings extend previous work by highlighting that such heterogeneity is evident not only *in vivo*, but also when comparing regionally patterned astrocytes side-by-side *in vitro*.

Cytokine and chemokine expression profiles of CTX- and VM-Astros revealed striking regional differences at both protein and gene level. *IL6*, a cytokine central to neuroinflammation, was significantly upregulated in VM-Astros across all stimulation groups, with TIC having the strongest effect on *IL6* mRNA release. CTX-Astros have shown a similar upregulation, but only post-IL-1 α and TIC stimulation. At the protein level, the IL-6 release mirrored transcriptional regulation, with both regions secreting very high levels post-stimulation with IL-1 α and TIC, but VM-Astros also produced IL-6 in TNF α +IL-17A stimulated group, releasing substantially more protein than CTX. Similar is observed by MacMahon Copas *et al.* (2024) in VM-Astros post-TNF α +IL-17A stimulation, by McComish *et al.* (2024) in CTX-Astros stimulated with TIC and 10-fold dilution, as well as by Barbar *et al.* (2020) in CTX-Astros stimulated with TIC and TNF α +IL-1 β . CXCL10, a potent chemokine involved in the immune cell recruitment, followed a similar pattern. At gene level, VM-Astros expressed very high levels of *CXCL10* mRNA across all stimuli, while CTX-Astros responded mainly to stimulation with TIC, with a weaker *CXCL10* mRNA expression

post-IL-1 α stimulation. CXCL10 chemokine release was very robust in both regions, although VM-Astros released over twice as much CXCL10 as CTX-Astros post TNF α +IL-17A stimulation. Substantial amounts of CXCL10 at protein and gene level were released by TNF α alone and TNF α +IL-17A stimulation in VM-Astros, as per MacMahon Copas *et al.* (2024). Similarly, potent CXCL10 mRNA release was reported in CTX-Astros post-TIC stimulation, but the cytokine levels were not assessed (Barbar *et al.*, 2020; McComish *et al.*, 2024). Szpakowski *et al.* (2022) however observed an extremely high CXCL10 release in primary human cortical astrocytes stimulated with TIC.

CCL5 (a gene encoding RANTES), a chemoattractant involved in immune cell recruitment, was expressed at low levels at baseline, but stimulation with IL-1 α and TIC significantly increased its expression in both regions. Protein release again paralleled gene expression, with VM-Astros producing more RANTES than CTX across all stimulation groups. Similar expressions of CCL5 at gene and protein level post-stimulation were observed in numerous studies (Barbar *et al.*, 2020; Crompton *et al.*, 2023; MacMahon Copas *et al.*, 2024; McComish *et al.*, 2024), including stimulation with TIC, TNF α +IL-17A, TNF α +IL-1 β , TNF α alone or IL-1 β alone, just to name a few.

Finally, CSF2 (a gene encoding GM-CSF) was selectively upregulated at gene level by TIC stimulation, with CTX-Astros expressing roughly 3-fold more CSF2 mRNA than VM. At protein level, stimulation with IL-1 α and TIC induced GM-CSF release in both regions, though the response to TIC stimulation was the strongest, and CTX-Astros secreted substantially more GM-CSF than VM. This was previously reported in CTX-Astros stimulated with TIC or TNF α +IL-1 β (Barbar *et al.*, 2020; McComish *et al.*, 2024), but to our knowledge, CSF2/GM-CSF in astrocyte reactivity across different brain regions is a lot less studied. Together, these results demonstrate that while both cortical and midbrain astrocytes can produce inflammatory cytokines and chemokines, VM-Astros show broader responsiveness to multiple stimuli, particularly TNF α +IL-17A, whereas CTX-Astros mount a more selective, but sometimes substantially stronger response like in the case of CSF2/GM-CSF. This

suggests that astrocytes from different brain regions employ distinct inflammatory strategies, which may differentially affect local environments.

Among the additional inflammatory mediators assessed, *PTX3*, *TLR4* and *IL1B* showed regionally distinct patterns. *PTX3*, an innate immune pattern-recognition molecule involved in complement activation and inflammatory cascades, was expressed at relatively high baseline levels, with a trend toward higher expression in CTX-Astros. Significant upregulation was only observed post-IL-1 α and TIC stimulation, with the expression being higher in CTX-Astros than in VM. This is very similar to the report by McComish *et al.* (2024) who observed an increase in *PTX3* mRNA expression post-TIC stimulation and 10-fold dilution in CTX-Astros, while MacMahon Copas *et al.* (2024) observed a slight increase in *PTX3* expression post TNF α +IL-17A stimulation, which was non-significant. Zamanian *et al.* (2012) also observed a *PTX3* induction in neuroinflammation, both after LPS stimulation and post-TBI injury. Similarly, Rodriguez-Grande *et al.* (2014) reported an increased expression of *PTX3* at protein and gene level post-acute brain ischemia at the injury site, but not in other regions.

In contrast, *TLR4*, a pattern-recognition receptor (PRR) responding to endogenous danger signals or DAMPs, which drives the NF- κ B signalling, was consistently expressed at higher levels by VM-Astros at baseline and in pro-inflammatory conditions. Like *PTX3*, it was selectively upregulated by IL-1 α and TIC stimulation in both regions. This selectivity in upregulation of *TLR4* depending on the stimuli is supported by other studies, such as Zamanian *et al.* (2012), where *TLR4* upregulation is observed in neuroinflammation post-TBI injury in adult mice, but not in LPS stimulation.

Finally, *IL1B*, a potent pro-inflammatory cytokine that amplifies downstream signalling cascades was robustly induced in CTX-Astros after IL-1 α and TIC stimulation, whereas VM-Astros showed only a non-significant trend in the same direction. Gomes *et al.* (2025) have also reported similar results in their iPSC-derived cortical spheroids containing neurons, astrocytes and oligodendrocytes. In their study, spheroids were stimulated with TIC, resulting in a number of upregulated genes, including *IL1B*. McComish *et al.* (2024) have also observed

significant upregulation in *IL1B* mRNA expression post-TIC stimulation. In contrast to our results, MacMahon Copas *et al.* (2024) observed a small, albeit significant increase in *IL1B* mRNA expression in VM-Astros post-TNF α +IL-17A stimulation, which was non-significant in our study. Taken together, these findings suggest that while cortical astrocytes potentially upregulate pro-inflammatory effectors (*PTX3*, *IL1B*), ventral midbrain astrocytes maintain higher expression of upstream PRRs such as *TLR4*, indicating region-specific inflammatory strategies.

In addition to cytokine secretion, astrocytes regulate neuroinflammation by modulating immune cell adhesion and receptor interactions. *ICAM1*, a key adhesion molecule mediating peripheral immune cell transport across the BBB and facilitating interactions between T-cells and astrocytes (Dong and Benveniste, 2001; Huber *et al.*, 2006), was expressed at low levels in both CTX and VM-Astros at baseline. This expression increased across all stimulated groups, but significance was only reached post-IL-1 α and TIC-stimulations, with no regional differences observed. This aligns with prior reports that astrocytic *ICAM1* is robustly induced by IL-1 β and TNF α (Frohman *et al.*, 1989; Zamanian *et al.*, 2012). Similarly, *ITGAM* which encodes the integrin α M (Cd11b) and more typically is associated with microglia, was only detectable in stimulated CTX-Astros, with the highest expression observed following TNF α +IL-17A and TIC stimulation groups. Non-significant trends towards induction were observed in VM-Astros under the same conditions, but the expression remained at least 10-fold lower than in CTX. To our knowledge, no other studies have detected expression of *ITGAM* mRNA in reactive astrocytes. While some studies (Batiuk *et al.*, 2020; Sokolova *et al.*, 2024) report very low or essentially non-existent levels of *ITGAM* mRNA in astrocytes, none of these studies looked at it post-stimulation with pro-inflammatory factors.

IL17RA mRNA expression was also low overall, but higher in CTX-Astros than VM, both at baseline and post-stimulation. Stimulation in CTX-Astros resulted in an upregulation of *IL17RA* mRNA across all groups, while in VM-Astros, this was only true for TNF α +IL-17A and TIC-stimulated groups. Notably, CTX-Astros' higher *IL17RA* mRNA expression did not translate into stronger TNF α +IL-17A responsiveness across most readouts. This is plausible because IL-17 signalling

depends on the *IL17RA* receptor pairing with IL17RC as well as the cross-talk between NF- κ B and MAPK signalling pathways, thus showing that receptor abundance alone is not sufficient to predict functional responsiveness of the cells (Zamanian *et al.*, 2012; Crompton *et al.*, 2023).

IFNB1, a type I interferon crucial for antiviral responses, was moderately upregulated by pro-inflammatory stimulation, particularly after IL-1 α and TIC, with the expression consistently higher in CTX-Astros than VM. In contrast, *IFNA2* mRNA expression remained very low across all groups and showed little evidence of region-specific regulation. Although the expression of *IFNA2* and *IFNB1* in astrocytes stimulated with pro-inflammatory cytokines is not well characterised, prior studies demonstrate that astrocytes are competent to mount type I interferon responses. For instance, astrocytes can strongly activate IFN α/β pathways during viral infection (Lin *et al.*, 2015; Jorgačevski and Potokar, 2023) and can produce IFN β in brain metastasis (Ma *et al.*, 2023). iPSC-derived astrocytes have also shown spontaneous activation of type I interferons in models of Aicardi-Goutières syndrome, which is a type of monogenic inflammatory encephalopathy (Giordano *et al.*, 2022). Furthermore, astrocytic IFN α/β signalling plays a crucial role in antiviral defence, immune crosstalk and neuronal support (Viengkhou and Hofer, 2023). Thus, while most evidence stems from infection or pathological models, *IFNB1* upregulation in stimulated CTX- and VM-Astros highlights their versatility and adaptability, extending the relevance of type I interferon pathways beyond infectious disease.

Taken together, these findings suggest that adhesion and receptor genes such as *ICAM1*, *ITGAM* and *IL17RA* are induced in iPSC-derived astrocytes, but their regulation differs between brain regions. The moderate induction of *IFNB1* further highlights that astrocytic immune programming extends beyond classical cytokines to include interferon pathways, underscoring the ability of astrocytes in shaping region-specific inflammatory responses.

3.4.4. Functional Astrocyte Genes

While adhesion molecules and interferon pathways highlight astrocytes' roles in immune surveillance and inflammatory crosstalk, these functions operate alongside their fundamental contributions to CNS homeostasis. To assess whether regional identity also shapes such supportive roles, we examined transcripts linked to neurotransmitter clearance, intercellular communication, and neuronal support, including *SLC1A2*, *SLC1A3*, *GJA1*, *GLUL*, *DRD2*, and *ITGA6*.

Core astrocytic support functions were broadly preserved across regions, with notable differences. *SLC1A2* (EAAT2) mRNA expression was similar between CTX and VM-Astros at baseline and across most stimuli, with a modest increase in CTX-Astros post-TIC stimulation and a decrease post-IL-1 α stimulation. In contrast, VM-Astros stimulated with TNF α +IL-17A have a higher *SLC1A2* mRNA expression compared to control, while the IL-1 α and TIC-stimulated groups induce *SLC1A2* downregulation. This contradicts the reports that EAAT2 is highly sensitive to pro-inflammatory environments, often showing robust downregulation (Pajarillo *et al.*, 2019). In contrast, *SLC1A3* (EAAT1) mRNA expression was robust in both regions, but approximately 2-fold higher in CTX-Astros than VM, both at baseline and post-stimulation, in line with EAAT1's recognised enriched expression in forebrain astrocytes (Danbolt, 2001). However, in CTX-Astros at gene level, *SLC1A3* mRNA levels increase when stimulated with IL-1 α or TNF α +IL-17A, while EAAT1 immunostaining intensity decreased with stimulation, thus suggesting post-transcriptional regulation, which has also been suggested in prior studies of glutamate transporter regulation under stress.

GJA1, encoding the astrocytic gap junction protein connexin 43 (Cx43), likewise showed disparity between gene and protein expression, with the mRNA expression being higher in CTX-Astros, while Cx43 intensity was higher in VM-Astros. As well as that, the mRNA was downregulated post-stimulation with IL-1 α and TIC, especially in CTX-Astros, while TNF α +IL-17A stimulation upregulated *GJA1* mRNA. Cx43 staining intensity decreased regardless of stimulation. This discrepancy echoes previous reports of stimuli- and context-dependent regulation of connexins, where inflammatory cues often suppress protein abundance despite gene-level

upregulation (Canals *et al.*, 2018). More recent work in iPSC-derived astrocytes similarly showed cytokine-specific modulation of Cx43, highlighting the complexity of its regulation (Perriot *et al.*, 2018; McComish *et al.*, 2024).

GLUL (glutamine synthetase) was consistently higher in CTX-Astros and mRNA expression increased further across all stimulation groups, while in VM-Astros this was only observed post-TNF α +IL-17A stimulation, thus implying greater glutamate-glutamine cycling capacity in CTX. This is consistent with early studies identifying glutamine synthetase as a defining marker of astrocytic glutamate metabolism (Norenberg and Martinez-Hernandez, 1979).

Moreover, *ITGA6/CD49f* mRNA, an astrocyte-associated integrin involved in cell adhesion and extracellular matrix interactions, was 4-fold higher in VM-Astros at baseline and significantly decreased with stimulation. However, in CTX-Astros, stimulation with pro-inflammatory factors upregulated *ITGA6* mRNA, especially post-TIC stimulation. At protein level, CD49f intensity was consistently higher in CTX-Astros, which again points to post-transcriptional differences between regions. Prior studies have described *ITGA6* as enriched in forebrain-derived astrocytes compared to spinal cord or other regions (Bradley *et al.*, 2019; Barbar *et al.*, 2020), thus supporting our protein-level data.

Among dopaminergic-related genes, *DRD2* mRNA expression was higher in CTX-Astros at baseline and post-IL-1 α stimulation compared to VM but decreased across all stimulation groups. *SLC6A3* mRNA, on the other hand, remained very low with only a marginal increase in TIC-stimulated CTX-Astros. Although astrocytic *DRD2* expression has been reported in some contexts (Crompton *et al.*, 2023), and sporadic evidence for *SLC6A3* in astrocytes also exists (Xin *et al.*, 2019), the overall low abundance and weak regulation suggests that these transcripts likely reflect residual regional imprinting rather than major contributors to astrocyte reactivity *in vitro*.

Together, these findings show that while core astrocytic functions such as glutamate uptake, gap junction communication, and glutamine synthesis are preserved across regions, cortical astrocytes generally display higher baseline and

stimulus-induced expression of functional support genes than their midbrain counterparts. These regional differences suggest that astrocytes' developmental origin not only imprints their inflammatory profiles but also shapes their homeostatic capacity, potentially influencing how effectively they sustain neuronal networks under stress.

3.4.5. Metabolic and Stress-Response Genes

In addition to their homeostatic roles, astrocytes are central regulators of brain energy metabolism, relying heavily on glycolysis and lactate production to support neurons (Pellerin and Magistretti, 1994; Bélanger, Allaman and Magistretti, 2011). Increasing evidence indicates that astrocytes undergo metabolic reprogramming during reactivity, shifting towards glycolysis in response to inflammatory or pathological stimuli (Calì *et al.*, 2024; McComish *et al.*, 2024). To investigate whether regional identity contributes to such metabolic adaptations, we examined glycolysis- and metabolism-related genes, including *LDHA*, *HK2*, *PFKFB3*, *SLC2A1*, and *PPARG*, in cortical and ventral midbrain astrocytes.

Glycolysis-associated gene expression was low at baseline but showed region- and stimuli-dependent regulation. *LDHA* mRNA was higher in CTX-Astros than VM, and increased with stimulation, especially TIC, albeit still within a low absolute range (~0.1-0.4 RQ), consistent with astrocytic *LDHA*'s role in lactate support to neurons (Yao *et al.*, 2023). A similar pattern was followed by hexokinase 2 (*HK2*) mRNA, which catalyses the first committed step of glycolysis through phosphorylation of glucose. *HK2* expression was significantly upregulated following IL-1 α and TIC stimulation in CTX-Astros but remained unchanged in VM-Astros. This aligns with *HK2*'s role as a glycolytic gatekeeper (Howden *et al.*, 2025), although astrocyte-specific literature on this topic remains limited.

PFKFB3 mRNA, a key glycolytic regulator that promotes fructose-2,6-bisphosphate production and glycolytic flux, increased in both regions in IL-1 α and TIC-stimulated groups, but not TNF α +IL-17A stimulation, with expression remaining significantly higher in CTX-Astros. This aligns with previous studies implicating *PFKFB3*-driven glycolysis in reactive astrocytes under inflammatory and metabolic stress

conditions (Lv *et al.*, 2015; Xiong, Tang and Yang, 2022). In contrast, *SLC2A1* (GLUT1) was slightly higher in VM-Astros at baseline but decreased post-IL-1 α and TIC in both regions, with higher expression in stimulated CTX-Astros than VM, which is consistent with reported regional differences in glucose handling and transporter signalling (Nehlig, Wittendorp-Rechenmann and Lam, 2004).

Finally, *PPARG* mRNA decreased with stimulation in both regions, but at baseline, VM-Astros express approximately 3-fold higher levels of *PPARG* mRNA than CTX. This is consistent with earlier immunohistochemical evidence that PPAR γ is expressed in astrocytes throughout the CNS, supporting its role as a regulator of glial metabolic and inflammatory function (Moreno, Farioli-Vecchioli and Cerù, 2004). Functionally, PPAR γ activation suppresses pro-inflammatory cytokine production, inhibits NF κ B signalling and promotes mitochondrial and lipid metabolic homeostasis in astrocytes, making it an important modulator of reactive astrocyte phenotypes. More recently, Fernandez *et al.* (2017) reported that astrocytes express PPAR γ at higher levels than neurons, including a change in astrocytic functional phenotype in PPAR γ knock-out experiment, while Warden *et al.* (2016) confirmed widespread astrocytic PPAR γ expression across brain regions, arguing for a maintained role in glial metabolism and inflammation.

Together, this data indicates a more pronounced expression of glycolytic genes in CTX-Astros (*LDHA*, *HK2*, *PFKFB3*) and a greater baseline metabolic gene expression in VM-Astros (*PPARG*, *SLC2A1*), which is consistent with Seahorse analysis discussed below. The observed upregulation of *PFKFB3*, *HK2* and *LDHA* in stimulated CTX-Astros aligns with the concept of metabolic reprogramming in reactive astrocytes (Liu *et al.*, 2024), where enhanced glycolysis helps meet the demand for additional energy. In pathological settings, such as neurodegeneration, reactive astrocytes often shift towards a glycolytic metabolism (Calì *et al.*, 2024), and the gene expression of key glycolytic enzymes is also altered in neuroinflammation (Cantando *et al.*, 2024; McComish *et al.*, 2024). These precedents support the idea that the transcriptional changes we see in CTX-Astros are a part of a broader, astrocyte-intrinsic metabolic shift in response to an inflammatory challenge.

3.4.6. Functional Metabolism: Glycolytic Stress Testing

To test whether the transcriptional differences in glycolytic and metabolic genes were reflected at the functional level, we assessed extracellular acidification rates (ECAR) using the Seahorse Glycolytic Stress Test. Astrocytes are metabolically flexible cells that provide critical bioenergetic support to neurons, and shifts towards glycolysis are a hallmark of reactive states (Pellerin and Magistretti, 1994; Bélanger, Allaman and Magistretti, 2011; Calì *et al.*, 2024). By directly measuring glycolytic flux and capacity, this assay allowed us to determine whether cortical and ventral midbrain astrocytes differ not only in their gene expression but also in their metabolic performance under inflammatory stimulation.

CTX-Astros exhibited significantly higher glycolytic activity when stimulated, with all pro-inflammatory groups showing elevated glycolysis and glycolytic capacity compared to the control. TIC stimulation consistently produced the strongest effect, followed by IL-1 α , while TNF α +IL-17A increased glycolysis only before and immediately after glucose injection. Notably, the TNF α +IL-17A effect resolved following oligomycin injection, indicating that glycolytic reserve was not significantly altered under this condition. In contrast, VM-Astros displayed a similar pattern but at much lower levels. No differences were evident before glucose injection; however, TIC-stimulated cells showed higher glycolysis post-glucose injection and both IL-1 α and TIC increased glycolysis following oligomycin injection, with TIC again being the most potent stimulus.

Across all conditions, CTX-Astros had higher glycolytic rates than VM-Astros, both at baseline and after stimulation, with the largest regional differences observed in TIC-stimulated groups. Quantitative analysis revealed that basal glycolysis and glycolytic capacity were consistently higher in CTX-Astros, reaching peak levels with TIC stimulation. Glycolytic reserve followed the same trend, significantly increasing in CTX-Astros after IL-1 α and TIC, but only in VM-Astros post-TIC stimulation. Non-glycolytic acidification was also higher in CTX-Astros, though stimulation effects were modest and non-significant.

These findings support the view that reactive astrocytes undergo metabolic reprogramming, shifting towards glycolysis in response to inflammatory stimuli. This is consistent with prior reports that human iPSC-derived astrocytes increase glycolytic flux under pro-inflammatory stimulation (McComish *et al.*, 2024), and with the broader evidence that inflammation drives astrocytes into a more glycolytic, less oxidative metabolic profile (Bélanger, Allaman and Magistretti, 2011; Cali *et al.*, 2024). The higher glycolytic rate and reserve in CTX-Astros aligns with the notion that cortical astrocytes are metabolically more flexible than midbrain astrocytes, an idea also supported by studies showing regional differences in astrocytic energy metabolism (Endo *et al.*, 2022; Hasel *et al.*, 2023; Bocchi *et al.*, 2025). Thus, Seahorse data complement our transcriptional analysis (*LDHA*, *HK2*, *PFKFB3*, *PPARG*) by confirming that regional identity imprints lasting differences on astrocyte metabolic capacity.

3.4.7. Summary of Key Findings

Table 3.4.1 Summary of key regional differences between CTX- and VM-Astros

Experimental area	Main findings	Key markers/features affected	Overall interpretation
Regional patterning of NPCs	CTX- and VM-NPCs expressed distinct regional markers at protein and gene level	CTX markers: TBR1; VM markers: LMX1A, FOXA2, EN1	Successful generation of region-specific neural progenitors
Astrocyte differentiation and identity	Both CTX- and VM-Astros expressed canonical astrocyte markers	GFAP, S100 β , EAAT1, Cx43, CD49f positive	Both populations successfully differentiated into astrocytes
Astrocyte-associated protein expression	Expression intensity differed between regions and stimulation conditions	CTX $\uparrow$ S100 β , EAAT1, CD49f; VM $\uparrow$ GFAP, Cx43;	Astrocyte identity and reactivity are region-dependent
Morphological analysis	CTX- and VM-Astros displayed distinct morphologies	CTX $\uparrow$ Cell area, perimeter, Feret diameter	Regional identity influences astrocyte structure
Region-specific gene expression	Distinct transcriptional signatures observed between CTX- and VM-Astros	Both express region-specific genes	Astrocytes retain developmental regional identity <i>in vitro</i>
Reactive marker expression	Reactive stimuli altered astrocyte reactivity markers differently between regions	CTX $\uparrow$ <i>GFAP</i> , <i>LCN2</i> , <i>TIMP1</i> VM $\uparrow$ <i>C3</i> , <i>SERPINA3</i> , <i>VIM</i>	Reactive astrocyte profiles are heterogeneous and region-specific
Cytokine and chemokine release	VM-Astros generally exhibited stronger inflammatory responses	VM $\uparrow$ IL-6, CXCL10, RANTES; CTX $\uparrow$ GM-CSF release after TIC	Regional identity shapes inflammatory responsiveness
Inflammatory gene expression	CTX- and VM-Astros responded differently to inflammatory stimulation at transcriptional level	VM $\uparrow$ <i>IL6</i> , <i>CXCL10</i> , <i>CCL5</i> , <i>TLR4</i> ; CTX $\uparrow$ <i>CSF2</i> , <i>IL17RA</i> , <i>PTX3</i> , <i>IL1B</i> , <i>ITGAM</i> , <i>IFNB1</i> ;	Distinct inflammatory signalling programs exist between regions
Functional gene expression	Functional astrocyte genes differed between CTX- and VM-Astros	CTX $\uparrow$ <i>SLC1A2</i> , <i>SLC1A3</i> , <i>GJA1</i> , <i>GLUL</i> , <i>DRD2</i> ; VM $\uparrow$ <i>ITGA6</i> ;	Functional specialisation is retained <i>in vitro</i>
Metabolic and stress-response genes	Regional differences in metabolic gene expression were observed	CTX $\uparrow$ <i>LDHA</i> , <i>HK2</i> , <i>PFKFB3</i> , <i>SLC6A3</i> ; VM $\uparrow$ <i>SLC2A1</i> , <i>PPARG</i> ;	Astrocyte metabolism differs according to developmental origin
Seahorse glycolytic analysis	CTX-Astros exhibited greater glycolytic activity and reserve capacity	CTX $\uparrow$ Basal glycolysis, glycolytic capacity and reserve	Cortical astrocytes are metabolically more flexible than VM-Astros

3.4.8. Conclusion

This chapter demonstrates substantial differences between cortical and midbrain ventral astrocytes in cytokine release, protein and gene expression, and metabolic activity, despite both populations being differentiated under identical conditions from iPSC-derived neural progenitors. These findings strongly suggest that astrocyte heterogeneity is shaped by developmental origin, which imprints lasting functional differences. *In vivo* (and co-culture systems *in vitro*) this intrinsic diversity is further modulated by interactions with neurons or microglia (Gomes *et al.*, 2001; Sakimoto *et al.*, 2012). Ultimately, it is this diversity that likely contributes towards the region-specific vulnerability observed in neurodegenerative diseases such as AD or PD (Molofsky *et al.*, 2012; Phatnani and Maniatis, 2015).

In summary, iPSC-derived astrocytes preserve key aspects of their regional identity, which in turn shape their morphology, molecular signatures, reactivity and metabolic activity. Cortical and midbrain astrocytes displayed divergent transcriptional and functional responses, reflecting patterns of heterogeneity described *in vivo*. These findings underscore the importance of developmental imprinting in defining astrocyte behaviour and its relevance for region-specific disease susceptibility. By modelling such diversity *in vitro*, this work highlights the value of region-specific astrocyte systems for dissecting the mechanisms of human neuropathology and for guiding the development of targeted therapeutic approaches.

Chapter 4. Modelling Alzheimer's Disease in iPSC-Derived Astrocytes: Effects of APOE Genotype and β -Amyloid Stimulation

4. Introduction

AD is characterised by progressive cognitive decline, synaptic failure, neuroinflammation, and the accumulation of A β peptides and tau pathology. Although neuronal dysfunction is central to the clinical phenotype, astrocytes are increasingly recognised as active contributors to AD pathogenesis. As key regulators of CNS homeostasis, astrocytes maintain neurotransmitter balance, provide metabolic support, modulate synaptic transmission, and coordinate immune responses (Sofroniew and Vinters, 2010). In AD, these homeostatic functions become disrupted, and astrocytes undergo profound morphological and molecular changes collectively referred to as “reactivity”. Reactive astrocytes typically upregulate intermediate filaments such as GFAP and vimentin, alter cytokine and chemokine release, remodel their processes, and shift their metabolic state (Escartin *et al.*, 2021). Initially protective, prolonged or dysregulated astrocyte reactivity can exacerbate inflammation, oxidative stress, and neuronal vulnerability (Liddelow and Barres, 2017).

Among genetic risk factors for late-onset AD, Apolipoprotein E (APOE) stands out as the strongest and most reproducible predictor of disease risk and age of onset. The three common APOE isoforms, APOE2, APOE3 and APOE4, differ by single amino acid substitutions that alter their lipid-binding properties, receptor interactions and immunomodulatory functions (Mahley and Huang, 2012). APOE4 increases risk and lowers age of onset in a dose-dependent manner, whereas APOE2 is protective (Holtzman, Herz and Bu, 2012; Liu *et al.*, 2013). Although APOE is expressed in multiple cell types, astrocytes are its predominant source in the healthy brain (Yamazaki *et al.*, 2019). This positions astrocytes at the intersection of lipid metabolism, innate immunity and neurodegeneration, the three biological pathways in which APOE plays a pivotal role.

Growing evidence suggests that APOE genotype modulates astrocyte biology in ways that may predispose the brain to AD pathology. APOE4 astrocytes exhibit deficits in cholesterol efflux and membrane repair, reduced glutamate uptake, heightened oxidative stress, and altered inflammatory responses (Zhao *et al.*, 2017;

Lin *et al.*, 2018). They release more pro-inflammatory cytokines, show impaired phagocytosis, and demonstrate transcriptional signatures indicative of metabolic and immune dysregulation (Jones *et al.*, 2017; Wang *et al.*, 2021; de Leeuw *et al.*, 2022). By contrast, APOE2 astrocytes often retain more homeostatic and anti-inflammatory profiles, consistent with their protective association in AD (de Leeuw *et al.*, 2022). These findings underscore the importance of investigating APOE genotype effects within human astrocytes, where APOE biology is known to differ from rodent systems.

Human iPSC-derived astrocytes provide a powerful platform for dissecting APOE-dependent mechanisms in a human-specific context. The use of isogenic APOE2/2, APOE3/3 and APOE4/4 iPSC lines removes donor variability and enables precise comparisons between genotypes. iPSC-derived astrocytes recapitulate many key features of primary human astrocytes, including cytokine responsiveness, glutamate uptake, calcium signalling, and metabolic properties (Serio *et al.*, 2013; Barbar *et al.*, 2020). Importantly, they capture human-specific APOE biology that cannot be fully modelled in non-human systems, making them an ideal tool for mechanistic studies of AD-associated genetic risk.

A β accumulation is a defining pathological feature of AD, yet the way astrocytes respond to A β is complex. Soluble A β peptides, particularly A β ₁₋₄₀ and A β ₁₋₄₂, can modulate astrocyte inflammatory activity, impair glutamate transport, and induce oxidative stress (García-Matas *et al.*, 2010). However, several studies indicate that A β alone may be insufficient to fully activate astrocytes, instead requiring cooperation with inflammatory cytokines or microglia-derived signals to induce robust reactivity (Perez-Nievas and Serrano-Pozo, 2018). This is consistent with the broader view that AD pathogenesis reflects interactions between protein aggregation, innate immunity, and glial dysfunction (Ferrari-Souza *et al.*, 2025). Understanding how APOE genotype shapes astrocyte responses to A β is therefore critical for clarifying the gene–environment interactions that drive early AD pathology.

Reactive astrocytes also undergo metabolic remodelling, shifting towards glycolysis to support inflammatory signalling and adapt to cellular stress (Allaman,

Bélanger and Magistretti, 2011). APOE genotype and A β exposure may both influence these metabolic states. APOE4 astrocytes have been reported to show impaired mitochondrial respiration, altered lipid handling and reduced redox capacity, which may limit their ability to maintain neuronal support during stress (Lin *et al.*, 2018). Measuring oxidative phosphorylation and glycolysis using Seahorse extracellular flux analysis thus provides valuable insight into how A β shapes astrocyte metabolic competence.

Taken together, APOE genotype and A β pathology represent two converging factors that shape astrocyte function and dysfunction in AD. By combining isogenic APOE iPSC-derived astrocytes with A β stimulation, this chapter aims to clarify how genetic risk modifies astrocyte reactivity, inflammatory signalling and metabolic adaptation. Understanding these interactions is essential for identifying mechanisms of vulnerability in AD and for informing therapeutic strategies that target astrocyte-driven pathology.

4.1. Aims and Hypothesis

Hypothesis: APOE genotype influences the magnitude and nature of astrocyte reactivity, with APOE4 astrocytes exhibiting a dysregulated inflammatory and metabolic profile in response to A β and cytokine stimulation.

Aims and Objectives:

1. Generate and characterise cortical astrocytes from isogenic APOE2/2, APOE3/3, and APOE4/4 iPSC lines.
2. Assess how APOE genotype modulates cytokine release, morphology, and gene expression following TIC stimulation.
3. Evaluate the effects of A β ₁₋₄₀ and A β ₁₋₄₂, alone or in combination with IL-1 α , on astrocyte reactivity and metabolism.
4. Examine glycolytic activity and metabolic shifts associated with A β -induced reactivity.

4.2. Methods

4.2.1. Cell Lines Utilised

The following cell lines were used in the study described in this chapter:

- BIONi037-A – APOE3/3 iPSC parent line along with its isogenic offspring APOE2/2 and APOE4/4 iPSC lines to generate CTX NPC and Astros
- NAS2 iPSC-derived CTX-Astros were used to investigate the potential effects of A β -stimulation on astrocyte reactivity

4.2.2. Quantification of the Proportion of Positive and Negative Staining

To quantify the proportion of cells staining positive or negative for a neuronal marker TUJ1, the cortical marker TBR1, or the astrocyte marker GFAP, the raw data obtained in CellProfiler for the nuclear integrated intensity were used. A 'cut off' point for each individual set of cells was determined by plotting the cells on a scatter plot, with the y-axis stretched out so that a clear indentation or break was seen, as seen in Fig. 4.2.1 below. For example, a cut-off point of 40 A.U. was used in all APOE NPCs to determine TUJ1-positive cells (nuclear intensity >40 A.U.) and TUJ1-negative cells (nuclear intensity <40 A.U.), while 15 A.U. was the cut-off point used for TBR1-positive (nuclear intensity >15 A.U.) and TBR1-negative (nuclear intensity <15 A.U.) cells. The number of positive and negative cells for each stain was then converted to a percentage.

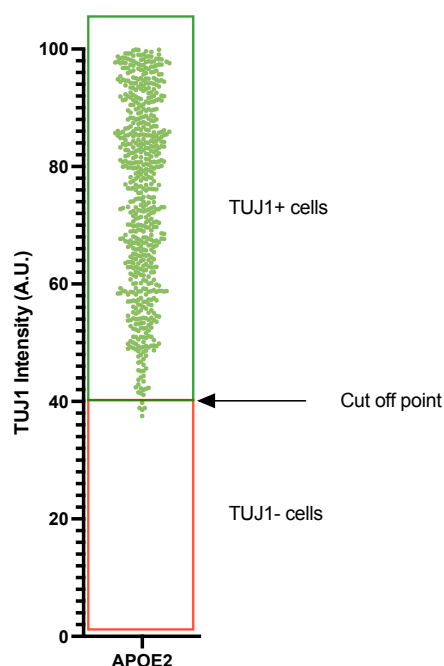


Figure 4.2.1 Scatter plot to determine the ‘cut-off’ point for TUJ1 intensity.

Individual data points represent nuclei integrated intensity (A.U.). A clear break was observed at 40 A.U. and was determined to be the divide between TUJ1-positive (TUJ1+; green box) cells with the nuclear integrated intensity above 40 A.U. and TUJ1-negative (TUJ1-; red box) cells with the nuclear integrated intensity below 40 A.U. Scatter plots were generated for the total number of cells for each n ($N=3$). The number of positive and negative cells was then transformed into a percentage for a clearer observation of the level of purity for each batch of cells.

4.2.3. Manufacture of A β ₁₋₄₀ and A β ₁₋₄₂ Oligomers

Human Amyloid β 1-40 (A β ₁₋₄₀) and 1-42 (A β ₁₋₄₂) peptides (AnaSpec, USA) were reconstituted and frozen at -70°C for long-term storage as monomers according to the manufacturer’s instructions. To make oligomers, the day before stimulation, A β peptide aliquots were thawed on ice and diluted down to 100 μ M in ice-cold PBS, as per the Stine *et al.* (2011) protocol. These aliquots were then stored at 4°C for 24 h to allow for A β oligomerisation before being used in experiments.

4.2.4. Stimulation with A β ₁₋₄₀ and A β ₁₋₄₂ Oligomers

Mature CTX Astros were subjected to stimulation with 1 μ M and 2 μ M concentrations of A β ₁₋₄₀ and A β ₁₋₄₂ oligomers, with and without the addition of 0.3 ng/mL IL-1 α , one of the microglial pro-inflammatory factors (Liddelow *et al.*,

2017), to induce a reactive profile in astrocytes and assess their response to A β stimulation. A β concentrations and the 24 h incubation period were selected based on previous studies demonstrating that low micromolar concentrations of soluble A β oligomers reliably induce inflammatory and metabolic responses in human iPSC-derived astrocytes without excessive cytotoxicity (Elsworthy *et al.*, 2025), together with preliminary optimisation experiments performed in-house. A β oligomers and IL-1 α were diluted in AMM media to the required concentrations. Prepared dilutions were then gently mixed by inversion to prevent further aggregation of soluble A β oligomers to insoluble fibrils. Media was aspirated from wells, and the cells were gently washed with PBS to remove any remaining cell debris. Experimental wells were administered the stimulation cocktails containing 1 μ M A β_{1-40} , 2 μ M A β_{1-40} , 1 μ M A β_{1-40} +IL-1 α , 2 μ M A β_{1-40} +IL-1 α , 1 μ M A β_{1-42} , 2 μ M A β_{1-42} , 1 μ M A β_{1-42} +IL-1 α , 2 μ M A β_{1-42} +IL-1 α and IL-1 α alone, while the control wells were administered AMM media alone. The cells were then incubated for 24 h (37°C; 5% CO₂) before supernatant collection and cell lysis.

4.2.5. Immunocytochemistry

ICC and subsequent analysis were performed on CTX- and VM- NPC, APC and Astros as described in Chapter 2, Section 2.9. The primary and secondary antibodies used are listed in Table 3.2.1. and Table 3.2.2. below.

Table 4.2.1 Primary Antibodies Used for Immunocytochemistry.

Primary Antibody	Manufacturer	Dilution
Rabbit anti- α Connexin-43	Sigma-Aldrich; C6219	1:1000
Rabbit anti-EAAT1	Bio-Techne; NBPI-2	1:1000
Rabbit anti-FOXA2	ThermoFisher Scientific; 9H5L7	1:1000
Chicken anti-GFAP	Merck; AB5541	1:1000
Hoechst 33342	Cell Signalling Technology; 4082	1:500
Rabbit anti-LMX1A	Merck; AB10533	1:1000
Rabbit anti-TBR1	Abcam; AB31940	1:300
Mouse anti-TUJ1	BioLegend; BL-801202	1:1000
Mouse anti-S100 β	Sigma-Aldrich; S2532	1:1000

Table 4.2.2 Secondary Antibodies Used for Immunocytochemistry.

Secondary Antibody	Manufacturer	Dilution

Goat Anti-chicken 488	Abcam; ab150169	1:500
Donkey Anti-mouse 488	ThermoFisher Scientific	1:500
Donkey anti-mouse 594	ThermoFisher Scientific	1:500
Donkey Anti-mouse 647	ThermoFisher Scientific	1:500
Donkey Anti-rabbit 594	ThermoFisher Scientific	1:500
Donkey Anti-rabbit 647	ThermoFisher Scientific	1:500

4.2.6. qPCR

qPCR analysis was performed on the control and stimulated CTX- and VM-Astros as described in detail in Chapter 2, Section 2.6. The relative quantification (RQ) of each target gene for CTX- and VM-Astros was calculated relative to iPSC to avoid bias toward either region. All primers were manufactured by ThermoFisher Scientific, USA. VIC fluorescent reporter dye was used for GAPDH and FAM fluorescent reporter dye was used for all target primers. The endogenous and target primers used in this study are listed in Table 3.2.3. below.

Table 4.2.3 qPCR Primers Used in the Study.

Primers	Gene Name	Assay ID
C3	complement component 3	Hs00163811_m1
IL6	interleukin 6	Hs00174131_m1
CXCL10	C-X-C motif chemokine ligand 10	Hs00171042_m1
CCL5	C-C motif chemokine ligand 5	Hs00982282_m1
CSF2	colony stimulating factor 2	Hs00929873_m1
ITGAM	integrin subunit alpha M	Hs00167304_m1
SLC1A2	solute carrier family 1 member 2	Hs01102423_m1
SLC1A3	solute carrier family 1 member 3	Hs00904823_g1
GLUL	glutamate-ammonia ligase	Hs00365928_g1
LDHA	lactate dehydrogenase A	Hs01378790_g1
HK2	hexokinase 2	Hs00606086_m1
PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	Hs00998698_m1
SLC2A1	solute carrier family 2 member 1	Hs00892681_m1
SLC6A3	solute carrier family 6 member 3	Hs00997374_m1

4.3. Results

To investigate the effects of APOE genotype on immune responses in Alzheimer's disease, the APOE3/3 iPSC parent line and its isogenic offspring APOE2/2 and APOE4/4 iPSC lines were utilised. These lines were differentiated into cortical neural progenitors, followed by cortical astrocyte progenitors, and finally, fully mature functional astrocytes using the in-lab protocol adapted from several established protocols (Chambers *et al.*, 2009, 2012; Shi, Kirwan and Livesey, 2012; Serio *et al.*, 2013; Nistor *et al.*, 2015; Nolbrant *et al.*, 2017), as outlined in Sections 2.4-5. Identity and purity were verified at each stage via ICC. Mature CTX-Astros were subsequently exposed to pro-inflammatory cytokines TNF α , IL-1 α and C1q (TIC) as per the Liddelow *et al.* (2017) protocol. We then examined APOE genotype-dependent differences in astrocyte marker expression, morphology, cytokine and chemokine release, as well as the expression of inflammatory, reactive and functional genes. The results are presented below in the order of experimental aims.

4.3.1. Differentiation of CTX NPC from iPSC

As described above, isogenic iPSC lines carrying the APOE2/2, APOE3/3 and APOE4/4 genotypes were differentiated into cortical neural progenitors (CTX-NPC) as the first step toward assessing the effect of APOE genotype on astrocyte immune responses. Neural induction was achieved using a Dual SMAD inhibition protocol adapted from previously published methods (see Section 2.4 in Ch. 2), which has been successfully implemented in this and other studies, e.g. McComish *et al.* (2024). Representative images of the iPSC colonies used for differentiation are shown in Figure 4.3.1 below.

Post-neural induction, cells were characterised by ICC for the expression of the neuronal marker TUJ1 (β -Tubulin III) and the cortical marker TBR1 (Fig. 4.3.2). Morphologically, all APOE lines exhibited the expected neuronal progenitor phenotype, with small, dense cell bodies and extensive, interconnected processes.

The intensity of staining and the proportion of marker-positive cells were then quantified for each of the neural progenitor cultures.

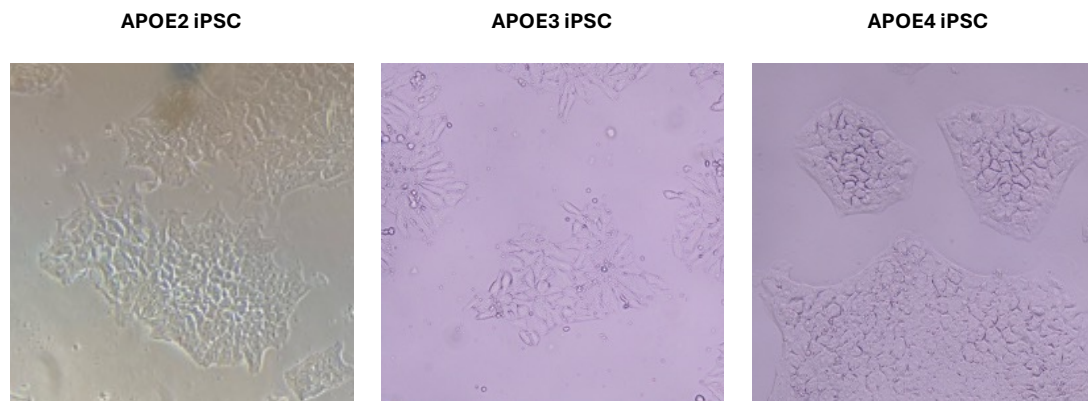


Figure 4.3.1 Representative images of iPSC colonies with APOE2, APOE3 or APOE4 phenotype.

BIONi037 APOE2/2 (left), APOE3/3 (middle), and APOE4/4 (right) cell lines form the characteristic iPSC colonies that are rounded and slightly raised before they are ready for a passage or neural differentiation. Magnification: x20.

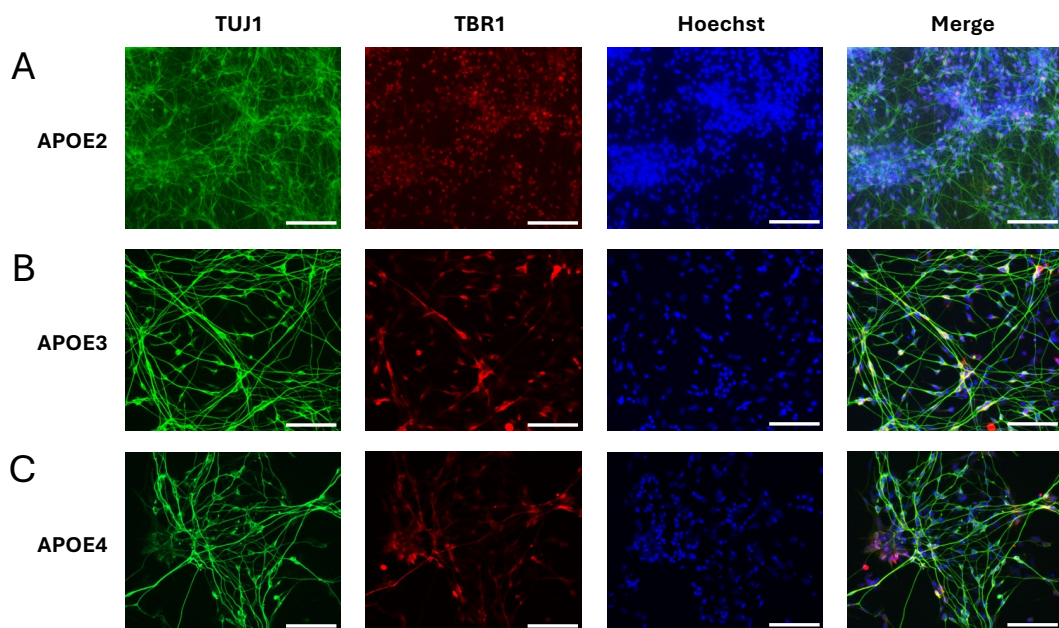


Figure 4.3.2 Cortical NPC differentiated from APOE iPSC lines stain positive for regional and neuronal markers.

Representative images of APOE2 (A), APOE3 (B) and APOE4 (C) CTX NPC stained positive for TUJ1 (green), a classical neuronal marker, and TBR1 (red), a cortical marker, as well as the nuclear stain Hoechst (blue). Scale bars: 100 μ M. Magnification: x20.

TUJ1 nuclear intensity of APOE2 cells was quantified as a mean integrated intensity of 140.84 ± 1.597 A.U., with 99.65% of APOE2 cells staining positive for TUJ1 and only 0.35% staining negative, with the percentage of TUJ1-positive cells being significantly higher compared to the TUJ1-negative APOE cells (**** $p < 0.0001$), as seen in Fig. 4.3.A and B below. TUJ1 nuclear intensity of APOE3 cells was quantified as a mean integrated intensity of 421.66 ± 10.34 A.U., with 94.65% and 5.35% of APOE3 cells staining positive and negative for TUJ1, respectively (see Fig. 4.3.A and B below). The percentage of TUJ1-positive APOE3 cells was significantly higher compared to TUJ1-negative cells (**** $p < 0.0001$). In the case of APOE4 cells, TUJ1 nuclear intensity had a mean integrated intensity value of 193.72 ± 2.73 A.U. 99.65% of APOE4 cells stained positive for TUJ1, while only 0.35% stained negative (Fig. 4.3.A and B), with the percentage of TUJ1-positive APOE4 cells being significantly higher compared to TUJ1-negative cells (**** $p < 0.0001$). Among the three lines, APOE3 NPC expressed the highest levels of TUJ1 in terms of the mean intensity, compared to APOE2 and APOE4 cells (#### $p < 0.0001$ for both). TUJ1 intensity of the APOE4 CTX NPC is also significantly higher compared to the APOE2 ones (++++ $p < 0.0001$). The percentage of TUJ1-positive cells was also significantly higher in the APOE2 (# $p = 0.0455$) and APOE4 (# $p = 0.0453$) cells compared to the APOE3 cells. Similarly, the percentage of the TUJ1-negative cells was significantly lower in the APOE2 (# $p = 0.0455$) and APOE4 (# $p = 0.0453$) cells, compared to the APOE3s.

The mean TBR1 nuclear intensity of the APOE2 cells was quantified as 43.88 ± 0.48 A.U. The percentage of TBR1-positive APOE2 cells was significantly higher compared to TBR1-negative cells (**** $p < 0.0001$), with 98.58% of cells staining positive and 1.42% staining negative for TBR1, as seen in Figure 4.3.C and D. In APOE3 cells, the mean TBR1 nuclear intensity was 41.97 ± 0.67 A.U. 98.83% of APOE3 cells stained positive for TBR1, while only 1.17% were TBR1-negative, with the percentage of TBR1-positive cells being significantly higher compared to those that stained negative (**** $p < 0.0001$; Fig. 4.3.C and D below). APOE4 cells had the highest mean TBR1 nuclear intensity at 51.12 ± 0.7 A.U. The percentage of TBR1-positive APOE4 cells was significantly higher compared to TBR1-negative

cells (**** $p < 0.0001$), with 99.3% of APOE4 staining positive for TBR1, and only 0.7% being TBR1-negative. The TBR1 intensity was significantly higher in the APOE4 cells compared to the APOE2 (++++ $p < 0.0001$) and APOE3 (#### $p < 0.0001$). No significant differences between the three lines were found in the percentage of cells staining positive or negative for TBR1.

Collectively, this data confirms successful neural induction and cortical identity of all three APOE lines, validating the use of this protocol for generating high-purity cortical neural progenitors suitable for further differentiation into astrocyte progenitors.

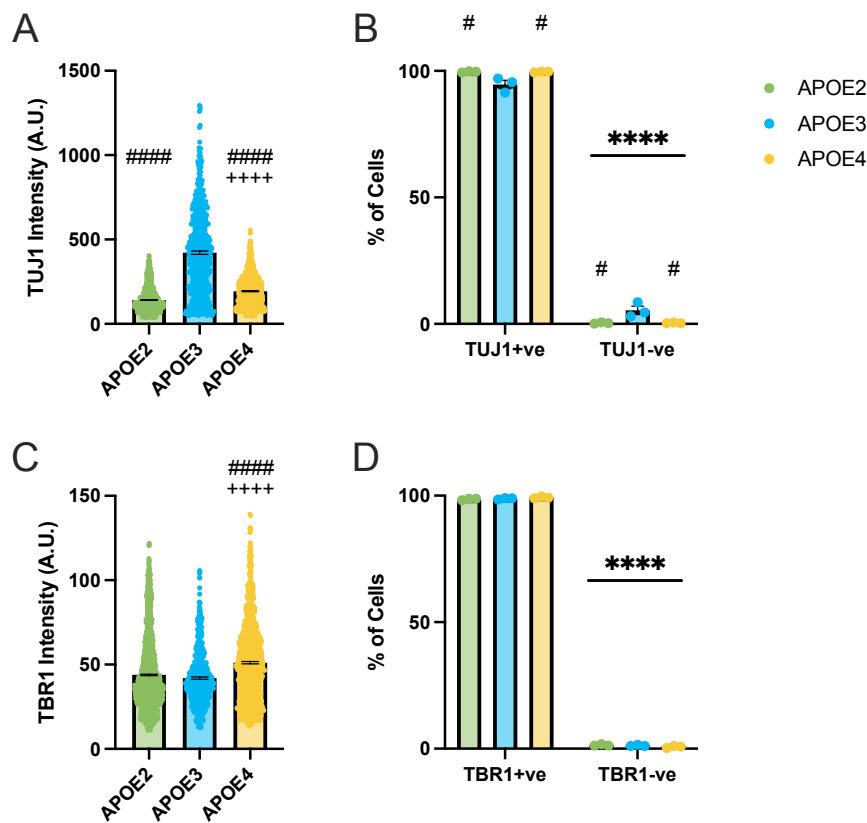


Figure 4.3.3 iPSC-derived APOE CTX-NPC express regional and neuronal markers.

(A) APOE3 cells express the highest intensity of TUJ1 staining, compared to APOE2 and APOE4s. (B) Nearly 99% of APOE2 and APOE4 and approximately 95% of APOE3 NPC stain positive for TUJ1. (C) These same NPC also express TBR1, a cortical marker, with the APOE4 cells expressing highest intensity of TBR1. (D) Over 98% of APOE2, APOE3 and APOE4 cells stain positive for TBR1. Data expressed as mean $\pm$ SEM (N=3; 100 cells per N for ICC). **** $p < 0.0001$ vs positive stain; ## $p < 0.01$, #### $p < 0.0001$ vs APOE3. ++++ $p < 0.0001$ vs APOE2. One-way (A, C) and two-way (B, D) ANOVA with Tukey's post hoc.

4.3.2. Glial switch protocol yields cortical astrocyte progenitors

Astrocytes play key roles in maintaining brain homeostasis and regulating neuroinflammation, processes that are central to neuronal survival and disease progression. To study APOE genotype-specific effects on neuroinflammation in AD, cortical astrocytes were generated, as they are the principal cell type expressing *APOE* in the brain. To confirm astrocytic identity, APOE2, APOE3 and APOE4 CTX APC were immunostained for GFAP and counterstained for TUJ1.

GFAP, one of the most commonly used astrocyte markers, was used in this study to confirm the astrocyte identity of the cells carrying different APOE genotypes post-differentiation from cortical neural progenitors. GFAP was selected at this stage in combination with TUJ1 to confirm astrocytic lineage commitment while simultaneously excluding neuronal contamination within the cultures. S100 β staining was used at later stages of differentiation to assess mature astrocyte identity, while concurrent TUJ1/S100 β co-labelling was limited by antibody host-species compatibility. As shown in **Figure 4.3.4.A-B** below, the three sets of APOE cells expressed very high GFAP intensity and very low TUJ1 intensity. Morphologically, the cell bodies and nuclei of these cells were larger, and the cells adopted a more stellate shape than the cortical NPC displayed in **Figure 4.3.2.A-B**. Mean GFAP nuclear intensity in APOE2 cells was quantified as 499.69 ± 9.19 A.U. The percentage of GFAP-positive APOE2 cells was significantly higher compared to the GFAP-negative APOE2 cells (**** $p < 0.0001$), with the values of 96.37% and 3.63%, respectively (see **Fig. 4.3.5.A-B**). In APOE3 cells, the mean GFAP nuclear intensity was at 315.12 ± 5.60 A.U. The percentage of GFAP-positive APOE3 cells was calculated as 99.81%, which was significantly higher than the GFAP-negative APOE3 cells at 0.19% (**** $p < 0.0001$; **Fig. 4.3.5.A-B**). The mean GFAP nuclear intensity of APOE4 cells was quantified as 518.99 ± 3.99 A.U., with 95.76% of GFAP-positive cells and 4.24% of GFAP-negative cells, as seen in **Fig. 4.3.5.A-B** below. The percentage of GFAP-positive APOE4 cells was significantly higher compared to GFAP-negative cells (**** $p < 0.0001$). Among the three lines, the APOE2 cells expressed the highest levels of GFAP intensity, compared to the APOE3 and APOE4 cells (++++ $p < 0.0001$ for both). The APOE4 cells had a significantly lower percentage

of GFAP-positive (#p=0.0451) and higher percentage of GFAP-negative (#p=0.0451) cells compared to APOE3s.

The same cells were counter-stained for TUJ1 to ensure that the protocol yielded a culture that is highly astrocytic, with little to no neuronal contamination. Indeed, all three cell lines show low levels of TUJ1 expression, with most cells not exceeding the nuclear intensity of 100 A.U., as seen in **Figure 4.3.5.C**. The mean TUJ1 nuclear expression in APOE2 cells was quantified as 43.88 ± 0.62 A.U., with 96.38% of APOE2 cells staining negative for TUJ1 and only 3.62% of cells staining positive. The percentage of TUJ1-negative APOE2 cells was significantly higher compared to TUJ1-negative cells (****p<0.0001; **Fig. 4.3.5.D**). The mean TUJ1 nuclear intensity of APOE3 cells was quantified as 13.69 ± 0.24 A.U., with 99.49% of APOE3 staining negative for TUJ1, and only 0.51% staining positive, as seen in **Fig. 4.3.5.C-D**). The percentage of TUJ1-negative APOE3 cells was significantly higher compared to the percentage of TUJ1-positive cells (****p<0.0001). The mean TUJ1 nuclear intensity of APOE4 cells was quantified as 20.59 ± 0.18 A.U., as seen in **Fig. 4.3.5.C** below. The percentage of TUJ1-negative APOE3 cells (97.9%) was significantly higher than the percentage of TUJ1-negative cells at 2.1% (****p<0.0001; see **Fig. 4.3.5.D**). Although the APOE2 cells expressed the highest intensity of TUJ1 compared to the APOE3 and APOE4 cells (++++p<0.0001), these levels were still mostly below 100 A.U. The APOE3 APC expressed the lowest TUJ1 intensity out of the three lines, with the APOE4 APCs expressing significantly more TUJ1 (####p<0.0001) compared to the APOE3s. There were no significant differences observed in the percentage of cells staining positive or negative for TUJ1 between the three cell lines.

Collectively, these results confirm the successful generation of high-purity cortical astrocyte progenitors from all three APOE isogenic iPSC lines. The strong GFAP and low TUJ1 expression patterns demonstrate that the glial switch protocol robustly yields astrocyte lineage-committed cells suitable for further maturation and functional analysis.

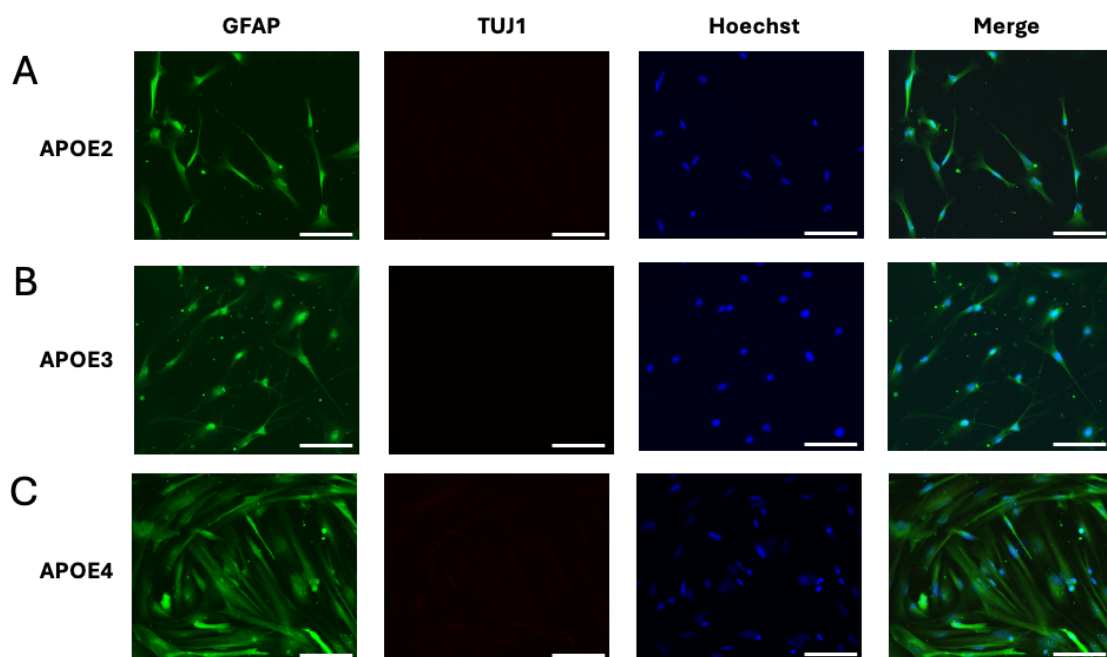


Figure 4.3.4 APC differentiated from CTX NPC stain positive for GFAP, but not TUJ1.

Representative images of APOE2 (**A**), APOE3 (**B**) and APOE4 (**C**) CTX APC stained positive for an astrocyte marker GFAP (green) and nuclear stain Hoechst (blue), but not the neuronal marker TUJ1 (red). Scale bars: 100 μ m. Magnification: x20.

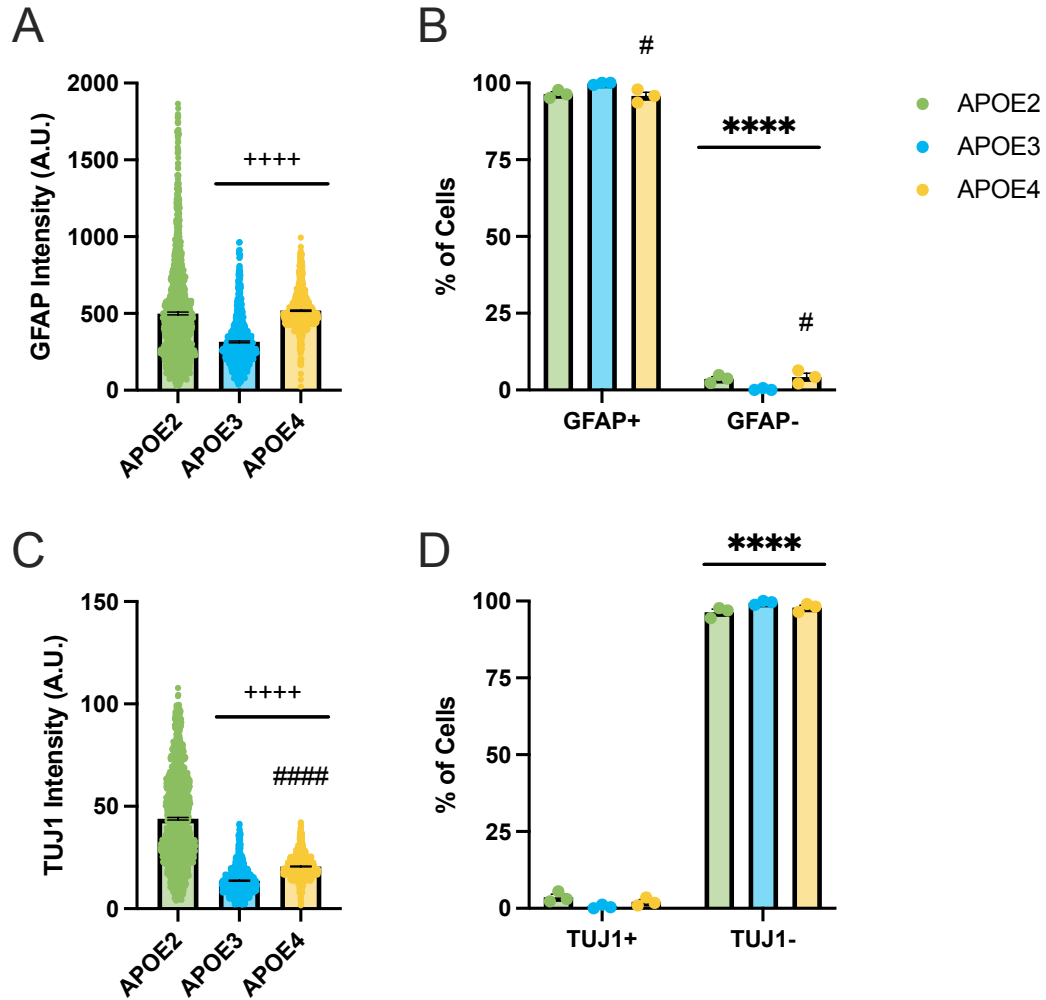


Figure 4.3.5 APC differentiated from CTX NPC express a classical astrocyte marker.

The expression of GFAP, an astrocyte marker, and TUJ1, a neuronal marker, was quantified in the APOE2, APOE3 and APOE4 astrocyte progenitors to confirm their identity. (A) APOE2 CTX-APC express highest levels of GFAP among the three lines. (B) Although all three lines are 95%+ positive for GFAP, the highest positivity is expressed by the APOE3 cells, with 99% of cells staining positive. (C) CTX APC were also counter-stained for TUJ1 to ensure high purity of the astrocyte progenitor population, with very low TUJ1 intensity. (D) CTX APC carrying the APOE2, APOE3 and APOE4 genotype are 95%, 99% and 96% negative for TUJ1, respectively. Data expressed as mean $\pm$ SEM (N=3; 100 cells per N). **** p <0.0001 vs positive stain. One-way (A, C) and two-way (B, D) ANOVA with Tukey's post hoc.

4.3.3. Mature Cortical Astrocytes Express Astrocyte Markers and Astrocyte-Associated Proteins

The glial switch protocol aims to differentiate neural progenitors into astrocyte progenitors. However, astrocyte progenitors are immature and therefore not fully functional. To fully characterise cells as astrocytes, they have to successfully undergo a maturation process and demonstrate the ability to enter reactive states, releasing pro-inflammatory cytokines/chemokines and expressing inflammatory genes. In this study, mature APOE CTX-Astros were stained for GFAP, S100 β , EAAT1, Cx43 and CD49f (**Fig. 4.3.6**), and staining intensity was quantified across all three APOE lines (**Fig. 4.3.7** below).

In the APOE2 CTX-Astros, stimulation with TIC resulted in a significant decrease in GFAP (* $p=0.0209$), S100 β (*** $p=0.0008$), EAAT1 (*** $p=0.0001$) and Cx43 (* $p=0.0133$) intensity compared to the control, as seen in **Fig. 4.3.7.A-D**. Similarly, the stimulation of APOE3 CTX-Astros with TIC resulted in a significant decrease of S100 β (** $p=0.0094$) and EAAT1 (*** $p=0.0002$) intensity compared to the control, as seen in **Fig. 4.3.7.B-C**. Although some decrease in GFAP intensity was also observed post-TIC stimulation, this result was non-significant (see **Fig. 4.3.7.A** below). However, when APOE3 CTX-Astros were stimulated with TIC, the intensity of Cx43 significantly increased (**** $p<0.0001$), as seen in **Fig. 4.3.7.D**.

In contrast, APOE4 CTX-Astros displayed a distinct expression pattern. While GFAP intensity decreased post-TIC stimulation compared to the control (**** $p<0.0001$) as seen in **Fig. 4.3.7.A**, the intensity of other proteins increased. A significant increase in EAAT1 and Cx43 (**** $p<0.0001$ for both) was observed in APOE4 CTX-Astros stimulated with TIC, compared to the control, as seen in **Fig. 4.3.7.C-D**. Although a trend towards an increase is also observed in the case of S100 β intensity (see **Fig. 4.3.7.B**), this was non-significant.

Direct comparison between genotypes revealed several baseline and stimulation-dependent differences. GFAP intensity (**Fig. 4.3.7.A**) was significantly lower in the APOE4 controls compared to the APOE2 control (+++ $p=0.0001$), as well as in the TIC-stimulated APOE4 CTX-Astros compared to the stimulated

APOE2 (++++p<0.0001) and APOE3 (####p<0.0001) cells. Although the S100 β intensity (**Fig. 4.3.7.B**) was significantly lower in the APOE4 controls compared to the APOE3 control (#p=0.0232), the APOE4 cells stimulated with TIC had a significantly higher S100 β intensity compared to the TIC-stimulated APOE2 cells (+p=0.0118). EAAT1 intensity (**Fig. 4.3.7.C**) is significantly higher in both APOE3 and APOE4 cells compared to APOE2, regardless of stimulation (++++p<0.0001 for all groups). However, the APOE4 control cells have a significantly lower EAAT1 intensity compared to the APOE3 control (####p<0.0001). In contrast, the EAAT1 intensity of TIC-stimulated APOE4 cells is significantly higher compared to the TIC-stimulated APOE3 CTX-Astros (####p<0.0001). APOE2 CTX-Astros have a significantly higher Cx43 intensity (**Fig. 4.3.7.D**) compared to the APOE3 and APOE4 cells in both control and TIC-stimulated groups (++++p<0.0001 for all groups). Cx43 intensity of TIC-stimulated APOE4 cells is also significantly higher compared to the TIC-stimulated APOE3 cells (##p=0.0036).

Overall, these findings demonstrate that while all APOE genotypes yield mature astrocytes expressing canonical astrocyte markers, their responses to inflammatory stimulation differ markedly. APOE2 and APOE3 astrocytes show a reduction in marker expression upon stimulation, indicative of cytoskeletal or metabolic downregulation, whereas APOE4 astrocytes exhibit increased expression of several astrocyte-associated proteins, particularly EAAT1 and Cx43. This suggests that APOE astrocytes undergo distinct reactive remodelling, potentially reflecting an altered or heightened response to inflammatory cues. Together, these results confirm that the maturation and reactive profiles of APOE astrocytes are genotype-dependent, setting the stage for subsequent analyses of cytokine release and inflammatory gene expression.

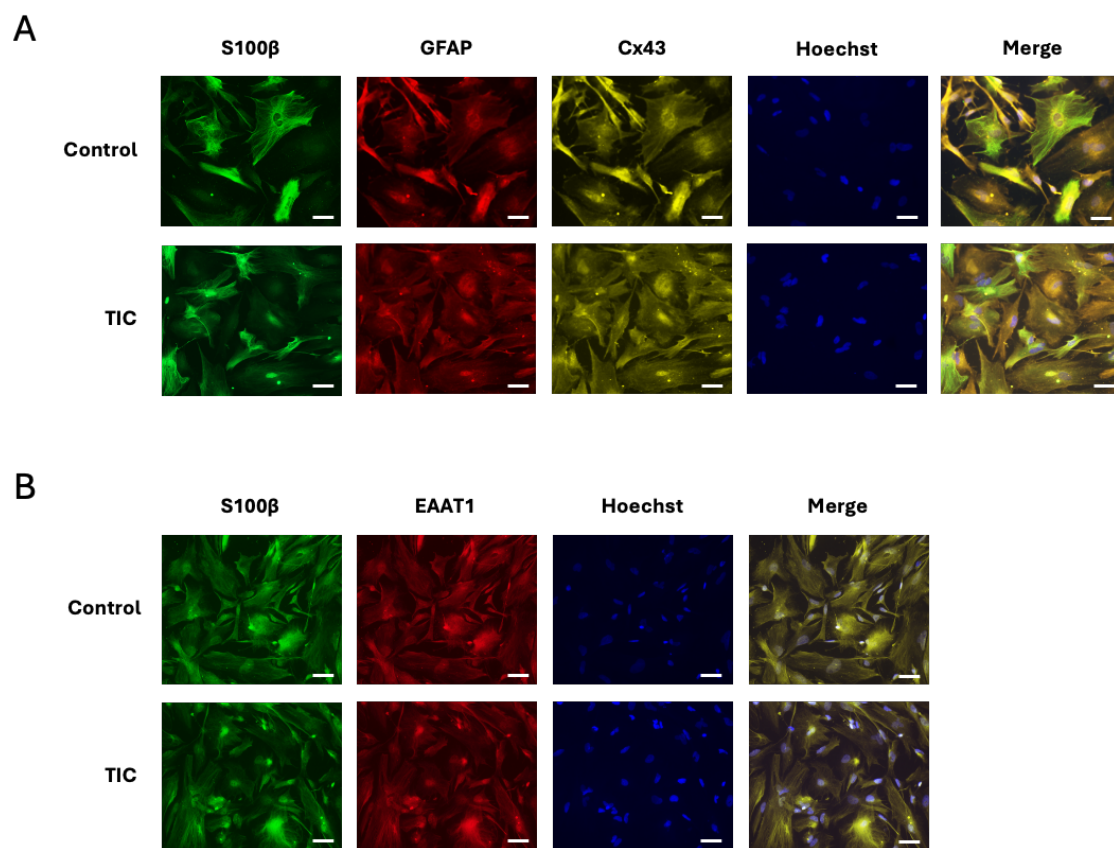


Figure 4.3.6 Mature cortical astrocytes stain positive for astrocyte markers and astrocyte-associated proteins.

Representative images of APOE2 APC with and without TIC stimulation staining positive for **(A)** S100 β (green), GFAP (red) and Cx43 (yellow) and **(B)** S100 β (green), EAAT1 (red) and CD49f (yellow). All CTX-Astros were also stained for nuclear stain Hoechst (blue). Scale bars: 100 μ m. Magnification: x20.

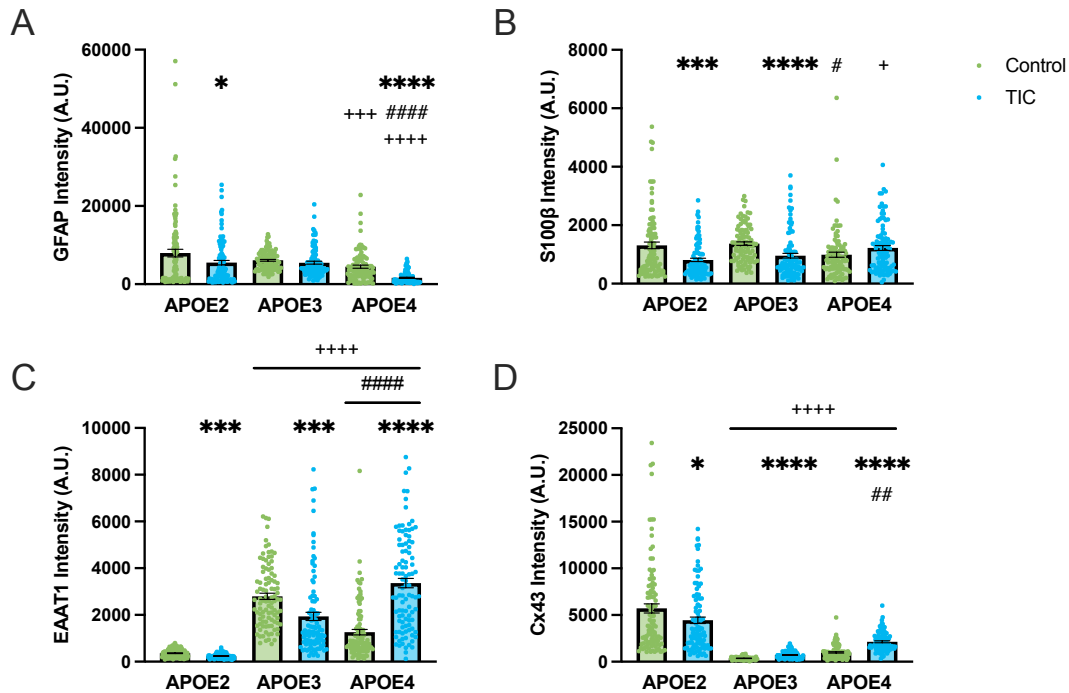


Figure 4.3.7 Staining intensity of astrocyte markers differs between APOE CTX-Astros.

The expression of (A) GFAP, (B) S100 β , (C) EAAT1, and (D) Cx43 was quantified in APOE CTX-Astros. (A) A decrease in GFAP was observed across the three lines post-TIC stimulation. (B) S100 β intensity was decreased in TIC-stimulated APOE2 and APOE3 CTX-Astros only. (C) EAAT1 intensity decreased in stimulated APOE2 and APOE3 CTX-Astros, while an increase was observed in TIC-stimulated APOE4 CTX-Astros compared to the control and the other two lines. (D) A decrease in Cx43 intensity was observed in stimulated APOE2 CTX-Astros, while it increased in stimulated APOE3 and APOE4 CTX-Astros, with the intensity being highest overall in APOE2 cells. Data expressed as mean $\pm$ SEM (N=3; 100 cells per N). * p <0.05, *** p <0.001, **** p <0.0001 vs control. # p <0.05, ## p <0.01, #### p <0.0001 vs APOE3 line. + p <0.05, +++ p <0.001, ++++ p <0.0001 vs APOE2 line. Unpaired two-tailed Student's t -Test and two-way ANOVA with Tukey's post hoc test.

4.3.4. Morphology Changes in Reactive Astrocytes Vary with the APOE Genotype

One of the hallmarks of astrocyte reactivity is a change in cell morphology. To assess this, morphological parameters were quantified using Hoechst (nuclear) and S100 β (cytoplasmic) staining in control and TIC-stimulated astrocytes. Measurements included cell area, perimeter, circularity, eccentricity, as well as

max and min Feret diameter, providing a comprehensive overview of size and shape changes associated with reactivity across APOE2, APOE3 and APOE4 CTX-Astros.

When APOE2 CTX-Astros were stimulated with TIC, a significant decrease in the cell area (**** $p < 0.0001$), perimeter (*** $p = 0.0001$), max Feret diameter (* $p = 0.0187$) and min Feret diameter (* $p = 0.0118$) were observed compared to the control (**Fig. 4.3.8.A, B, E, F**). In contrast, cell circularity increased when APOE2 CTX-Astros were stimulated with TIC (* $p = 0.0453$), compared to the control (see **Fig. 4.3.8.C**). No changes were observed in cell eccentricity (**Fig. 4.3.8.D**) between the two groups of APOE2 CTX-Astros.

In APOE3 CTX-Astros, TIC stimulation produced the opposite trend, with a decrease in cell circularity and an increase in cell eccentricity (**** $p < 0.0001$ for both), compared to the control, as seen in **Fig. 4.3.8.C and D**. Although a minimal decrease in cell area and an increase in max Feret diameter were also observed post-TIC stimulation (**Fig. 4.3.8.A and E**), these results were non-significant. No significant changes or trends were observed in the cell perimeter (**Fig. 4.3.8.B**) or min Feret diameter (**Fig. 4.3.8.F**) of the APOE3 CTX-Astros, regardless of stimulation.

The morphological changes in APOE4 CTX-Astros post-TIC stimulation also differed from those of the APOE2 or the APOE3 cells. A significant increase in the cell area (**** $P < 0.001$), perimeter (**** $P < 0.001$), max Feret diameter (**** $P < 0.001$) and min Feret diameter (**** $P < 0.001$) were observed in TIC-stimulated APOE4 CTX-Astros compared to the control (**Fig. 4.3.8.A, B, E and F** below). However, no changes were observed in cell circularity or eccentricity of the APOE4 CTX-Astros, regardless of stimulation, as seen in **Fig. 4.3.8.C and D**.

Overall, the APOE2 control CTX-Astros had the lowest cell area (++ $p = 0.0010$ vs APOE3; ++ $p = 0.0038$ vs APOE4), cell perimeter (+ $p = 0.0180$ vs APOE3), cell circularity (++++ $p < 0.0001$), max Feret (+ $p = 0.0106$) and min Feret (++ $p = 0.0016$) diameters compared to the APOE3 and APOE4 control CTX-Astros. Cell eccentricity, however, was significantly higher in the APOE2 CTX-Astro controls compared to the APOE3 (++++ $p < 0.0001$) and APOE4 (++ $p = 0.0091$) controls. Similar

was observed post-TIC stimulation where the cell area (+++p=0.0001 vs APOE3; ++++p<0.0001 vs APOE4), cell perimeter (++++p<0.0001 for both), circularity (++++p<0.0001 for APOE3; +++p=0.0004 for APOE4), max Feret (++++p<0.0001 for both) and min Feret (++++p<0.0001 for both) diameter were significantly smaller in APOE2 CTX-Astros compared to the stimulated APOE3 and APOE4 cells. Cell eccentricity was also significantly higher in the TIC-stimulated APOE2 CTX-Astros compared to the stimulated APOE3 (+p=0.0488) and APOE4 (+p=0.0320) cells. The control APOE4 CTX-Astros also exhibited a lower cell circularity (####p<0.0001) and higher eccentricity (##p=0.001) than the APOE3 control group. In the TIC-stimulated groups, APOE4 CTX-Astros were the largest of the three lines, with a significantly increased cell area (####p<0.0001, ++++p<0.0001), cell perimeter (####p<0.0001, ++++p<0.0001), max Feret (####p<0.0001, ++++p<0.0001) and min Feret ((####p<0.0001, ++++p<0.0001) diameters, compared to the APOE2 and APOE3 TIC-stimulated CTX-Astros.

In summary, APOE genotype strongly influences astrocyte morphology and its modulation under inflammatory stress. APOE2 astrocytes become smaller and more rounded following TIC stimulation, indicative of cytoskeletal retraction. APOE3 astrocytes, while maintaining intermediate size, become more elongated and polarised, reflecting potential structural remodelling. In contrast, APOE4 astrocytes exhibit a hypertrophic response, with a significant increase in cell size and Feret diameters, suggesting a distinct reactive phenotype associated with cellular enlargement rather than retraction. Together, these findings highlight genotype-specific morphological signatures of astrocyte reactivity, reinforcing the broader theme of APOE-dependent heterogeneity in astrocyte behaviour.

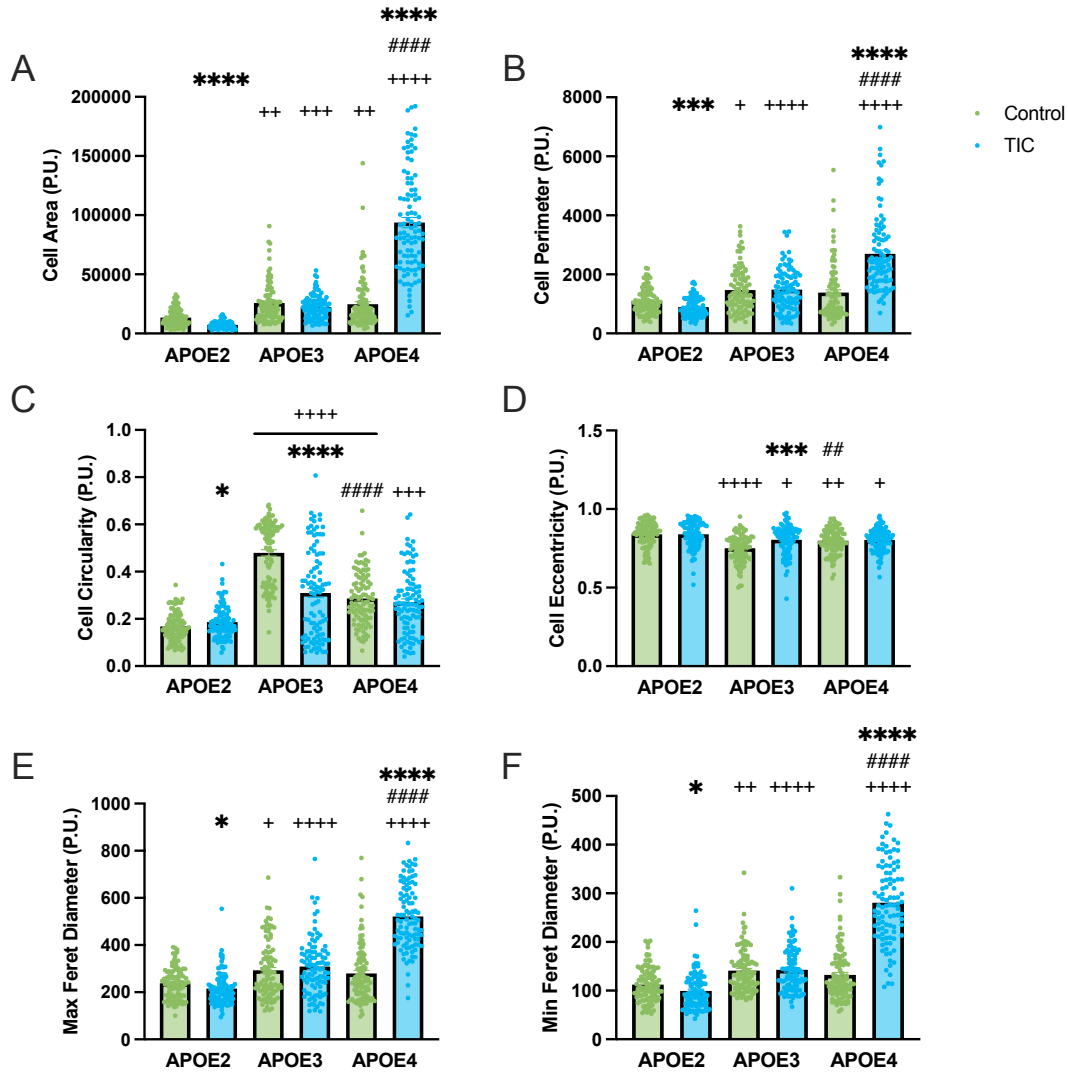


Figure 4.3.8 Different morphology changes are observed in reactive CTX-Astros depending on the APOE phenotype.

Cell area (A) and cell perimeter (B) are significantly decreased in stimulated APOE2 CTX-Astros and increased in stimulated APOE4 CTX-Astros. (C) Cell circularity increased in stimulated APOE2 CTX-Astros but decreased in the APOE3. (D) Cell eccentricity increased in stimulated APOE3 CTX-Astros only. Max (E) and Min (F) Feret diameters both decreased in stimulated APOE2 CTX-Astros but increased in stimulated APOE4 CTX-Astros. Overall, APOE4 cells were larger in size, but cell circularity and eccentricity changed the most in the APOE3 CTX-Astros. Data expressed as mean $\pm$ SEM (N=3; 100 cells per N). * p <0.05, *** p <0.001, **** p <0.0001 vs control. # p <0.05, ## p <0.01, ### p <0.001, #### p <0.0001 vs APOE3 line. ++ p <0.01, +++ p <0.0001 vs APOE2 line. Unpaired two-tailed Student's t -Test and two-way ANOVA with Tukey's post hoc test.

4.3.5. Microglial Factors Induce Pro-Cytokine and Chemokine Release in Cortical Astrocytes Carrying Different APOE Genotypes

Morphological remodelling of astrocytes is a key feature of the transition from a surveying to a reactive phenotype, but this change is often accompanied by the secretion of cytokines and chemokines that amplify neuroinflammatory signalling. To evaluate the functional reactivity of APOE2, APOE3 and APOE4 cortical astrocytes, the release of pro-inflammatory mediators was quantified following stimulation with the microglia-derived cytokines (TIC). These factors have been shown to induce a robust A1-like reactive astrocyte phenotype (Liddelow *et al.*, 2017) and, therefore, provide a relevant model for studying APOE genotype-dependent differences in inflammatory signalling.

Stimulation with TIC significantly increased the release of IL-6 by the APOE2 (**** $p < 0.0001$), APOE3 (**** $p < 0.0001$) and APOE4 (**** $p < 0.0001$) CTX-Astros, compared to the control, as seen in **Fig. 4.3.9.A**. The stimulated APOE3 and APOE4 cells release less IL-6 compared to the TIC-stimulated APOE2 CTX-Astros (++++ $p < 0.0001$ for both), with 4,000 pg/mL, 2,800 pg/mL and 2,950 pg/mL of IL-6 released by the APOE2, APOE3 and the APOE4 cells, respectively.

CXCL10 (see **Fig. 4.3.9.B** below) was also released by the APOE2 (**** $p < 0.0001$), APOE3 (** $p = 0.0070$) and APOE4 (**** $p < 0.0001$) cells post TIC-stimulation compared to the control. The stimulated APOE2 CTX-Astros released the highest levels of CXCL10 at 8,400 pg/mL, compared to the APOE3 (++++ $p < 0.0001$) and APOE4 (+ $p = 0.0289$) cells. APOE3 CTX-Astros released the lowest levels of CXCL10 post-TIC stimulation at 1,000 pg/mL, compared to the APOE2 (++++ $p < 0.0001$) and the APOE4 (#### $p < 0.0001$) cells. The APOE4 cells released approximately 7,700 pg/mL of CXCL10 post-TIC stimulation.

Significant levels of RANTES are released by all three cell lines post-TIC stimulation compared for the control (**** $p < 0.0001$ for APOE2, APOE3 and APOE4s), as seen in **Fig. 4.3.9.C** below. Approximately 1,950 pg/mL and 1,800 pg/mL of RANTES are

released by both APOE2 and APOE3 CTX-Astros stimulated with TIC respectively. APOE4 CTX-Astros, however, release nearly 3,300 pg/mL of RANTES post-TIC stimulation, which is significantly higher compared to the TIC-stimulated APOE2 (++++p<0.0001) and APOE3 (####p<0.0001) CTX-Astros.

Stimulation of the APOE CTX-Astros with TIC also resulted in a significant increase of GM-CSF by the APOE2 (***p=0.0003) and APOE3 (*p=0.0237) cells compared to the control (**Fig. 4.3.9.D**). TIC-stimulation results in a 260 pg/mL release of GM-CSF in the APOE2 CTX-Astros, which is significantly higher than the 115 pg/mL released by the APOE3 cells (++p=0.0057) or the 66 pg/mL released by the APOE4 CTX-Astros (++++p<0.0001).

In summary, all APOE genotypes exhibited robust cytokine and chemokine responses to microglial factors, confirming successful induction of a reactive state. However, the magnitude and pattern of these responses were genotype-dependent: APOE2 astrocytes were the most reactive overall, releasing the highest levels of IL-6, CXCL10 and GM-CSF; APOE4 astrocytes were characterised by selective hypersecretion of RANTES; and APOE3 astrocytes displayed a comparatively attenuated response, particularly for CXCL10. These results indicate that the APOE genotype not only shapes astrocyte morphology but also influences the intensity and profile of their inflammatory secretome.

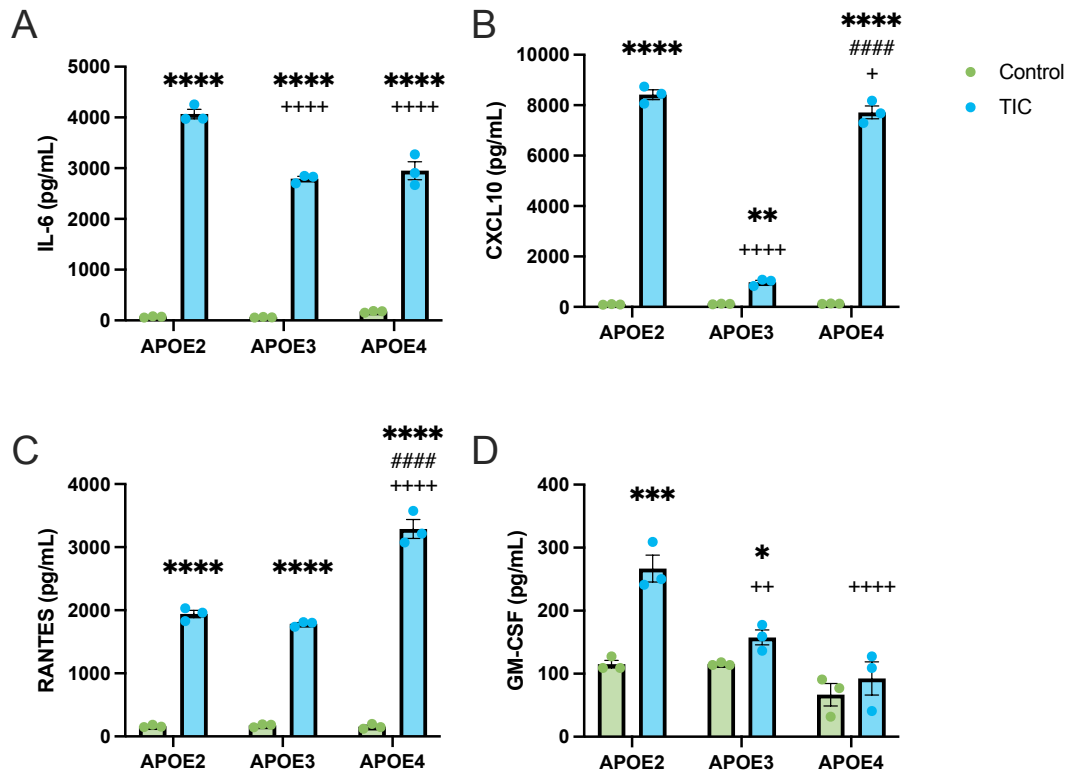


Figure 4.3.9 TIC-stimulated CTX-Astros release higher concentrations of pro-inflammatory cytokines and chemokines regardless of APOE phenotype.

Stimulated APOE2 and APOE3 CTX-Astros release significantly higher concentrations of CXCL10 (A), IL-6 (B), RANTES (C) and GM-CSF (D) compared to control. Stimulated APOE4 CTX-Astros release significantly higher levels of CXCL10 (A), IL-6 (B) and RANTES (C) only. Data expressed as mean $\pm$ SEM (N=3, r=2-3). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs control. ##### $p < 0.0001$ vs APOE3 line. + $p < 0.05$ ++ $p < 0.01$, +++ $p < 0.0001$ vs APOE2 line. Unpaired two-tailed Student's t-Test and two-way ANOVA with Tukey's post hoc test.

4.3.6. APOE2 and APOE4 CTX-Astros Express High Levels of Inflammatory and Reactive Genes Post Pro-Inflammatory Stimulation

The cytokine release assays demonstrated that APOE genotype influences the severity and profile of astrocyte secretory responses. To determine whether these differences reflect changes at the transcriptional level, we next assessed the expression of key inflammatory, reactive and functional genes following TIC stimulation. These included *IL6*, *CXCL10*, *CCL5*, *CSF2*, *C3* and *ITGAM*, which are

genes associated with classical A1-like reactivity, as well as *SLC1A2*, *SLC1A2* and *GLUL*, which encode proteins involved in glutamate uptake and metabolism.

APOE2 CTX-Astros expressed significantly higher levels of *IL6* (**** $p < 0.0001$), *CXCL10* (**** $p < 0.0001$), *CCL5* (**** $p < 0.0001$), *CSF2* (* $p = 0.0150$), *C3* (**** $p < 0.0001$) and *ITGAM* (* $p = 0.0108$) mRNA post-stimulation with TIC, compared to the control, as seen in **Fig. 4.3.10.A-F** below.

In contrast, the APOE3 CTX-Astros were a lot less responsive to the TIC stimulation. The only significant increase in the expression of inflammatory or reactive genes by the APOE3 cells post-TIC stimulation was observed in terms of the expression of *ITGAM* mRNA (**** $p < 0.0001$), compared to the control. Although a small increase in the expression of *IL6*, *CCL5* and *CSF2* mRNA was also observed, these results were non-significant. No increase in the expression of *CXCL10* and *C3* mRNA was observed in the TIC-stimulated APOE3 CTX-Astros.

Similarly to the APOE2s, TIC-stimulated APOE4 CTX-Astros also expressed significantly more *IL6* (**** $p < 0.0001$), *CXCL10* (* $p = 0.0117$), *CCL5* (**** $p < 0.0001$), *CSF2* (**** $p < 0.0001$) and *ITGAM* (*** $p = 0.0003$) mRNA compared to the control (**Fig. 4.3.10.A-F**). However, no changes in *C3* mRNA expression were observed in the APOE4 CTX-Astros, regardless of stimulation.

The levels of mRNA expression of inflammatory and reactive genes differed between the three APOE lines. TIC-stimulated APOE2 CTX-Astros expressed highest levels of *IL6* (++++ $p < 0.0001$ for both), *CXCL10* (++++ $p < 0.0001$ for APOE3; ++ $p = 0.0097$ for APOE4) and *C3* (++++ $p < 0.0001$ for both) mRNA, compared to the APOE3 and APOE4 cells. The levels of *CCL5* mRNA expression were significantly higher in the stimulated APOE2 CTX-Astros compared to APOE3s (++++ $p < 0.0001$) but were comparable with the levels of *CCL5* mRNA expression in the APOE4s post-TIC stimulation. The stimulated APOE3 CTX-Astros expressed very low levels of all the inflammatory genes assessed, however, a significantly higher level of *ITGAM* mRNA expression was observed in the APOE3 cells compared to the TIC-stimulated APOE2s (++ $p = 0.0049$) and although it was also higher than the *ITGAM* expression in the APOE4 CTX-Astros stimulated with TIC, this result was non-

significant. APOE4 CTX-Astros stimulated with TIC express intermediate levels of inflammatory genes with most expression being significantly lower than the APOE2s but significantly higher than the APOE3s. The *IL6* gene expression by stimulated APOE4 cells was significantly higher than the APOE3 cells (### $p=0.0001$), and significantly lower than APOE2s (++++ $p<0.0001$). *CXCL10* mRNA expression was also significantly higher in the TIC-stimulated APOE4 CTX-Astros compared to the stimulated APOE3s (# $p=0.0121$), but significantly lower compared to the APOE2s (++ $p=0.0097$). However, while the expression of *CCL5* mRNA was still significantly higher in the APOE4s compared to the stimulated APOE3 CTX-Astros (#### $p<0.0001$), this result was not significantly lower than the APOE2s. The *CSF2* mRNA expression by TIC-stimulated APOE4 CTX-Astros was significantly higher compared to both APOE2s (++++ $p<0.0001$) and APOE3s (#### $p<0.0001$). No significant differences were observed between TIC-stimulated APOE4 and APOE3 CTX-Astros in terms of *C3* and *ITGAM* mRNA expression. To summarise, both APOE2 and APOE4 CTX-Astros expressed higher levels of inflammatory and reactive genes post stimulation with pro-inflammatory cytokines. However, the APOE3 CTX-Astros were not as responsive to the same stimulation.

The same cells were also analysed for their expression of *SLC1A2* (EAAT1), *SLC1A3* (EAAT2) and *GLUL* mRNA with and without the TIC stimulation, as seen in **Fig. 4.3.11.A-C** below. Although a slight increase was observed across the board post-stimulation, none of these results were significant. No significant differences were observed between the different APOE lines either.

In summary, APOE2 and APOE4 astrocytes both mounted strong transcriptional responses to pro-inflammatory stimulation, while APOE3 astrocytes remained comparatively unresponsive. The alignment between gene expression and cytokine release profiles reinforces that APOE genotype modulates both the magnitude and nature of astrocyte reactivity, shaping their inflammatory and metabolic state. Together, these findings confirm that APOE genotype influences astrocyte behaviour at multiple functional levels, from morphology and cytokine secretion to gene expression.

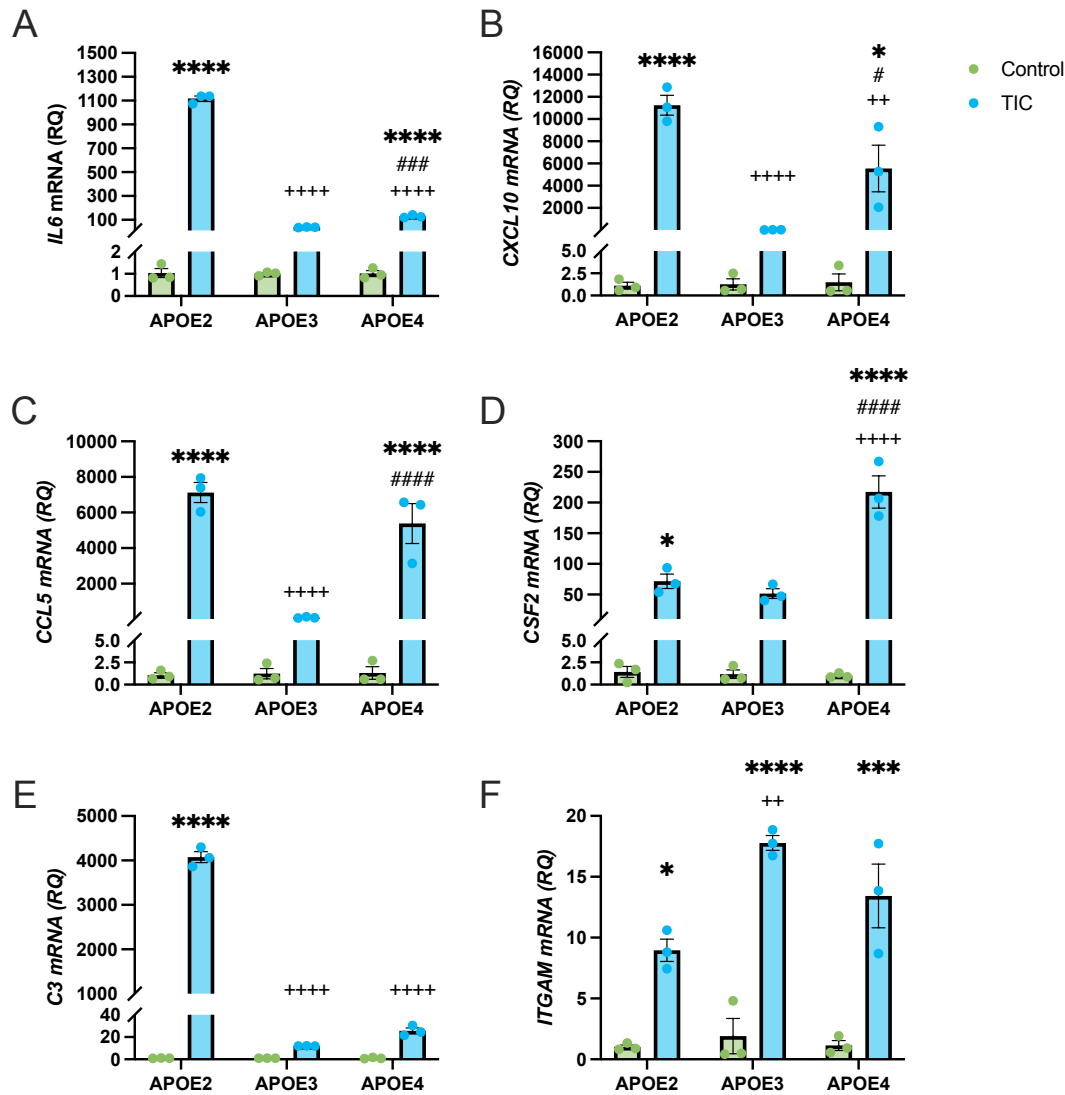


Figure 4.3.10 Reactive APOE2 CTX-Astros express higher levels of inflammatory and reactive genes are differentially expressed in CTX-Astros depending on stimulation and APOE phenotype, with most expression observed in reactive APOE2 CTX-Astros.

APOE2, APOE3 and APOE4 CTX-Astros stimulated with TIC expressed significantly higher levels of IL6 (A), CCL5 (C), CSF2 (D), ITGAM (E) and C3 (F) mRNA, compared to their control. An upregulation of CXCL10 (B) mRNA expression post-TIC stimulation was observed in APOE2 and APOE3 CTX-Astros only. Overall, a more substantial upregulation of the gene expression was observed in APOE2, with the exception of CSF2 and ITGAM. Highest expression of CSF2 (D) mRNA was observed in stimulated APOE4 CTX-Astros, while ITGAM (E) mRNA exhibited the highest upregulation in stimulated APOE3 CTX-Astros. Data expressed as mean $\pm$ SEM (N=3, r=2-3). * p <0.05, *** p <0.001, **** p <0.0001 vs control. # p <0.05, ### p <0.001, #### p <0.0001 vs APOE3 line. ++ p <0.01, +++ p <0.0001 vs APOE2 line. Unpaired two-tailed Student's t -Test and two-way ANOVA with Tukey's post hoc test.

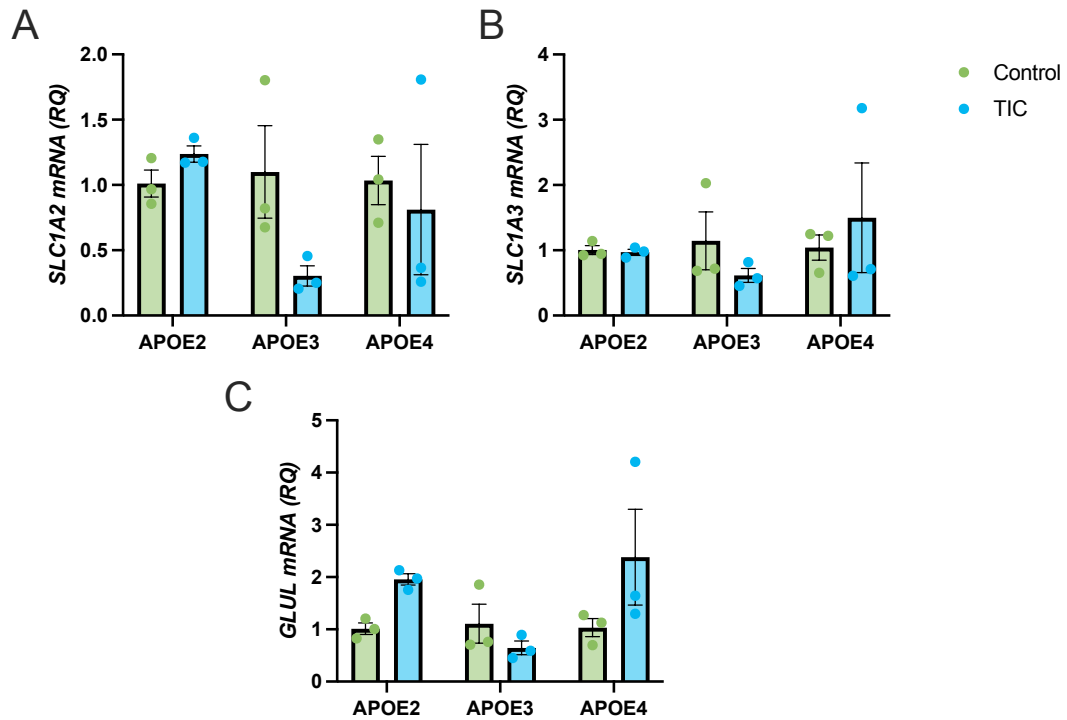


Figure 4.3.11 No differences were observed in the expression of functional genes between CTX-Astros carrying different APOE genotypes.

Quiescent and reactive APOE2, APOE3 and APOE4 CTX-Astros were analysed for their expression of functional genes SLC1A2 (**A**), SLC1A3 (**B**) and GLUL (**C**). Overall, the expression of these genes was very low, regardless of the APOE phenotype or stimulation. No significant changes were observed in (**A**) SLC1A2, (**B**) SLC1A3 or (**C**) GLUL mRNA expression, regardless of stimulation of APOE genotype. Data expressed as mean $\pm$ SEM (N=3, r=2-3). Two-way ANOVA.

4.3.7. Stimulation with Amyloid Beta 1-40 and 1-42 Does Not Have an Effect on Cell Viability

To determine whether A β oligomers directly modulate astrocyte viability or reactivity, NAS2 cortical astrocytes, a validated iPSC-derived line previously optimised for inflammatory and metabolic assays in this study, were used for all A β stimulation experiments.

Astrocytes were stimulated with 1 μ M or 2 μ M of A β ₁₋₄₀ or A β ₁₋₄₂, with or without 0.3 ng/mL IL-1 α , to model combined amyloid and cytokine exposure. Cell viability was assessed using the alamarBlue assay to measure metabolic activity and the LDH assay to quantify cytotoxicity.

No significant changes were observed in the metabolic activity following A β ₁₋₄₀ stimulation (**Fig. 4.3.12.A** below). Although a slight decrease in the metabolic activity was observed in the 2 μ M A β ₁₋₄₀+IL-1 α stimulation, this result was non-significant. Similarly, no change in the cell toxicity was observed, regardless of A β ₁₋₄₀ concentration used or whether it was combined with IL-1 α or not (**Fig. 4.3.12.C**).

Stimulation with A β ₁₋₄₂ also did not have a significant effect on the metabolic activity of CTX-Astros, as seen in **Figure 4.12.B** below. Although a trend towards a decrease in metabolic activity was observed when CTX-Astros were stimulated with a 2 μ M concentration of A β ₁₋₄₂, this was non-significant. No changes were observed in cell toxicity of the CTX-Astros stimulated with A β ₁₋₄₂, with or without the addition of IL-1 α (**Fig. 4.3.12.D**).

In summary, exposure to A β ₁₋₄₀ or A β ₁₋₄₂ at 1-2 μ M did not compromise CTX-Astros viability either alone or in combination with IL-1 α . These findings indicate that the selected concentrations are non-cytotoxic and are potentially suitable for downstream analyses of astrocyte reactivity and gene expression.

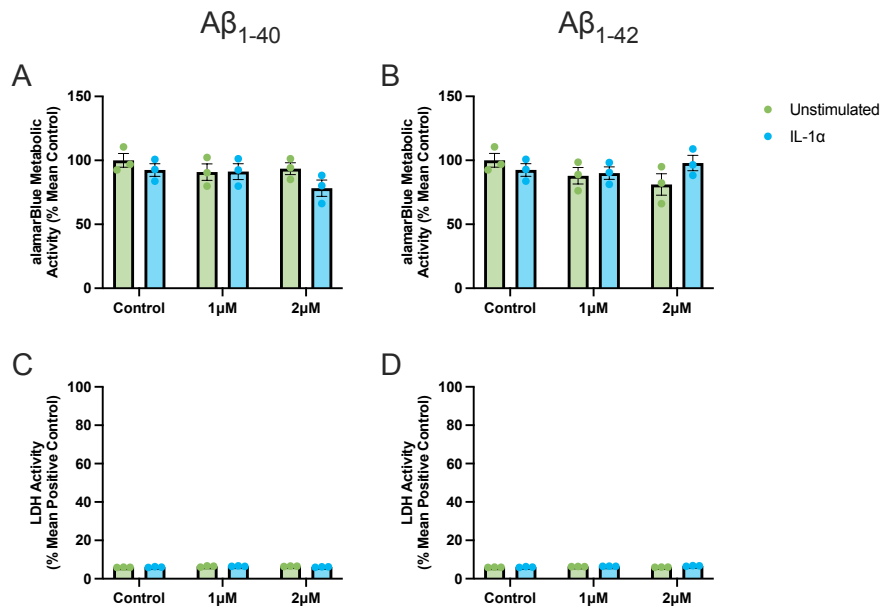


Figure 4.3.12 Stimulation of CTX-Astros with Aβ does not affect the viability of these cells.

CTX-Astrocytes were stimulated with various doses of Aβ₁₋₄₀ and Aβ₁₋₄₂ with and without the addition of IL-1α. Stimulation with Aβ₁₋₄₀ (A) or Aβ₁₋₄₂ (B) does not affect the cells metabolic activity measured by the alamarBlue assay. Likewise, Aβ₁₋₄₀ (C) or Aβ₁₋₄₂-stimulation (D) does not increase the LDH activity of these cells which is a measure of cell cytotoxicity. Data expressed as mean ± SEM (N=3, r=2-3). Two-way ANOVA.

4.3.8. Stimulation with Aβ alone is not sufficient to induce a reactive phenotype in CTX-Astros

Aβ oligomers are central to Alzheimer's disease pathology and are known to influence astrocyte function and reactivity *in vivo* (Liddel et al., 2017; Verkhatsky et al., 2023). However, their direct effects on human astrocytes remain debated, with some studies suggesting that Aβ alone induces only mild or transient activation unless combined with pro-inflammatory cytokines (García-Matas et al., 2010; LaRocca et al., 2021). To evaluate this, NAS2 cortical astrocytes were stimulated with Aβ₁₋₄₀ or Aβ₁₋₄₂ oligomers at 1 μM and 2 μM concentrations, with or without 0.3 ng/mL IL-1α, to determine whether Aβ oligomer exposure alone is sufficient to trigger cytokine release and a reactive phenotype.

Stimulation with Aβ₁₋₄₀ oligomers alone at either concentration did not alter the release of pro-inflammatory cytokines, as seen in Fig. 4.3.13.A-D. However, when combined with 0.3 ng/mL of IL-1α, some additional effects were observed. Namely,

a higher release of RANTES (**Fig. 4.3.13.C**) was observed post-stimulation with 1 μ M $A\beta_{1-40}$ +IL-1 α , compared to the unstimulated control (**** $p<0.0001$) and IL-1 α alone (## $p=0.0021$). Although a slight increase in RANTES was also observed with the 2 μ M concentration, this was non-significant. A similar trend towards an increase in the release of CXCL10 was observed when 1 μ M $A\beta_{1-40}$ +IL-1 α was used, but this was also non-significant, as seen in **Fig. 4.3.13.B** below. No additional significant increase in IL-6 or GM-CSF (**Fig. 4.3.13.A and D**) were observed when $A\beta_{1-40}$ was combined with IL-1 α . Moreover, increasing the concentration of $A\beta_{1-40}$ from 1 μ M to 2 μ M resulted in a significant decrease in the CXCL10 release compared to the 1 μ M $A\beta_{1-40}$ +IL-1 α (++++ $p<0.0001$), thus further supporting the potential use of 1 μ M concentration of $A\beta_{1-40}$ in combination with IL-1 α .

Similar results were obtained when the 1 μ M and 2 μ M concentrations of $A\beta_{1-42}$ were combined with 0.3 ng/mL of IL-1 α . The release of RANTES (**Fig. 4.3.13.G**) was significantly increased when $A\beta_{1-42}$ was combined with IL-1 α , compared to the unstimulated control (**** $p<0.0001$) and IL-1 α alone (# $p=0.0227$ for 1 μ M; #### $p<0.0001$ for 2 μ M). While no additional effect was observed on combining $A\beta_{1-42}$ with IL-1 α in terms of IL-6 (**Fig. 4.3.13.E**) or GM-CSF (**Fig. 4.3.13.H**) release, a significant decrease in CXCL10 release (# $p=0.0276$ for 1 μ M; ## $p=0.0016$ for 2 μ M) was observed, compared to IL-1 α alone, as seen in **Fig. 4.3.13.F** below. Therefore, despite a higher increase in RANTES release when combining 2 μ M $A\beta_{1-42}$ with IL-1 α compared to the 1 μ M concentration (+++ $p=0.0008$), the higher decrease in the CXCL10 release at that concentration suggests that higher concentrations of $A\beta$ may be detrimental to astrocyte function.

Preliminary experiments assessing $A\beta_{1-40}$ and $A\beta_{1-42}$ oligomers alone had minimal effects of astrocyte activity, with no significant induction of inflammatory markers or viability changes. In contrast, combining $A\beta$ oligomers with IL-1 α increased chemokine release, most notably RANTES. Therefore, only the combined stimulation conditions (1 μ M $A\beta_{1-40}$ +IL-1 α and 1 μ M $A\beta_{1-42}$ +IL-1 α) were used for subsequent qPCR and Seahorse analyses.

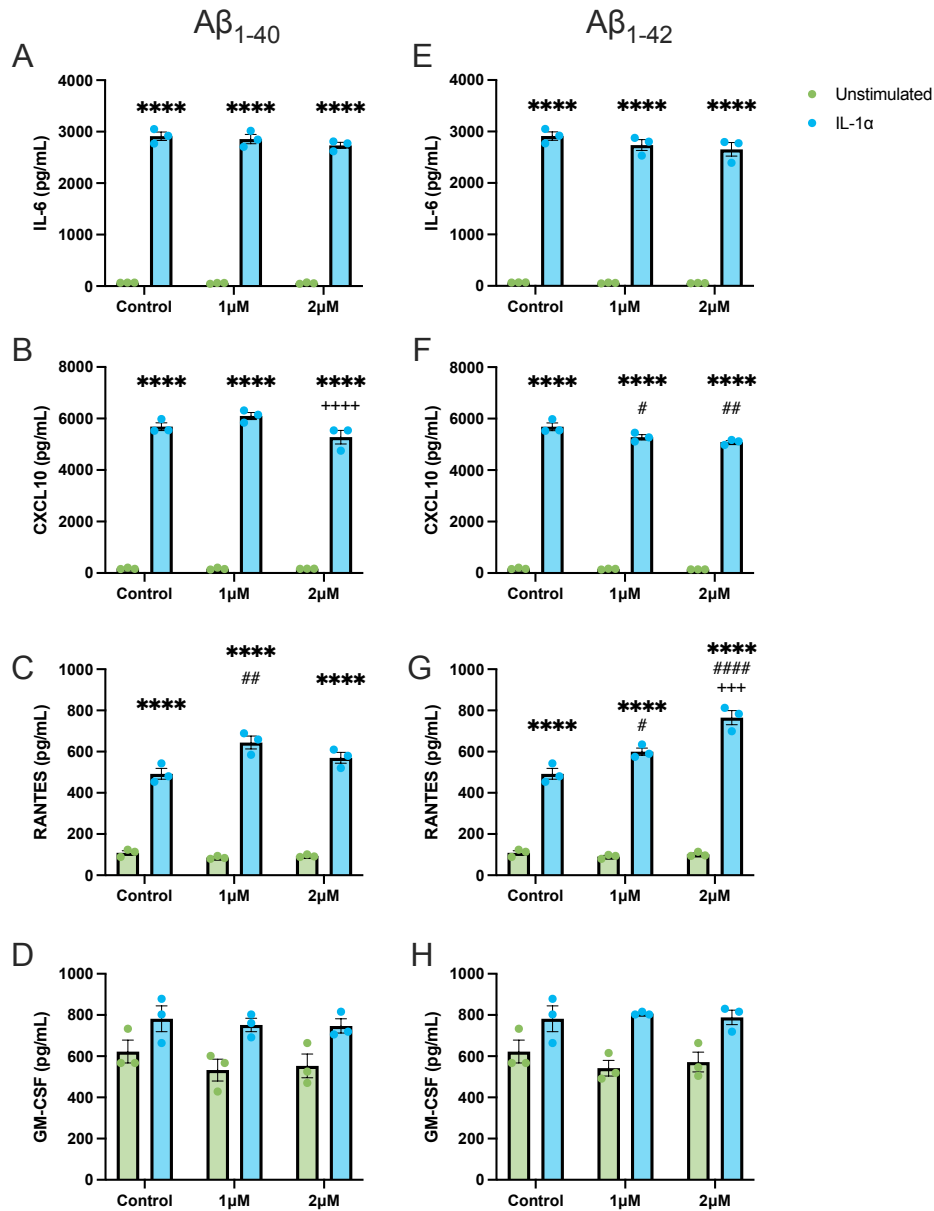


Figure 4.3.13 Aβ stimulation alone is not enough to induce a reactive profile in CTX-Astros.

CTX-Astros stimulated with Aβ₁₋₄₀ and Aβ₁₋₄₂ (with and without the addition of IL-1α) were assessed for their release of pro-inflammatory cytokines. Stimulation with Aβ alone did not have any effect on the release of pro-inflammatory cytokines (**A-H**). Combination of Aβ₁₋₄₀ with IL-1α significantly increased the release of RANTES (**C**) compared to the control and IL-1α stimulation alone. Similarly, stimulation with Aβ₁₋₄₂ and IL-1α significantly increased the release of RANTES (**G**) compared to the control and IL-1α. However, Aβ₁₋₄₂+IL-1α stimulation also significantly decreased the release of CXCL10 (**F**) compared to IL-1α alone. The combination of Aβ₁₋₄₀ or Aβ₁₋₄₂ with IL-1α did not have any additional effects on the release of IL-6 (**A, E**) or GM-CSF (**D, H**). Based on these results 1μM concentration for both Aβ₁₋₄₀ and Aβ₁₋₄₂ combined with IL-1α were chosen for further experiments. Data expressed as mean ± SEM (N=3, r=2-3). ****p<0.0001 vs control. #p<0.05, ##p<0.01, ###p<0.0001 vs IL-1α alone. +++p<0.001, ++++p<0.0001 vs 1μM Aβ. Two-way ANOVA with Tukey's post hoc test.

4.3.9. A β +IL-1 α stimulation upregulates the inflammatory gene expression in CTX-Astros

To determine whether combined A β and cytokine stimulation influences the transcriptional reactivity of human astrocytes, CTX-Astros were analysed for the expression of inflammatory, functional and metabolic genes following exposure to A β ₁₋₄₀+IL-1 α and A β ₁₋₄₂+IL-1 α . Given that A β alone did not elicit a strong cytokine response, this experiment focused on whether the addition of IL-1 α could enhance astrocytic gene expression associated with inflammation, complement activation and metabolic regulation.

Stimulation with A β ₁₋₄₀+IL-1 α results in a significant upregulation of several inflammatory genes in CTX-Astros, as seen in **Fig. 4.3.14.A-E** below. Namely, the expression of *IL6* (*p=0.0168), *CXCL10* (*p=0.0155), *CCL5* (*p=0.0361), *C3* (**p=0.0009) and *ITGAM* (*p=0.0126) mRNA is significantly increased compared to the control. Although a trend towards an upregulation of *CSF2* mRNA is also observed post-A β ₁₋₄₀+IL-1 α stimulation, this result was non-significant (see **Fig. 4.3.14.F**).

Similarly, stimulation with A β ₁₋₄₂+IL-1 α also results in a significant upregulation of the same inflammatory genes in CTX-Astros, as seen in **Fig. 4.3.14.A-E** below. The expression of *IL6* (*p=0.02), *CXCL10* (**p=0.0041), *CCL5* (RANTES; **p=0.0044), *C3* (**p=0.0009) and *ITGAM* (**p=0.002) mRNA is significantly increased in the A β ₁₋₄₂+IL-1 α stimulated cells compared to the control. Although the expression of *CSF2* mRNA is also trending towards an increase, this result was non-significant (see **Fig. 4.3.14.F** below). No significant differences in mRNA expression of inflammatory genes were observed between the A β ₁₋₄₀+IL-1 α and the A β ₁₋₄₂+IL-1 α -stimulated groups.

The same cells were also investigated for their mRNA expression of several functional and metabolic genes. Although trends toward an increase in the expression of *SLC1A2*, *SLC1A3*, *GLUL*, *HK2*, *LDHA* and *SLC2A1* mRNA were observed post-stimulation with A β ₁₋₄₀+IL-1 α or A β ₁₋₄₂+IL-1 α , none of these results were significant (**Fig. 4.3.15.A-D, F and G**). Stimulation with A β ₁₋₄₀+IL-1 α or the

A β ₁₋₄₂+IL-1 α did not have any effect on the expression of *PFKFB3* or *SLC6A3* mRNA in CTX-Astros, as seen in **Fig. 4.3.15.E and H** below.

Overall, these findings demonstrate that co-stimulation with A β and IL-1 α is sufficient to trigger a reactive transcriptional program in CTX-Astros, characterised by the robust upregulation of inflammatory genes such as *IL6*, *CXCL10* and *C3*, without concurrent induction of functional or metabolic genes.

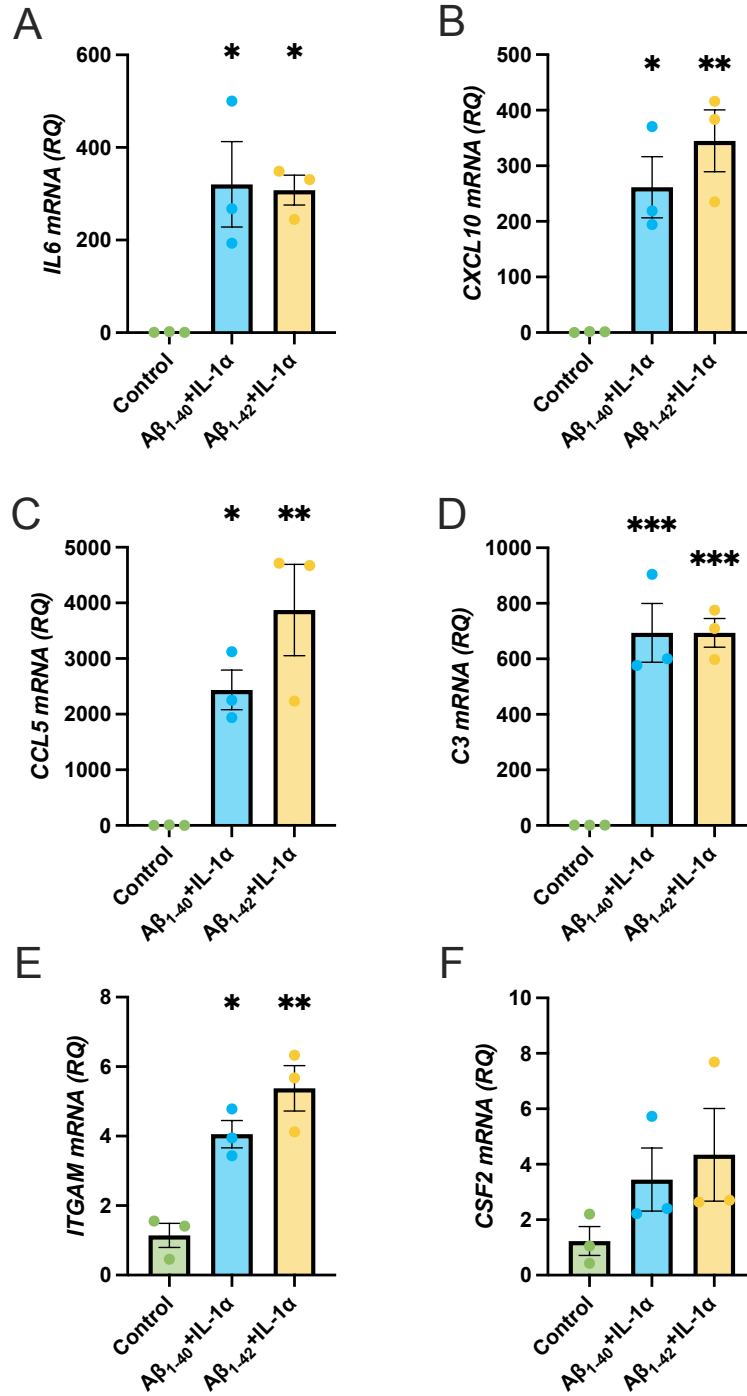


Figure 4.3.14 Aβ+IL-1α stimulation upregulates inflammatory gene expression in CTX-Astros.

CTX-Astros stimulated with 1 μM of Aβ₁₋₄₀+IL-1α or Aβ₁₋₄₂+IL-1α were used to assess the gene expression of inflammatory and reactive genes. Both stimulation cocktails induced a significant upregulation of IL6 (A), CXCL10 (B), CCL5 (C), C3 (D) and ITGAM (E) mRNA expression. Although a slight increase of CSF2 (F) mRNA expression was also observed post stimulation, this was non-significant. No significant differences were observed between stimulation with Aβ₁₋₄₀ versus Aβ₁₋₄₂ in combination with IL-1α. Data expressed as mean ± SEM (N=3, r=2-3). *p<0.05, **p<0.01, ***p<0.001 vs control. One-way ANOVA with Tukey's post hoc test.

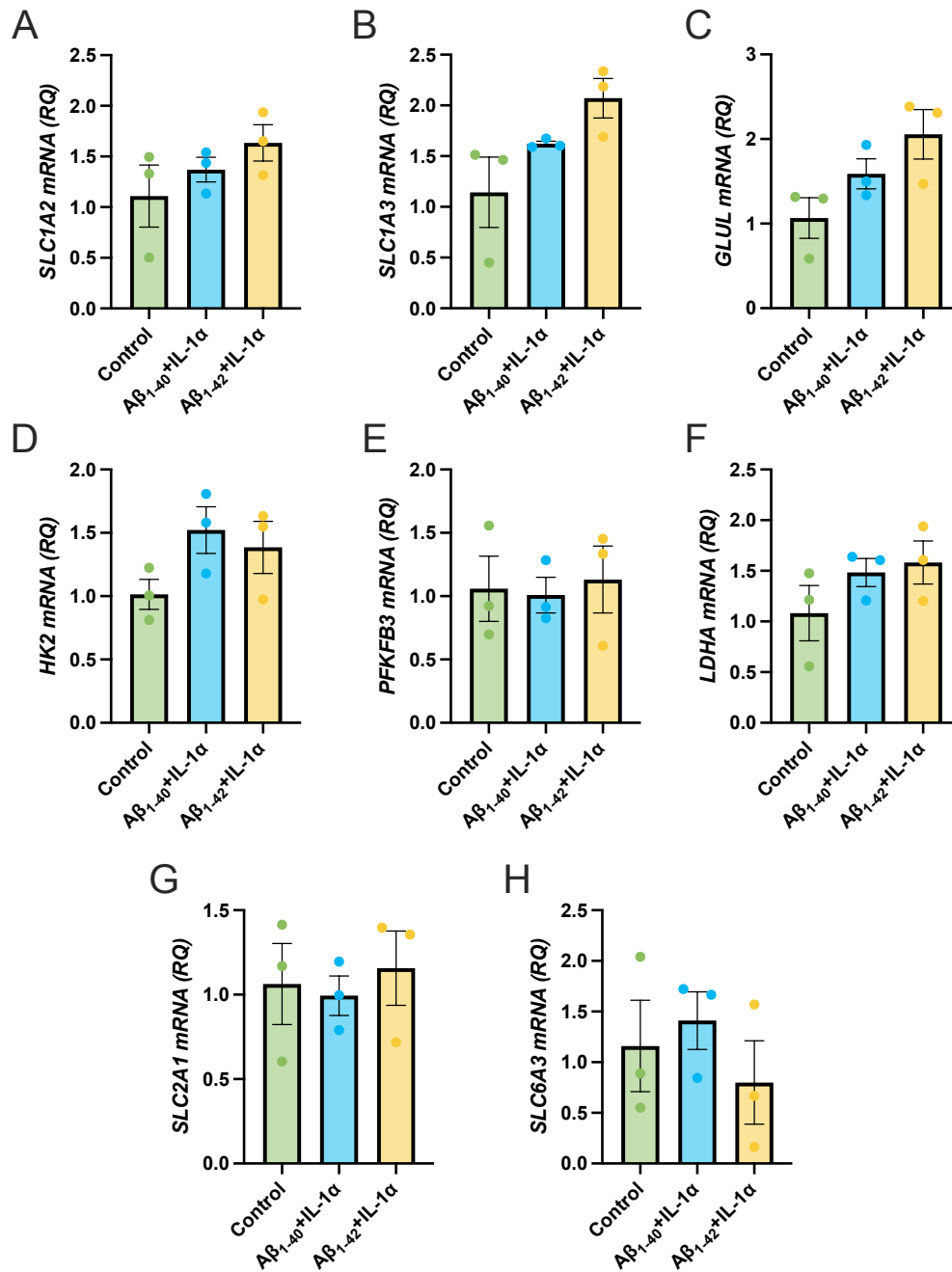


Figure 4.3.15 Stimulation with Aβ+IL-1α does not affect the expression of functional and metabolic genes in CTX-Astros.

CTX-Astros stimulated with 1 μM of Aβ₁₋₄₀+IL-1α or Aβ₁₋₄₂+IL-1α were used to assess the gene expression of functional and metabolic genes. Although a trend towards an increase of SLC1A2 (A), SLC1A3 (B), GLUL (C), HK2 (D), LDHA (F), and SLC6A3 (H) mRNA was observed post-stimulation with Aβ₁₋₄₀+IL-1α or Aβ₁₋₄₂+IL-1α compared to the control, these results were non-significant. The expression of PFKFB3 (E) and SLC2A1(G) mRNA did not change at all with stimulation. Data expressed as mean ± SEM (N=3, r=2-3). One-way ANOVA.

4.3.10. Stimulation with $A\beta_{1-40}+IL-1\alpha$ increases the basal glycolysis and glycolytic capacity of CTX-Astros

To evaluate whether $A\beta$ -driven inflammatory activation also alters the metabolic profile of astrocytes, extracellular acidification rate (ECAR) was measured using the Seahorse Glycolytic Stress Test on the control and stimulated NAS2 CTX-Astros.

Stimulation of CTX-Astros with $A\beta_{1-40}+IL-1\alpha$ or $A\beta_{1-42}+IL-1\alpha$ did not have an effect on the basal ECAR rate during the Glycolytic Stress Test assay via Seahorse, as seen in **Fig. 4.3.16.A** below. However, once glucose was injected, the $A\beta_{1-40}+IL-1\alpha$ -stimulated cells had a significantly increased ECAR rate compared to the control (* $p=0.0111$ at 21 min, ** $p=0.0098$ at 27 min, * $p=0.0154$ at 34 min). Similarly, once Oligomycin was injected, the $A\beta_{1-40}+IL-1\alpha$ -stimulated cells continued to exhibit a significantly increased ECAR rate compared to the control (** $p=0.0016$ at min 40, ** $p=0.0010$ at min 47, ** $p=0.0014$ at min 53) and the $A\beta_{1-42}+IL-1\alpha$ -stimulated (# $p=0.0147$ at min 40, ## $p=0.0099$ at min 47 and ## $p=0.0089$ at min 53) groups. The injection with 2-DG inhibited all glycolysis within the cells, and thus, the ECAR rate returned to baseline, regardless of stimulation.

Basal glycolysis (see **Fig. 4.3.15.B** below) was significantly increased in the CTX-Astros stimulated with $A\beta_{1-40}+IL-1\alpha$ compared to the control (** $p=0.0048$) and the $A\beta_{1-42}+IL-1\alpha$ -stimulated group (# $p=0.0174$). Similarly, the glycolytic capacity (**Fig. 4.3.16.C**) was also significantly increased in the $A\beta_{1-40}+IL-1\alpha$ -stimulated CTX-Astros compared to the control (* $p=0.0475$) and stimulation with $A\beta_{1-42}+IL-1\alpha$ (# $p=0.0424$). Although a trend toward an increase was observed in the glycolytic reserve post stimulation with $A\beta_{1-40}+IL-1\alpha$, this result was non-significant. Likewise, no significant differences were observed between the stimulation groups in terms of non-glycolytic acidification rate (see **Fig. 4.3.16.D and E** below).

In summary, $A\beta_{1-40}+IL-1\alpha$ stimulation increased both basal glycolysis and glycolytic capacity in CTX-Astros, indicating a metabolic shift toward enhanced glycolytic activity under inflammatory and amyloid-associated stress. In contrast, $A\beta_{1-42}+IL-1\alpha$ stimulation did not significantly alter glycolytic parameters, suggesting potential peptide-specific differences in how $A\beta$ modulates astrocyte energy metabolism.

These findings align with the concept that astrocyte activation involves metabolic reprogramming toward glycolysis and that $A\beta_{1-40}$, rather than $A\beta_{1-42}$, may be more effective at potentiating such responses in this *in vitro* model.

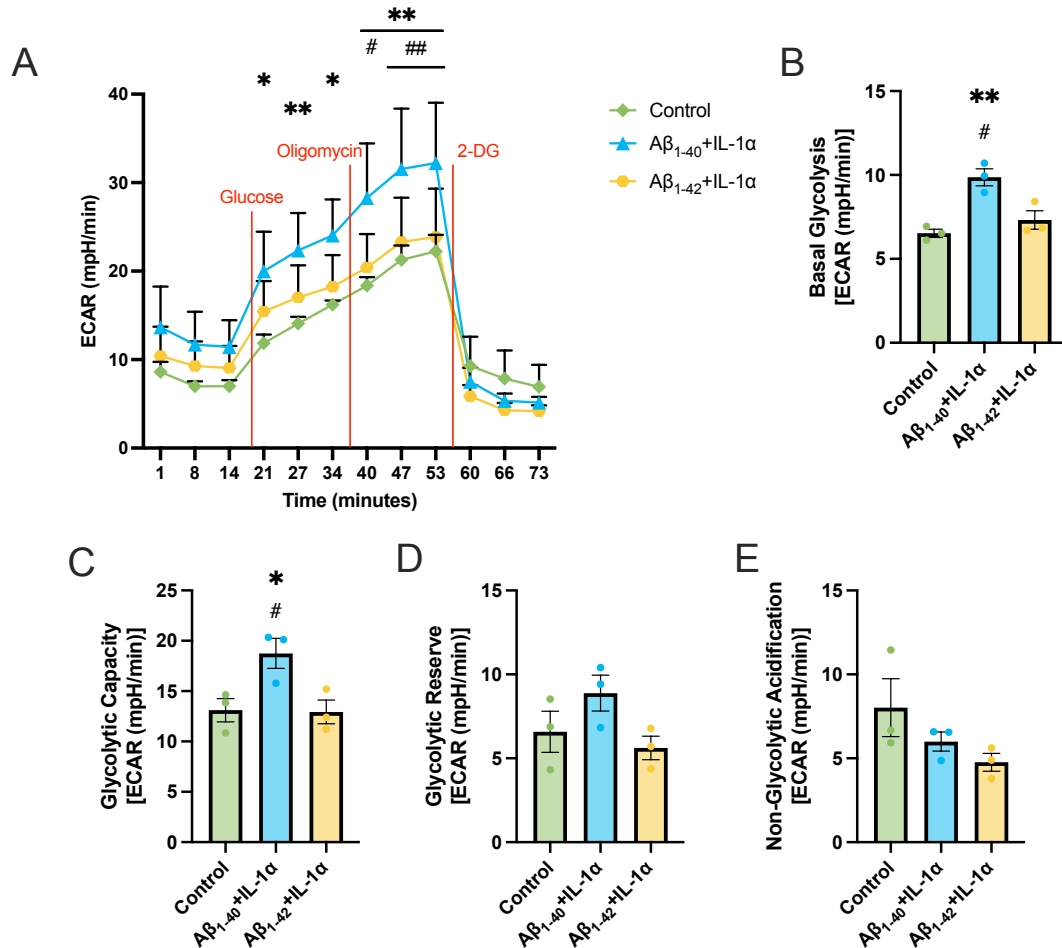


Figure 4.3.16 Stimulation with $A\beta_{1-40}+IL-1\alpha$ increases the basal glycolysis and the glycolytic capacity of CTX-Astros.

CTX-Astros stimulated with 1 μ M of $A\beta_{1-40}+IL-1\alpha$ or $A\beta_{1-42}+IL-1\alpha$ were subjected to the Glycolytic Stress Test via Seahorse. Stimulation with $A\beta_{1-40}+IL-1\alpha$ in CTX-Astros significantly increased the ECAR rate post-glucose and oligomycin injections (**A**), compared to the control. This rate was also significantly increased compared to the stimulation with $A\beta_{1-42}+IL-1\alpha$ once oligomycin was injected. The addition of 2-DG reduced the ECAR rate to the baseline, thus confirming that the metabolic rate of these cells is primarily glycolytic. $A\beta_{1-40}+IL-1\alpha$ -stimulated CTX-Astros exhibited an increased basal glycolysis (**B**) and glycolytic capacity (**C**) compared to the control and the $A\beta_{1-42}+IL-1\alpha$ stimulation. No significant differences were observed in the glycolytic reserve (**D**) or the non-glycolytic acidification rate (**E**), regardless of stimulation. Data expressed as mean $\pm$ SEM (N=3, r=2-3). * p <0.05, ** p <0.01 vs control. # p <0.05, ## p <0.01 vs $A\beta_{1-42}+IL-1\alpha$ stimulation. One-way ANOVA with Tukey's post hoc test.

4.4. Discussion

The findings presented in this chapter demonstrate that APOE genotype influences multiple aspects of human iPSC-derived astrocyte biology, including differentiation, morphology, inflammatory response and metabolism. All APOE isogenic lines used have successfully differentiated into cortical astrocytes; however, their responses to pro-inflammatory stimulation diverged markedly. APOE2 astrocytes were the most reactive, releasing higher levels of IL-6, CXCL10 and GM-CSF and showing stronger transcriptional induction of inflammatory genes. In contrast, APOE3 astrocytes exhibited a more restrained profile, while APOE4 cells displayed selective upregulation of chemokines such as RANTES. Moreover, A β alone was insufficient to drive astrocyte reactivity, but its combination with IL-1 α elicited robust transcriptional activation, accompanied by a metabolic shift towards increased glycolysis. Collectively, these results highlight that APOE genotype and inflammatory context both modulate astrocyte behaviour, supporting a model in which genetic background and local immune cues shape astrocytic contributions to Alzheimer's disease pathogenesis.

4.4.1. APOE genotype influences astrocyte identity and differentiation

The differentiation of iPSC-derived astrocytes carrying different APOE genotypes was successful, as confirmed by the expression of canonical astrocytic markers GFAP, S100 β , EAAT1 and Cx43. These findings indicate that the APOE isogenic lines were able to generate morphologically and phenotypically mature astrocytes comparable to those described in other iPSC-based differentiation protocols, such as Serio *et al.* (2013), as well as the ones used in the previous chapter investigating the regional differences in astrocytes.

The quantification of nuclear staining for TUJ1 and TBR1 during earlier stages of differentiation provided a reliable measure of neural and cortical identity. Further differentiation of these neural progenitors into astrocyte progenitor cells resulted in

a high expression of GFAP and a low expression of TUJ1. Low expression, rather than absence, of TUJ1 in astrocyte progenitors would be considered negative staining due to β Tubulin III's role in structural support, cell migration and division during development (Götz and Huttner, 2005; Dráberová *et al.*, 2008). Therefore, the low TUJ1 intensity observed here is consistent with non-neuronal, glial progenitor identity rather than contamination by neuronal cells.

Consistent with the findings in NAS2 CTX-Astros presented in the previous chapter, a significant decrease in S100 β intensity was observed in TIC-stimulated APOE2 and APOE3 Astros. Jones *et al.* (2017) also observed a reduced S100 β fluorescence in FAD and SAD patient-derived Astros. However, while the Jones *et al.* group report an S100 β decrease in the APOE4 genotype specifically, this was not observed in our study. Instead, a small, but non-significant, increase in S100 β expression was induced by stimulation of APOE4 Astros with TIC. Other studies, such as Hasel *et al.* (2021) and Clementi *et al.* (2023), suggest that the overexpression of S100 β is more closely associated with neurodegeneration than its downregulation. While the S100 β expression differs between the models and stimuli used, it is becoming more evident that S100 β plays an important role in neurodegeneration-associated neuroinflammation by stimulating the activation of pro-inflammatory cytokine release (Langeh and Singh, 2020), thus potentially having a detrimental effect on astrocyte reactivity and neuronal damage. This suggests that differential S100 β regulation across APOE genotypes could contribute to the modulation of neuroinflammation in AD.

GFAP, an intermediate filament protein expressed by astrocytes, is involved in a number of cellular processes, both in the healthy CNS and during cellular stress (Kamphuis *et al.*, 2014). Typically, an increase in GFAP is associated with astrogliosis and astrocytic damage (Amalia, 2021). Indeed, an upregulation of GFAP expression has been observed in both mouse and human AD samples (Kamphuis *et al.*, 2012, 2014), both at protein and gene levels. Other studies, however, did not observe any changes in GFAP expression in iPSC-derived APOE4 Astros (Jones *et al.*, 2017), and a reactive model of CTX Astros stimulated with TIC (McComish *et al.*, 2024) has even reported a decrease in GFAP expression. Both reports align with the

results observed in our study. While the APOE2 and APOE4 Astros exhibit a significantly reduced GFAP expression post-TIC stimulation, the GFAP intensity remains unchanged in the APOE3s. This suggests that the TIC-induced reactive profile in iPSC-derived astrocytes may present a distinct, non-canonical reactivity state that does not necessarily involve GFAP upregulation.

EAAT1, a glutamate transporter expressed in astrocytes, is crucial for maintaining glutamate levels, both intra- and extracellularly (Shigeri, Seal and Shimamoto, 2004), a disruption of which may contribute to excitotoxic neuronal injury and death (Rothstein *et al.*, 1996). While Jones *et al.* (2017) report a decrease in EAAT1 expression in APOE4 Astros, the opposite has been observed in our study, with a 2-fold increase in EAAT1 intensity in TIC-stimulated APOE4 Astros. However, a decrease in EAAT1 expression is observed in reactive APOE2 and APOE3 Astros. Notably, at baseline, the EAAT1 expression is higher in quiescent APOE3 Astros, but the TIC-stimulated APOE4 Astros express the highest levels of EAAT1 across the three APOE lines, with and without stimulation. This may indicate a compensatory upregulation of glutamate transport in APOE4 Astros under inflammatory stress, potentially linked to altered metabolic demands. However, excessive EAAT1 expression could also exacerbate excitotoxic stress by altering glutamate cycling, thus contributing to the APOE4-associated neuronal vulnerability observed in AD.

Cx43, a gap junction protein highly expressed in astrocytes, plays an important role in intracellular communication, metabolic support to neurons and modulation of synaptic plasticity, while also contributing to neurovascular signalling and blood-brain barrier maintenance through interactions with endothelial cells (Giaume and Liu, 2012; Boulay *et al.*, 2015). Although in our study, the Cx43 intensity is significantly decreased in TIC-stimulated APOE2 Astros, the opposite is true for the APOE3 and APOE4 Astros, which display an upregulation in Cx43 expression when reactive. This is consistent with an already published study reporting an increase in Cx43 puncta in reactive astrocytes in post-mortem AD brain tissue (Nagy *et al.*, 1996). Increased Cx43 expression in APOE3 and APOE4 astrocytes may reflect enhanced gap junction formation during reactivity, potentially facilitating propagation of calcium and inflammatory signals. Conversely, reduced Cx43

expression in the APOE2 astrocytes might limit such intercellular communication, contributing to their more autonomous but hyper-reactive cytokine release profile.

4.4.2. APOE genotype modulates astrocyte reactivity

The release of pro-inflammatory cytokines and chemokines following TIC stimulation differed hugely between APOE genotypes, indicating that APOE influences astrocyte immune reactivity. In this study, APOE2 Astros exhibited the highest secretion of IL-6, CXCL10 and GM-CSF following stimulation, while APOE4 astrocytes released the highest levels of RANTES. In contrast, APOE3 astrocytes produced comparatively lower cytokine levels across all measured markers, suggesting a less reactive phenotype despite equivalent pro-inflammatory conditions.

These results align with the emerging evidence that APOE isoforms differentially regulate astrocyte responses to immune activation. APOE4 has been associated with exaggerated inflammatory signalling and impaired resolution of inflammation in both human and murine models (Liao *et al.*, 2018; Wang *et al.*, 2021). However, the pattern observed here, where APOE2 Astros were most responsive, suggests that the relationship between APOE genotype and reactivity is context-dependent, potentially reflecting differences in the balance between pro- and anti-inflammatory mediators across stimuli or cell maturity.

The increased IL-6 and CXCL10 secretion by APOE2 astrocytes could indicate a hyper-reactive but transient inflammatory phenotype, as these cytokines are rapidly induced through NF- κ B and JAK/STAT signalling following cytokine exposure (Kumar *et al.*, 2016). Conversely, the selective upregulation of RANTES (CCL5) in APOE4 astrocytes may point to sustained chemotactic signalling that could enhance microglial and peripheral immune recruitment, consistent with findings that APOE4 carriers exhibit elevated glial chemokine expression in AD brain tissue (Fernandez *et al.*, 2019; Li *et al.*, 2021). The attenuated response in APOE3 Astros suggests a more balanced or regulated inflammatory profile, aligning with its well-documented role as the “neutral” isoform in AD risk (Mahley and Huang, 2012). This

comparatively moderate reactivity may contribute to maintaining homeostasis in the CNS environment following inflammatory challenge. Collectively, these findings support the notion that APOE genotype shapes astrocytic inflammatory programming, not only in terms of magnitude but also in cytokine profile, which may have downstream implications for neuron-glia communication, microglial recruitment and overall neuroimmune tone in AD.

The divergent cytokine and chemokine responses observed among APOE genotypes likely correspond to broader differences in astrocyte functional states. Inflammatory activation not only alters the secretory profile of astrocytes but also reshapes their morphology, reflecting cytoskeletal and metabolic adaptations linked to reactivity. Given that APOE has been implicated in cytoskeletal regulation and cellular architecture (Zhou, Zuo and Jiang, 2019; Gamba *et al.*, 2023), it was important to assess whether these genotypic differences in inflammatory responsiveness were accompanied by structural changes. To address this, we next examined how TIC stimulation influenced astrocyte morphology across APOE2, APOE3 and APOE4 lines.

4.4.3. Reactivity-induced morphological changes in APOE models

Astrocyte morphology is a well-established indicator of functional state and reactivity. In the healthy CNS, astrocytes display a fine, highly branched structure that facilitates neuronal support, neurotransmitter clearance, and metabolic exchange. In contrast, reactive astrocytes often undergo hypertrophy, increased GFAP expression, and reorganisation of the cytoskeleton (Pekny and Pekna, 2014). Such morphological remodelling reflects underlying transcriptional and metabolic changes that accompany inflammatory activation (Escartin *et al.*, 2021).

In this study, TIC stimulation induced distinct morphological responses depending on the APOE genotype. APOE2 astrocytes exhibited a reduction in cell area and perimeter alongside increased circularity, suggesting a mild contraction of the cytoskeleton and a shift towards a more compact morphology. In contrast, APOE4 astrocytes showed pronounced hypertrophy, with increases in area, perimeter, and

Feret diameters, which is generally indicative of a more reactive or stress-associated profile. APOE3 astrocytes displayed a unique pattern, maintaining a relatively stable cell size but showing increased eccentricity and reduced circularity, consistent with elongation or process retraction, rather than overall hypertrophy.

These genotype-specific differences may reflect divergent cytoskeletal regulation and inflammatory signalling pathways. Previous studies have shown that APOE isoforms influence astrocytic actin organisation and motility (Zhou, Zuo and Jiang, 2019; Wang *et al.*, 2021), with APOE4 in particular associated with altered RhoA–ROCK signalling and impaired cytoskeletal dynamics (Nuriel *et al.*, 2017). The observed hypertrophy of APOE4 astrocytes may therefore stem from cytoskeletal dysregulation and excessive reactivity, aligning with reports that APOE4 enhances NF- κ B activation and pro-inflammatory signalling (Fernandez *et al.*, 2017). Conversely, the smaller and more circular morphology of APOE2 astrocytes, even post-stimulation, could indicate a more controlled or protective form of reactivity, consistent with APOE2's generally anti-inflammatory and neuroprotective profile (Li *et al.*, 2020).

Altogether, these findings highlight that APOE genotype not only modulates astrocytic cytokine and gene expression but also translates these molecular differences into distinct morphological signatures of reactivity. The hypertrophic, enlarged morphology of APOE4 astrocytes supports the hypothesis that this genotype predisposes to maladaptive glial activation, potentially amplifying neuroinflammatory cascades and tissue stress in Alzheimer's disease. However, this heightened morphological reactivity was not consistently reflected by the mRNA expression of inflammatory markers, suggesting that structural remodelling and inflammatory transcriptional responses may be differently regulated across APOE genotypes.

4.4.4. APOE genotype modulates astrocyte inflammatory reactivity

Astrocytes carrying different APOE alleles exhibited clear genotype-dependent differences in their inflammatory responsiveness to TIC stimulation. Among the three isogenic lines, the APOE2 Astros were the most reactive, showing robust upregulation of *IL6*, *CXCL10*, *CCL5* and *C3* mRNA along with increased cytokine secretion. In contrast, APOE3 astrocytes were comparatively unresponsive, while APOE4 astrocytes displayed an intermediate profile characterised by strong RANTES release but less pronounced IL-6 and CXCL10 induction. These results indicate that APOE genotype shapes not only baseline astrocyte state but also the magnitude and pattern of reactivity following exposure to pro-inflammatory cytokines.

Such variation is consistent with accumulating evidence that APOE isoforms differentially influence astrocyte immune signalling. APOE4 astrocytes have been shown to exhibit heightened NF- κ B activation and greater basal expression of complement and inflammatory genes (Fernandez *et al.*, 2019; Wang *et al.*, 2021), which can promote a chronic, maladaptive form of astrogliosis. Conversely, APOE2 is typically associated with enhanced lipid metabolism, antioxidant capacity and neuroprotection (Li *et al.*, 2020), yet our data reveal that APOE2 astrocytes can mount a substantial acute inflammatory response. This may reflect a more flexible or efficient capacity to detect and respond to injury signals, rather than an inherently pro-inflammatory phenotype. APOE3, regarded as the “neutral” isoform, appears to maintain tighter regulation of cytokine output, consistent with its comparatively balanced transcriptional state (Zhou *et al.*, 2020).

Interestingly, the discordance between cytokine secretion and transcriptional responses among the APOE lines suggests that post-transcriptional or metabolic factors also contribute to astrocyte heterogeneity. Previous work has linked APOE4 to impaired mitochondrial function, redox imbalance and metabolic inflexibility (Mahley and Huang, 2012; Wang *et al.*, 2021). These changes could limit the energy available for acute inflammatory responses, explaining why APOE4 astrocytes released high levels of RANTES but exhibited less pronounced upregulation of IL-6 and CXCL10. The heightened *C3* mRNA expression in APOE2

astrocytes may also indicate enhanced complement activation capacity, echoing findings that complement genes are among the most responsive transcripts in reactive human astrocytes (Escartin *et al.*, 2021).

Together, these data reinforce the concept that APOE genotype dictates distinct astrocytic reactivity trajectories, with APOE2 favouring a strong but potentially transient inflammatory burst, APOE3 maintaining homeostatic restraint, and APOE4 exhibiting a more chronic, dysregulated activation state. This diversity in astrocytic immune programming may underlie region- and genotype-specific vulnerability in Alzheimer's disease, influencing how effectively astrocytes clear debris, modulate microglia, or protect neurons in the face of pathology.

4.4.5. A β -induced astrocyte responses and metabolic effects

While A β deposition is a hallmark of Alzheimer's disease (AD), its direct effects on astrocytes remain complex and context-dependent. In this study, stimulation of CTX-Astros with A β alone was insufficient to induce a reactive phenotype or trigger cytokine release, consistent with previous findings that A β exposure, in isolation, evokes only mild or transient activation in astrocytes (García-Matas *et al.*, 2010; Perez-Nievas and Serrano-Pozo, 2018). The addition of IL-1 α , however, markedly enhanced inflammatory gene expression and cytokine secretion, particularly for IL-6, CXCL10, and C3. This enhanced response in the presence of IL-1 α suggests that astrocytic reactivity in AD may depend more strongly on inflammatory priming by microglial-derived cytokines than on A β exposure alone, consistent with evidence that A β in isolation often induces only limited or transient astrocyte activation (Perez-Nievas and Serrano-Pozo, 2018). This aligns with recent evidence that microglial-derived IL-1 α , TNF α , and C1q are key drivers of astrocyte activation (Liddelow *et al.*, 2017; Ferrari-Souza *et al.*, 2025).

The observation that A β_{1-40} and A β_{1-42} exert similar transcriptional effects when combined with IL-1 α supports the notion that the magnitude of astrocytic activation depends more on inflammatory context than on A β species alone. This cooperative mechanism may better reflect the *in vivo* environment, where A β accumulation

coincides with chronic microglial activation and cytokine release. Such cross-talk may promote the transition from a surveillant to a reactive astrocyte phenotype, characterised by complement activation and cytokine secretion, as observed in this study.

In contrast to their transcriptional upregulation of inflammatory genes, A β +IL-1 α -stimulated CTX-Astros did not exhibit significant changes in metabolic or functional gene expression, including *SLC1A2*, *SLC1A3* (EAAT1/2) and *GLUL*. This indicates that inflammatory activation precedes, or occurs independently of, changes in classical homeostatic astrocyte functions. Nevertheless, the Seahorse assay revealed a significant increase in both basal glycolysis and glycolytic capacity following A β_{1-40} +IL-1 α stimulation, suggesting a metabolic shift toward glycolysis, which is a hallmark of reactive astrocyte states (Allaman, Bélanger and Magistretti, 2011). Such metabolic reprogramming likely supports the heightened energetic demands of cytokine synthesis and secretion.

Collectively, these findings reinforce the idea that A β alone does not elicit a robust reactive response in human astrocytes, but instead acts synergistically with microglial-derived cytokines to drive inflammatory activation and metabolic reprogramming. This interplay underscores the importance of studying astrocyte reactivity within a multicellular context, where the convergence of amyloid pathology and neuroinflammatory cues defines disease-relevant phenotypes.

4.4.6. Differential roles of A β_{1-40} and A β_{1-42} in astrocyte reactivity

Amyloid- β peptides exist predominantly as A β_{1-40} and A β_{1-42} , which differ by only two C-terminal amino acids yet display markedly distinct biochemical and pathological properties. A β_{1-42} is more hydrophobic, aggregates more readily, and is considered the principal driver of amyloid plaque formation in Alzheimer's disease, whereas A β_{1-40} is more abundant and has been suggested to play modulatory or even protective roles under certain conditions (Benilova, Karran and De Strooper, 2012; Selkoe and Hardy, 2016).

In the present study, A β ₁₋₄₀ and A β ₁₋₄₂ elicited largely comparable transcriptional responses in CTX-Astros when combined with IL-1 α , with no clear genotype-specific preference for one peptide over the other. This suggests that, at least in human iPSC-derived astrocytes, the inflammatory context outweighs intrinsic differences between A β species in determining astrocytic activation. It is also possible that IL-1 α acts as a priming signal, sensitising astrocytes to subsequent A β exposure and thereby lowering the threshold for inflammatory activation. These findings align with reports that soluble A β , regardless of isoform, is a relatively weak astrocyte activator in isolation and requires concomitant cytokine signalling to induce robust reactivity (Perez-Nievas and Serrano-Pozo, 2018).

Nevertheless, subtle functional distinctions between A β ₁₋₄₀ and A β ₁₋₄₂ may still be relevant in vivo. A β ₁₋₄₂ has been shown to preferentially induce oxidative stress, calcium dysregulation and mitochondrial impairment in glial cells, effects that may not be fully captured in short-term or simplified in vitro paradigms (Butterfield and Boyd-Kimball, 2018). Conversely, A β ₁₋₄₀ has been reported to interfere with A β ₁₋₄₂ aggregation and toxicity, potentially acting as a competitive modulator of amyloid pathology (Jan *et al.*, 2011). Such interactions may influence astrocyte behaviour indirectly by shaping plaque composition, microglial activation and local cytokine gradients rather than directly triggering astrocyte reactivity.

The lack of differential astrocytic responses to A β ₁₋₄₀ versus A β ₁₋₄₂ oligomers in this model may also reflect the use of soluble peptide preparations rather than fibrillar or plaque-associated A β , which are more potent drivers of chronic glial activation (Condello *et al.*, 2018). Astrocytes in the AD brain are exposed to heterogeneous A β assemblies over prolonged periods, alongside sustained microglial-derived inflammatory cues. Under such conditions, isoform-specific effects of A β may emerge indirectly through differential engagement of microglia, complement activation, or APOE-dependent clearance mechanisms (Ulrich and Holtzman, 2016).

Overall, the present findings support a model in which A β isoform alone is not the dominant determinant of astrocyte reactivity, but instead acts in concert with inflammatory signals to drive disease-relevant astrocytic states. While A β ₁₋₄₂

remains central to plaque formation and neurotoxicity, both A β ₁₋₄₀ and A β ₁₋₄₂ appear capable of contributing to astrocyte activation when embedded within a pro-inflammatory milieu. This highlights the importance of considering amyloid pathology and neuroinflammation as interdependent processes, rather than isolating the effects of individual A β species, when modelling astrocyte contributions to Alzheimer's disease.

4.4.7. Limitations and future directions

Although the findings presented in this chapter provide new insights into how APOE genotype and amyloid-related stimuli influence astrocyte reactivity, several limitations should be acknowledged.

Although staining for canonical markers (GFAP, S100 β , EAAT1 and Cx43) confirmed astrocytic identity, transcriptomic profiling (e.g., RNA-seq or single-cell RNA-seq) would provide a more comprehensive assessment of astrocyte subtype composition and functional heterogeneity.

Moreover, the A β studies were performed under simplified monoculture conditions, which do not fully recapitulate the cellular complexity of the human brain. *In vivo*, astrocyte activation is shaped by interactions with microglia, neurons, and endothelial cells. In particular, microglial-derived cytokines such as IL-1 α , TNF α , and C1q are critical for eliciting neurotoxic A1-like astrocyte states (Liddelow *et al.*, 2017; Ferrari-Souza *et al.*, 2025). Co-culture or organoid models incorporating microglia and neurons would therefore provide a more physiologically relevant system to investigate APOE- and A β -dependent signalling.

Finally, the concentrations and acute exposure time used for A β stimulation may not capture the chronic, low-grade stress characteristic of Alzheimer's pathology. Long-term, repeated exposure paradigms or 3D culture systems could better mimic disease progression and reveal cumulative metabolic and inflammatory changes.

Future studies should therefore aim to: validate genotype-specific findings across independent differentiation batches and donors; integrate transcriptomic and

proteomic profiling to identify APOE-dependent signalling networks; employ multi-cellular or organoid co-culture systems to model glial cross-talk; and investigate chronic A β exposure and metabolic resilience in APOE-specific astrocytes. Such approaches would strengthen the translational relevance of these findings and help clarify how APOE genotype shapes astrocyte contributions to Alzheimer's disease pathogenesis.

4.4.8. Summary of Key Findings

Table 4.4.1 Summary of APOE genotype and A β -dependent differences in astrocyte reactivity

Experimental area	Main findings	Key markers/features affected	Overall interpretation
Astrocyte differentiation and identity	APOE2, APOE3 and APOE4 lines successfully differentiated into cortical astrocytes	GFAP, S100 β , EAAT1, Cx43, CD49f expression	APOE genotype did not impair astrocyte differentiation
Astrocyte morphology	APOE genotypes displayed distinct morphological responses to TIC stimulation	APOE4: hypertrophy; APOE2: $\uparrow$ circularity; APOE3: elongation (eccentricity) changes	APOE genotype influences structural astrocyte reactivity
Reactive astrocyte marker expression	TIC altered astrocyte marker expression differently across APOE genotypes	$\downarrow$ GFAP (all) APOE2: $\downarrow$ S100 β APOE3: $\downarrow$ S100 β APOE4: $\uparrow$ S100 β	Reactive phenotypes differ according to APOE background
EAAT1 expression	APOE4 Astros showed increased EAAT1 intensity following TIC stimulation	APOE2: $\downarrow$ EAAT1 APOE3: $\downarrow$ EAAT1 APOE4: $\uparrow$ EAAT1	Suggests altered glutamate handling in APOE4 astrocytes
Cx43 expression	APOE3 and APOE4 Astros upregulated Cx43 following stimulation, whereas APOE2 showed reduced expression	APOE2: $\downarrow$ Cx43 APOE3: $\uparrow$ Cx43 APOE4: $\uparrow$ Cx43	APOE genotype affects astrocyte communication and neurovascular signalling
Cytokine and chemokine release	APOE2 Astros displayed strongest inflammatory secretory response following TIC stimulation	$\uparrow$ IL-6, CXCL10, RANTES, GM-CSF	APOE genotype modulates inflammatory responsiveness
Inflammatory gene expression	APOE-dependent transcriptional differences observed following stimulation	$\uparrow$ <i>IL6</i> , <i>CXCL10</i> , <i>CCL5</i> , <i>CSF2</i> , <i>C3</i> , <i>ITGAM</i> but at different levels	APOE genotype shapes reactive inflammatory programmes
Functional and metabolic gene expression	Limited genotype-specific changes observed in glutamate transport and metabolic genes	<i>SLC1A2</i> , <i>SLC1A3</i> , <i>GLUL</i> not differentially expressed across APOE lines	Inflammatory changes were more pronounced than metabolic alterations
A β stimulation alone	A β 1-40 and A β 1-42 alone produced minimal astrocyte activation	Minimal cytokine or viability changes	A β alone insufficient to induce robust astrocyte reactivity
A β + IL-1 α stimulation	Combined treatment enhanced inflammatory responses	$\uparrow$ CXCL10, RANTES (protein and gene)	Cytokine priming is required for robust A β -associated astrocyte activation
A β 1-40 versus A β 1-42	Both isoforms produced comparable astrocyte responses under inflammatory conditions	Similar inflammatory profiles	Inflammatory context outweighed isoform-specific effects

4.4.9. Concluding remarks

Overall, the findings presented in this chapter demonstrate that both genetic and environmental factors, specifically APOE genotype and exposure to amyloid and cytokine stimuli, shape the inflammatory and metabolic profiles of human iPSC-derived astrocytes. The successful differentiation of isogenic APOE2, APOE3 and APOE4 astrocytes enabled a controlled comparison of genotype-specific traits, revealing that APOE2 astrocytes exhibit a more pronounced acute inflammatory response, whereas APOE3 astrocytes maintain a restrained, homeostatic phenotype, and APOE4 astrocytes display an intermediate yet dysregulated reactivity profile. These results highlight the nuanced influence of APOE on astrocyte function, extending beyond lipid metabolism to encompass immune signalling and cellular morphology.

Notably, the data also underscore that A β alone is insufficient to drive robust astrocyte reactivity, reinforcing the concept that amyloid pathology acts in concert with microglial- or cytokine-derived signals to elicit a fully reactive phenotype. The synergistic effect of A β with IL-1 α , which is manifested by increased cytokine secretion and upregulation of inflammatory genes, mirrors the cooperative glial activation observed in Alzheimer's disease, where cross-talk between microglia and astrocytes amplifies neuroinflammation.

Finally, the observed increase in glycolytic activity following A β ₁₋₄₀+IL-1 α stimulation suggests a metabolic reprogramming that supports inflammatory activation. This metabolic shift aligns with emerging evidence that astrocytes undergo bioenergetic adaptation during reactivity, coupling increased glycolysis with cytokine production. Together, these findings position astrocytes not as passive responders but as active mediators of neuroinflammatory and metabolic imbalance in Alzheimer's disease.

Future work integrating multi-cellular co-culture and chronic stimulation paradigms will be essential to determine how APOE-dependent differences in astrocytic metabolism and inflammatory signalling contribute to disease progression and neuronal vulnerability in the ageing brain.

Chapter 5. Cannabinoid Modulation of Inflammatory Responses in Human iPSC-derived Astrocytes

5. Introduction

Neuroinflammation is now recognised as a key driver of pathology across neurodegenerative diseases, and astrocytes play a central role in shaping inflammatory responses within the CNS (Sofroniew, 2015; Liddel and Barres, 2017). Earlier chapters of this thesis demonstrated how human iPSC-derived astrocytes respond to inflammatory stimuli and genetic predispositions, including IL-1 α , TNF α +IL-17A, TIC, APOE genotype, and amyloid- β oligomers. While these findings describe how inflammatory phenotypes emerge, a critical question remains: can such reactivity be therapeutically modulated? This chapter therefore shifts from characterising astrocyte reactivity to exploring pharmacological intervention as a therapeutic strategy, focusing on cannabinoids and receptor-specific modulators.

Cannabinoids have long been associated with anti-inflammatory actions in immune and glial cells (Stella, 2004; Cabral and Griffin-Thomas, 2009). Among phytocannabinoids, cannabidiol (CBD) and Δ^9 -tetrahydrocannabinol (THC) are the most widely studied. THC primarily acts via the CB₁ receptor, which is expressed by human astrocytes and regulates intracellular signalling and gliotransmission (Navarrete and Araque, 2008; Stempel *et al.*, 2016; Bernal-Chico *et al.*, 2023; Martinez Ramirez *et al.*, 2023). CBD interacts with a broader set of molecular targets, including PPAR γ , TRPV1, and GPR55, enabling diverse immunomodulatory effects (Pertwee, 2008; Pisanti *et al.*, 2017). Both cannabinoids have anti-inflammatory propensity, and have been shown to reduce cytokine release, oxidative stress and NF κ B activation in glia and immune cells (Kozela *et al.*, 2011; Martín-Moreno *et al.*, 2011; Nichols and Kaplan, 2020; Cui Sun *et al.*, 2025; Hooshmand *et al.*, 2025). However, the effects of CBD, THC, and their combination on human iPSC-derived astrocytes, particularly regarding NF κ B signalling and transcriptional inflammatory responses, remain poorly defined.

Although cannabinoid action has been studied in rodent astrocytes and microglia, human astrocytes exhibit species-specific transcriptional regulation, especially within inflammatory networks (Zhang *et al.*, 2016; J. Li *et al.*, 2019). The extent to

which cannabinoids influence hallmark astrocyte inflammatory pathways, such as IL-6, CXCL10 and CCL5 release, complement activation, and NFκB- and interferon (IFN)-linked inflammatory signalling, has not been comprehensively assessed in human iPSC-derived models.

To clarify the mechanisms underlying cannabinoid signalling, this chapter employs receptor-specific pharmacological tools, including the PPARγ agonist Pioglitazone hydrochloride, the PPARγ antagonist T0070907, and the CB₁ antagonist SR141716. PPARγ is a well-established regulator of inflammatory gene transcription (Ogawa *et al.*, 2005), and previous studies suggest CBD may exert anti-inflammatory effects partly through PPARγ activation (Esposito *et al.*, 2011). CB₁ signalling, however, influences calcium dynamics and inflammatory crosstalk within astrocytes (Navarrete and Araque, 2008). Therefore, investigating the PPARγ and CB₁ receptors facilitates a systematic evaluation of whether cannabinoid-mediated modulation depends on these pathways, or whether additional, receptor-independent mechanisms are involved.

As a mechanistic comparator, the NLRP3 inhibitor MCC950 is included in this set of experiments. MCC950 potently inhibits inflammasome activation in microglia and macrophages (Coll *et al.*, 2015), but human astrocytes possess limited inflammasome activity due to lower CD14/TLR4 expression and reduced NLRP3 priming capacity (Gustin *et al.*, 2015; Zanoni *et al.*, 2017). Evaluating MCC950 alongside cannabinoids will help to determine whether suppression of astrocyte inflammation relies primarily on inflammasome-independent pathways.

The studies presented here therefore examined whether cannabinoids attenuate IL-1α-induced astrocyte reactivity, interrogating the signalling pathways involved, and determined whether receptor-specific modulation enhances or diminishes cannabinoid efficacy. Using cytokine profiling, qPCR, NFκB nuclear translocation analysis, and receptor pharmacology, this chapter provides a comprehensive assessment of cannabinoid-mediated modulation of human astrocyte inflammatory responses.

5.1. Aims and Objectives

The specific objectives of this chapter are to:

1. Assess the potential anti-inflammatory properties of CBD, THC and the combination of both on reactive astrocytes, including the effects of the cannabinoids on viability and cytokine/chemokine release.
2. Evaluate the impact of cannabinoids on NFκB nuclear translocation as a key inflammatory signalling event.
3. Determine how cannabinoids influence the expression of inflammatory genes, including *IL6*, *CXCL10*, *CCL5*, *C3* and *CSF2*.
4. Dissect the mechanistic contribution of PPARγ and CB₁ signalling through selective pharmacological agonists and antagonists.
5. Compare cannabinoid effects with NLRP3 inhibition using MCC950 to contextualise pathway specificity in astrocyte inflammatory modulation.

Together, these analyses define how cannabinoids and receptor-targeted interventions modulate astrocyte inflammatory states and highlight potential therapeutic strategies to mitigate neuroinflammation.

5.2. Methods

5.2.1. Cell Lines Utilised

The following cell lines were used in the study described in this chapter:

- NAS2 iPSC-derived CTX-Astros were used to investigate the potential effects of cannabinoids, cannabinoid-related receptor agonists and antagonists, as well as MCC950, on astrocyte reactivity

5.2.2. Stimulation with TIC, TNF α and IL-1 α

Mature CTX-Astros were subjected to stimulation with the combination of cytokines tumour necrosis factor-alpha (TNF α ; 30 ng/mL; Cell Signalling Technology, USA), interleukin-1 alpha (IL-1 α ; 3 ng/mL; PeproTech, UK) and complement component 1q (C1q; 400 ng/mL; MyBioSource, USA), or with IL-1 α (0.3 ng/mL) alone for 24h to induce a reactive phenotype in astrocytes. Concentrations of TNF α , IL-1 α and C1q were chosen based on an already established protocol in the lab which is based on a study carried out in mouse primary astrocytes (Liddelow *et al.*, 2017). The cells were either stimulated with these cytokines by themselves or in conjunction with cannabinoids, as described in section 5.2.3. below. Post-stimulation/treatment, supernatants was collected and stored at -20°C for the LDH assay, IFN α / β assay and ELISA, and the cells were used for the alamarBlue™ assay or lysed for qPCR analysis (described in Chapter 2, Sections 2.6-2.8).

5.2.3. Treatment with Cannabinoids

Mature CTX-Astros were subjected to treatment with a range of concentrations of CBD (10 μ M, 5 μ M, 1 μ M, 100 nM, 10 nM and 1 nM; Tocris, UK) diluted in AMM and incubated (37°C, 5% CO₂) for 24h. Given the general lack of cannabinoid research on iPSC-derived astrocytes, dose-response experiments were designed to assess cell viability, cytokine release and receptor gene expression post-CBD treatment. Based on these experiments, a 5 μ M CBD concentration was chosen for the

remaining experiments, including a 30-minute pre-treatment with CBD prior to 24h stimulation and treatment. Post-treatment, media was collected and stored at -20°C for the LDH assay, IFN α / β assay and ELISA. The cells were used for the alamarBlue™ assay or lysed for qPCR analysis (as described in Chapter 2, Sections 2.6-2.8). Ethanol (EtOH) was used as the cannabinoid vehicle, with a final concentration within the treatment media under 0.1%. Positive control (20% dimethyl sulfoxide; DMSO) was used in all studies.

5.2.4. Treatment with PPAR γ and CB $_1$ Agonists and Antagonists

Mature CTX-Astros were subjected to treatment with Pioglitazone hydrochloride (1 μ M; Sigma-Aldrich, UK), T0070907 (1 μ M; Cayman Chemical, USA) or SR141617 (1 μ M; Cayman Chemical, USA). Drug concentrations were selected based on previous studies demonstrating effective modulation of PPAR γ and CB $_1$ signalling in glial and neural cell models without inducing significant toxicity (Pertwee, 2005; Kendall and Yudowski, 2017; Ji *et al.*, 2018). Pioglitazone hydrochloride, a PPAR γ agonist, was first reconstituted in EtOH before being further diluted down to 1 μ M concentration in AMM media. T0070907, a PPAR γ antagonist, was also first reconstituted in EtOH before being further diluted down to 1 μ M in AMM media. SR141617, however, was first reconstituted in DMSO before being further diluted down to 1 μ M in AMM media. All three drugs were introduced to the cells 1 h before treatment with cannabinoids, during which the cells were incubated at 30°C, 5% CO $_2$. EtOH was used as the vehicle for Pioglitazone and T0070907 while DMSO was used as the vehicle for SR141617, with final vehicle concentrations maintained below 0.1% (v/v) in the treatment media, for both EtOH and DMSO. Positive control (20% DMSO) was used in all studies.

5.2.5. Treatment with MCC950

Mature CTX-Astros were subjected to treatment with a range of concentrations of MCC950 (10 μ M, 5 μ M and 0.5 μ M; Merck Millipore, USA) diluted in AMM and

incubated (37°C, 5% CO₂) for 24 h to induce a reactive phenotype in astrocytes. Post-treatment, supernatants were collected and stored at -20°C for the LDH assay and ELISA. The cells were used for the alamarBlue™ assay or lysed for qPCR analysis (as described in Chapter 2, Sections 2.6-2.8). DMSO was used as the vehicle, with a final DMSO concentration within the treatment media under 0.1%. Positive control (20% DMSO) was used in all studies.

5.2.6. IFN α / β assay

This study was performed with the help of Prof. Eric Downer and Melody Cui Sun using the cells and established protocol from Prof. Sarah Doyler and Dr Kiva Brennan (Brennan *et al.*, 2022). The IFN α / β reporter HEK blue cell line (HEK-293, InvivoGen, USA) originate from human embryonic kidney cells and were genetically modified to express a fully activated type I IFN signalling pathway. The stimulation of HEK-293 cells with human IFN α or IFN β results in the production of secreted embryonic alkaline phosphatase (SEAP). Levels of SEAP present in the cell treatment supernatants can then be used as a reporter gene assay to measure human IFN α / β expression.

HEK-293 cells were cultured in a flask in culture media containing DMEM (Merck, USA) with 10% FBS and 1% PenStrep, supplemented with blasticidin (30 μ g/mL, InvivoGen, USA) and zeocin (100 μ g/mL; InvivoGen, USA) while the cells were in culture. Cells were incubated in a sterile, humidified conditions (37°C, 5% CO₂) and passaged when fully confluent (every 3-4 days).

When ready for plating, the cells were gently scraped using a sterile cell scraper and plated in 96-well plates at 50,000 cells/well and topped up with 180 μ L of culture media. Following plating, 20 μ L of treatment supernatants and controls were added. DMEM alone was used as a negative control. Recombinant human INF α 2 α (Milenyi Biotec, Germany) at 50 U/mL was used as a positive control. The cells were incubated overnight (37°C, 5% CO₂). The next day, 20 μ L of supernatant from each well was added to a new 96-well plate containing 180 μ L of QuantiBlue solution (InvivoGen, USA) after which the plate was incubated for 30-60 min.

Following incubation, absorbance was measured at 630 nm. Data were normalised to the 50 U/mL IFN α / β control and expressed as a percentage of the mean of all positive control wells.

5.2.7. Immunocytochemistry

ICC and subsequent analysis were performed on CTX-Astros as described in Chapter 2, Section 2.9. The primary and secondary antibodies used are listed in **Table 5.2.1** and **Table 5.2.2** below.

Table 5.2.1 Primary Antibodies Used for Immunocytochemistry.

Primary Antibody	Manufacturer	Dilution
Chicken anti-GFAP	Merck; AB5541	1:1000
Rabbit anti-NF κ B p65	Cell Signalling Technology; 8242	1:500
Mouse anti-S100 β	Sigma-Aldrich; S2532	1:1000

Table 5.2.2 Secondary Antibodies Used for Immunocytochemistry.

Secondary Antibody	Manufacturer	Dilution
Goat Anti-chicken 488	Abcam; ab150169	1:500
Donkey Anti-mouse 647	ThermoFisher Scientific	1:500
Donkey Anti-rabbit 594	ThermoFisher Scientific	1:500

5.2.8. NF κ B Translocation via ICC

To assess the NF κ B translocation from the cytoplasm into the nucleus post-stimulation with a pro-inflammatory stimulus and the potential effect of cannabinoid treatment on such signalling, ICC staining was performed as described in detail in Chapter 2, Section 2.9.1., followed by image acquisition and CellProfiler analysis as outlined in Chapter 2, Sections 2.9.2-3. To quantify NF κ B translocation, the intensity of staining for NF κ B in the nucleus and the whole cell was determined. The raw data was exported to Excel where the intensity of nuclear NF κ B staining was taken away from the whole cell NF κ B staining intensity to quantify the cytoplasmic NF κ B intensity outside the nucleus. The nuclear intensity value was then divided by the cytoplasmic intensity value to obtain the Nuc:Cyt ratio. The ratio greater than 1 was determined as nuclear translocation while a ratio below 1 was considered cytoplasmic NF κ B. The percentage of cells with NF κ B

nuclear translocation was then calculated by dividing the number of cells with translocation by the total number of cells and multiplying it by 100.

5.2.9. qPCR

qPCR analysis was performed on the control, stimulated and treated CTX-Astros as described in detail in Chapter 2, Section 2.6. All primers were manufactured by ThermoFisher Scientific, USA. VIC fluorescent reporter dye was used for GAPDH and FAM fluorescent reporter dye was used for all target primers. The endogenous and target primers used in this study are listed in **Table 5.2.3** below.

Table 5.2.3 qPCR Primers Used in the Study.

Primers	Gene Name	Assay ID
C3	complement component 3	Hs00163811_m1
IL6	interleukin 6	Hs00174131_m1
CXCL10	C-X-C motif chemokine ligand 10	Hs00171042_m1
CCL5	C-C motif chemokine ligand 5	Hs00982282_m1
TLR4	toll like receptor 4	Hs00152939_m1
CSF2	colony stimulating factor 2	Hs00929873_m1
PPARG	peroxisome proliferator activated receptor gamma	Hs01115513_m1
PPARA	peroxisome proliferator activated receptor alpha	Hs00947536_m1
CNR1	cannabinoid receptor 1	Hs01038522_s1
CNR2	cannabinoid receptor 2	Hs05019229_s1

5.3. Results

5.3.1. The assessment of potential candidates for cannabinoid treatment and pro-inflammatory stimuli

To establish an appropriate experimental framework for evaluating the therapeutic potential of cannabinoids in modulating astrocyte reactivity, we first determined the optimal concentrations of CBD and suitable pro-inflammatory stimuli, which allowed us to establish the conditions used in all subsequent experiments. A range of CBD concentrations (1 nM-10 μ M) were assessed for their impact on the viability of CTX-Astros using alamarBlue assays (**Fig. 5.3.1.A** below) which measures the metabolic activity of the cells. Data presented herein revealed that all treatment groups had significantly higher metabolic activity, when compared to cells treated with the positive control (#### $p < 0.0001$). However, the 10 μ M concentration of CBD did significantly affect the metabolic activity of CTX-Astros, when compared to the control (**** $p < 0.0001$), reducing metabolic activity by approximately 40%. Similarly, LDH activity (**Fig. 5.3.1.B**), which measures cell cytotoxicity, was significantly lower across all treatment groups compared to the positive control (#### $p < 0.0001$). The 10 μ M CBD concentration also promoted a significant increase in LDH activity, when compared to the control cells (** $p = 0.0014$). This ~15% increase in LDH activity suggests that the treatment with 10 μ M CBD negatively impacts the viability of CTX-Astros.

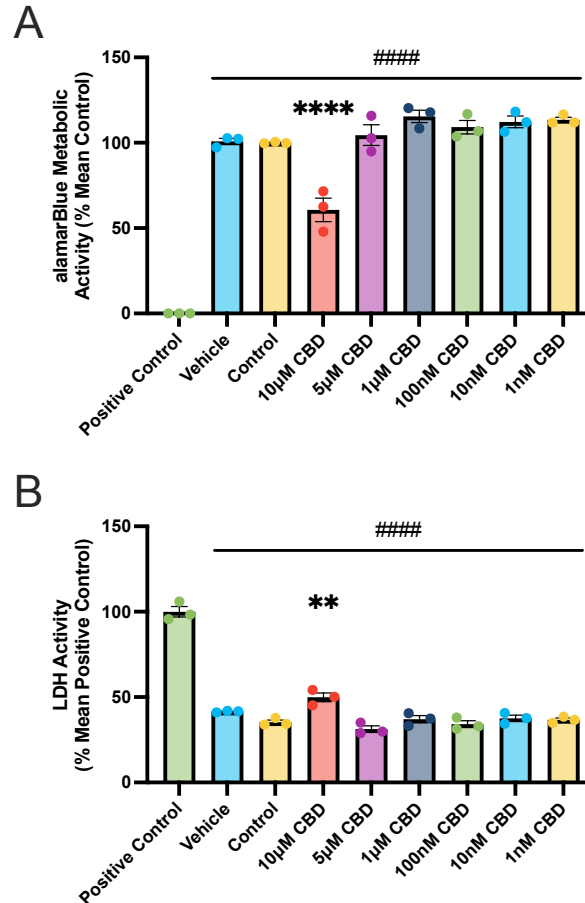


Figure 5.3.1 High concentrations of CBD reduce cell viability in CTX-Astros.

(A) AlamarBlue metabolic activity is significantly reduced by 10 μ M concentration of CBD. (B) Cell cytotoxicity, as determined by LDH assay, is significantly increased by treatment with 10 μ M CBD. No effect on cell viability was observed following treatment of cells with lower concentrations of CBD (1 nM – 5 μ M). Data expressed as mean $\pm$ SEM (N=3, r=3). ** p <0.01, **** p <0.0001 vs control; ##### p <0.0001 vs positive control. One-way ANOVA with Tukey's post hoc.

To assess the potential anti-inflammatory effect of CBD on astrocyte reactivity, CTX-Astros were stimulated with TIC. As already shown in Chapters 3 and 4, TIC is a very potent pro-inflammatory stimulant and is often used to induce reactivity in iPSC-derived astrocytes (McComish *et al.*, 2024). Indeed, stimulation of CTX-Astros with TIC significantly increased the release of IL-6 and CXCL10 compared to the control (**** p <0.0001 for both). However, when the TIC-stimulation was combined with CBD treatment, a reduction in IL-6 release was observed (Fig. 5.3.2.A). Although this reduction was present across all CBD concentrations, it was only

significant in cells treated with CBD at 1 μ M ($##p=0.0061$) and 1 nM ($#p=0.0129$) prior to TIC stimulation. A similar effect was observed with 5 μ M CBD which reduced CXCL10 release compared to TIC-stimulation alone ($p=0.0546$), as seen in **Fig. 5.3.2.B** below. Treatment of cells with CBD alone did not have any effect on IL-6 or CXCL10 release.

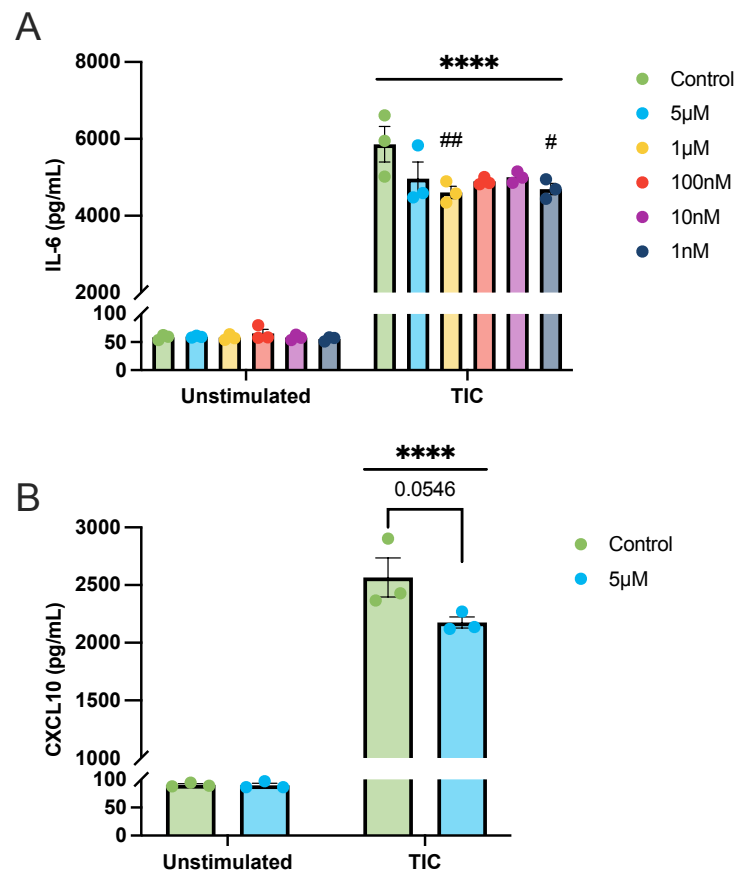


Figure 5.3.2 Treatment with CBD has the potential to reduce cytokine release at certain doses.

(A) Stimulation with TIC promotes a significant release of IL-6. However, treatment with CBD had a significant inhibitory effect on IL-6 release at 1 μ M and 1 nM concentrations. (B) 5 μ M CBD also significantly reduces IL-6 release in TIC-stimulated Astros. Overall, TIC stimulation is a potent pro-inflammatory stimulant. Data expressed as mean $\pm$ SEM ($N=3$, $r=3$). **** $p<0.0001$ vs control; # $p<0.05$, ## $p<0.01$ vs TIC stimulation. Two-way ANOVA with Tukey's post hoc.

Based on the above results, it was deduced that TIC is a very potent pro-inflammatory stimulant and is potentially masking the anti-inflammatory potential of cannabinoids. Therefore, we set out to assess the effect of treatment with IL-1 α or TNF α alone to determine a more appropriate stimulant that could sufficiently stimulate astrocytes to become more reactive thus causing them to initiate pro-inflammatory responses without masking the potential anti-inflammatory effect of the treatment with cannabinoids.

A range of IL-1 α and TNF α concentrations were assessed for their potential to stimulate astrocyte reactivity. Indeed, both ligands effectively induced the release of IL-6, CXCL10 and IFN α/β , but to a varying degree (**Fig. 5.3.3**). Stimulation with IL-1 α at final concentrations of 0.03 ng/mL (*p=0.0113), 0.3 ng/mL (****p<0.0001) and 3 ng/mL (****p<0.0001) significantly increased the release of IL-6 in CTX-Astros, with approximately 150, 2,000 and 2,750 pg/mL of IL-6 released, respectively (**Fig. 5.3.3.A**). A significant increase in CXCL10 release was observed when CTX-Astros were stimulated with 0.02 ng/mL (***p=0.0002), 0.03 ng/mL (****p<0.0001), 0.3 ng/mL (****p<0.0001) and 3 ng/mL (****p<0.0001) of IL-1 α , when compared to control cells (**Fig. 5.3.3.B**). The 0.3 ng/mL and 3 ng/mL IL-1 α concentrations also significantly increased the release of IFN α/β (**p=0.0015 and ****p<0.0001, respectively) with approximately a 30% and 75% increase determined, when compared to the control (**Fig. 5.3.3.C**).

Similarly, stimulation of CTX-Astros with a range of TNF α concentrations also induced a release of pro-inflammatory cytokines. Stimulation with TNF α at 3 ng/mL and 30 ng/mL significantly increased IL-6 release by CTX-Astros (****p<0.0001 for both) when compared to the control (**Fig. 5.3.3.D**), although the amount of IL-6 was lower than when CTX-Astros were stimulated with IL-1 α , with a range of 100-150 pg/mL of IL-6 determined. However, a substantial amount of CXCL10 was released by CTX-Astros stimulated with either of the three concentrations of TNF α (****p<0.0001 for all), as seen in **Fig. 5.3.3.E** below. The 3 ng/mL and 30 ng/mL of TNF α also induced a significant increase in IFN α/β release by CTX-Astros compared to the control. Stimulation with TNF α at 30 ng/mL increased IFN α/β

release by 90% (**** $p < 0.0001$), while stimulation with TNF α at 3 ng/mL increased IFN α/β release by approximately 20% (** $p = 0.0076$).

Overall, data herein indicate that IL-1 α stimulation was more successful at inducing IL-6 release in CTX-Astros than the stimulation with TNF α . Moreover, the 0.3 ng/mL concentration of IL-1 α , which is a 10-fold dilution of the concentration used in the TIC stimulation cocktail, significantly increased the release of CXCL10 and IFN α/β . Therefore, the 0.3 ng/mL IL-1 α concentration was chosen for further assessment of the potential therapeutic effects of cannabinoids on neuroinflammation.

Together, these results confirmed that CBD is well-tolerated by CTX-Astros up to and including a 5 μ M concentration and that a low dose of IL-1 α (0.3 ng/mL) reliably induces pro-inflammatory responses in these cells without overwhelming the potential treatment effects. These conditions were therefore carried forward for the detailed assessment of the anti-inflammatory effect(s) of cannabinoids.

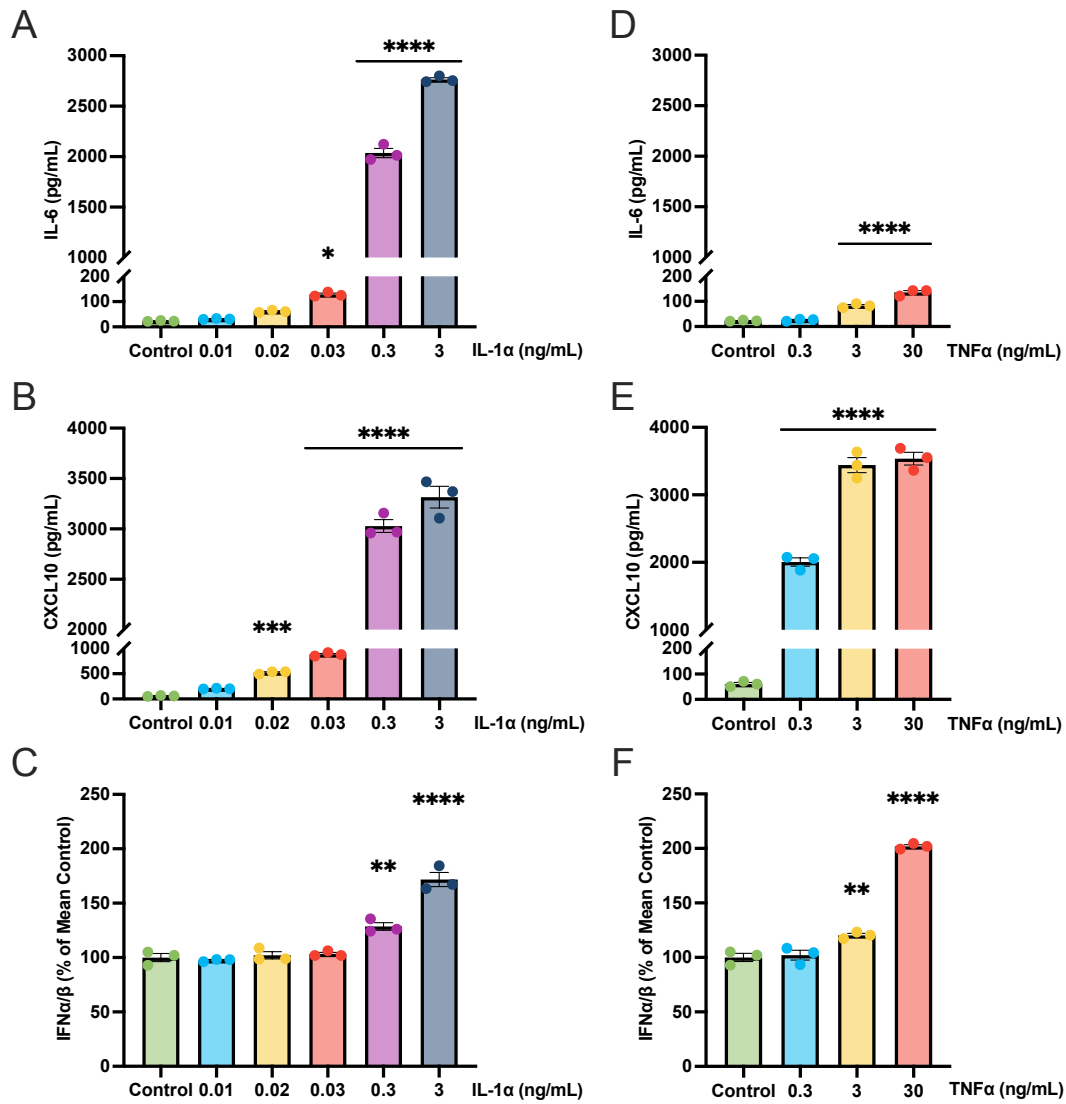


Figure 5.3.3 Stimulation with IL-1α or TNFα alone is sufficient to promote pro-inflammatory cytokine, chemokine and IFN production in CTX-Astros.

Stimulation with the 3 ng/mL or 0.3 ng/mL concentrations of IL-1α significantly increases the release of (A) IL-6, (B) CXCL10 and (C) IFNα/β by CTX-Astros. Similarly, CTX-Astros stimulated with 30 ng/mL or 3 ng/mL of TNFα also release significant levels of IL-6 (D), CXCL10 (E) and IFNα/β (F). Data expressed as mean ± SEM (N=3, r=3). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs control. One-way ANOVA with Tukey's post hoc.

5.3.2. Cannabinoids effectively reduce the reactive profile of CTX-Astros

To further assess the potential anti-inflammatory effects of cannabinoids on astrocyte reactivity, the 5 μ M concentration of CBD was supplemented with a 5 μ M concentration of THC to mimic sativex®, which is an oromucosal spray approved by European Medical Agency (EMA) for the treatment of spasticity in Multiple Sclerosis (MS), containing a roughly 1:1 combination of CBD and THC (Patti *et al.*, 2016). The treatment with CBD alone, THC alone and CBD+THC was first assessed on CTX-Astros without a pro-inflammatory stimulus to ensure that the treatments had no negative effect on cell viability or pro-inflammatory cytokine release. Indeed, treatment with THC and CBD alone and in combination had no impact on alamarBlue metabolic activity, LDH activity or the release of IL-6, CXCL10, RANTES, GM-CSF or IFN α/β , as seen in **Fig. 5.3.4** below.

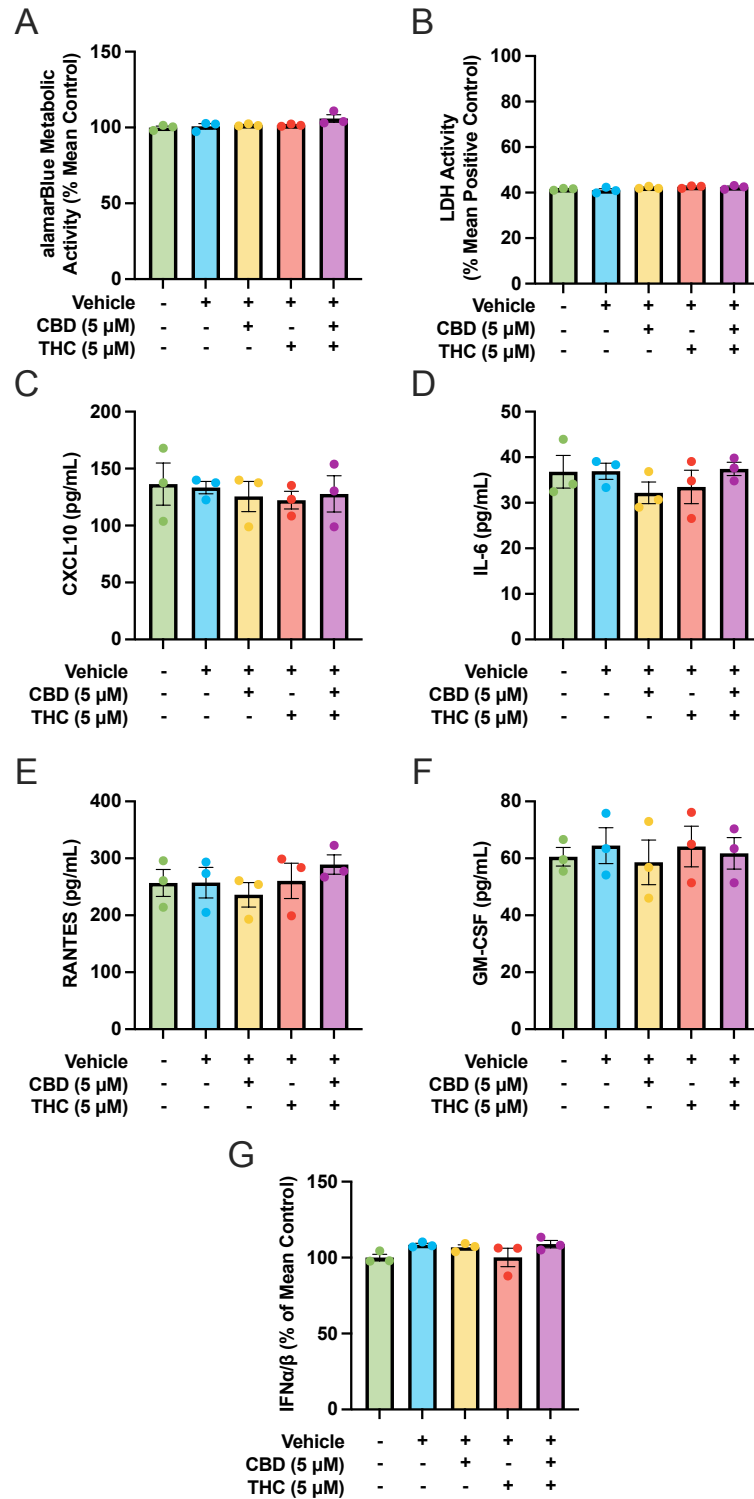


Figure 5.3.4 Treatment with cannabinoids does not have a negative effect on cell viability or cytokine release in CTX-Astros.

Treatment with 5 μ M CBD, 5 μ M THC or 1:1 combination of both does not alter the metabolic activity (A) or LDH release (B) of CTX-Astros. CTX-Astros treated with cannabinoids alone do not release significantly higher levels of IL-6 (C), CXCL10 (D), RANTES (E), GM-CSF (F) or IFN α / β (G). Data expressed as mean $\pm$ SEM (N=3, r=3). One-way ANOVA.

Furthermore, when cannabinoid treatment was introduced in IL-1 α -stimulated CTX-Astros, an anti-inflammatory effect was observed. Stimulation with 0.3 ng/mL of IL-1 α significantly increased the release of IL-6 (****p<0.0001), CXCL10 (****p<0.0001), RANTES (****p<0.0001) and GM-CSF (****p<0.0001), when compared to the control. An increase in IFN α / β release was also observed post-IL-1 α stimulation, however, this was not significant (p=0.0782). Importantly, the IL-1 α -stimulated release of IL-6 was significantly reduced by treatment with CBD (##p=0.0038; **Fig. 5.3.5.A**). Treatment with THC or CBD+THC did not affect the IL-6 release induced by IL-1 α stimulation. However, the IL-6 release in these groups was significantly increased compared to CBD treatment (++p=0.0012 for THC and +++p=0.0002 for CBD+THC).

Stimulated CTX-Astros treated with CBD (#p=0.0416), THC (##p=0.0083) or CBD+THC (###p=0.0002) released significantly less CXCL10 compared to IL-1 α stimulation alone (**Fig. 5.3.5.B**). The strongest anti-inflammatory effect was observed in CBD+THC-treated group, with the CXCL10 release significantly lower compared to treatment with CBD alone (+p=0.0144).

Treatment with CBD (##p=0.0052) or CBD+THC (#p=0.0140) also significantly decreased the release of RANTES compared to IL-1 α -stimulated CTX-Astros (**Fig. 5.3.5.C**). Although a small decrease in RANTES release was also observed post-THC treatment, this was not significant. In contrast, treatment with THC significantly decreased the release of GM-CSF (#p=0.0257) compared to IL-1 α stimulated group (**Fig. 5.3.5.D**). While a trend towards a decrease in GM-CSF release was observed in CTX-Astros treated with CBD or CBD+THC, these results were not significant. Similarly, although a trend towards a decrease in IFN α / β release was observed in CTX-Astros stimulated with cannabinoids, these results were also not significant (**Fig. 5.3.5.E**).

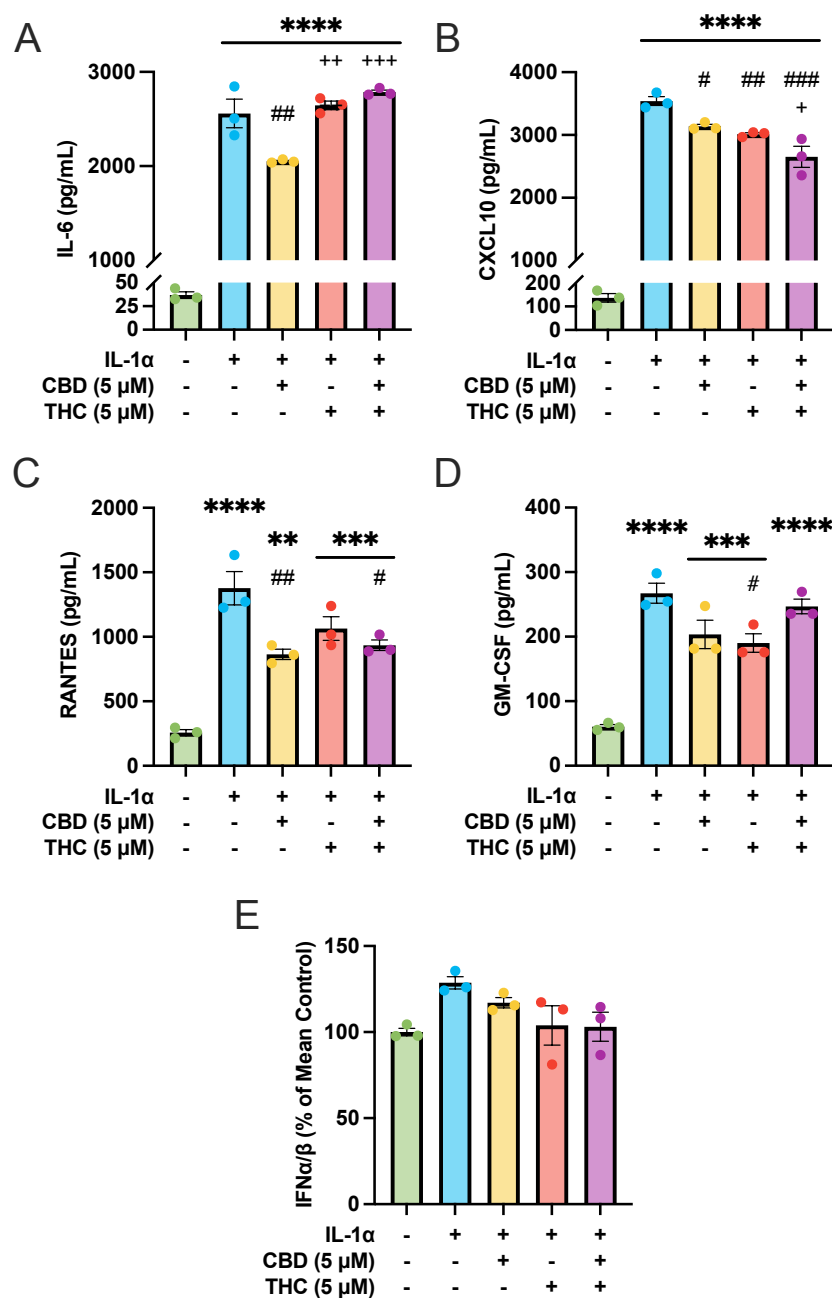


Figure 5.3.5 Cannabinoids effectively reduce the release of pro-inflammatory cytokines and chemokines post-stimulation with IL-1α.

(A) Treatment with CBD significantly reduces the release of IL-6 by CTX-Astros post-IL-1α stimulation. CXCL10 (B) release was significantly reduced by treatment with CBD, THC and CBD+THC. (C) Release of RANTES was significantly reduced by treatment with CBD and CBD+THC but not THC, while THC treatment significantly reduces the release of GM-CSF (D). (E) No significant effect on IFNα/β release was observed, regardless of stimulation or treatment. Data expressed as mean ± SEM (N=3, r=3). **p<0.01, ***p<0.001, ****p<0.0001 vs control; #p<0.05, ##p<0.01, ###p<0.001 vs IL-1α stimulation; +p<0.05, ++p<0.01, +++p<0.001 vs CBD treatment. One-way ANOVA with Tukey's post hoc.

In order to further elucidate the anti-inflammatory properties of cannabinoids, CTX-Astros were assessed for the NFκB translocation from the cytoplasm into the nucleus following IL-1α stimulation and/or cannabinoid treatment to determine whether the cytokine effects were reflected at the signalling level. As seen in **Fig. 5.3.6.A** below, ICC staining revealed a stronger presence of NFκB (red) in the nuclei of CTX-Astros stimulated with IL-1α, whereas in the control groups, NFκB presence is more equally dispersed across the cytoplasm. The quantification of NFκB translocation from the cytoplasm into the nucleus (**Fig. 5.3.6.B**) indeed confirmed that $12.89 \pm 1.04\%$ of IL-1α-stimulated CTX-Astros exhibited NFκB translocation into the nucleus, which was significantly higher compared to the $6.8 \pm 0.39\%$ in the control ($***p=0.0007$). Treating the cells with CBD or THC, but not the combination, significantly decreased the NFκB translocation compared to IL-1α stimulation alone ($####p<0.0001$ for CBD+IL-1α and $\#p=0.0263$ for THC+IL-1α). The NFκB translocation in stimulated CTX-Astros treated with CBD+THC+IL-1α was significantly higher compared to the treatment with CBD+IL-1α ($*p=0.0150$), thus making it non-effective at preventing the translocation of NFκB into the nucleus compared to the treatment of the same cells with CBD+IL-1α or THC+IL-1α.

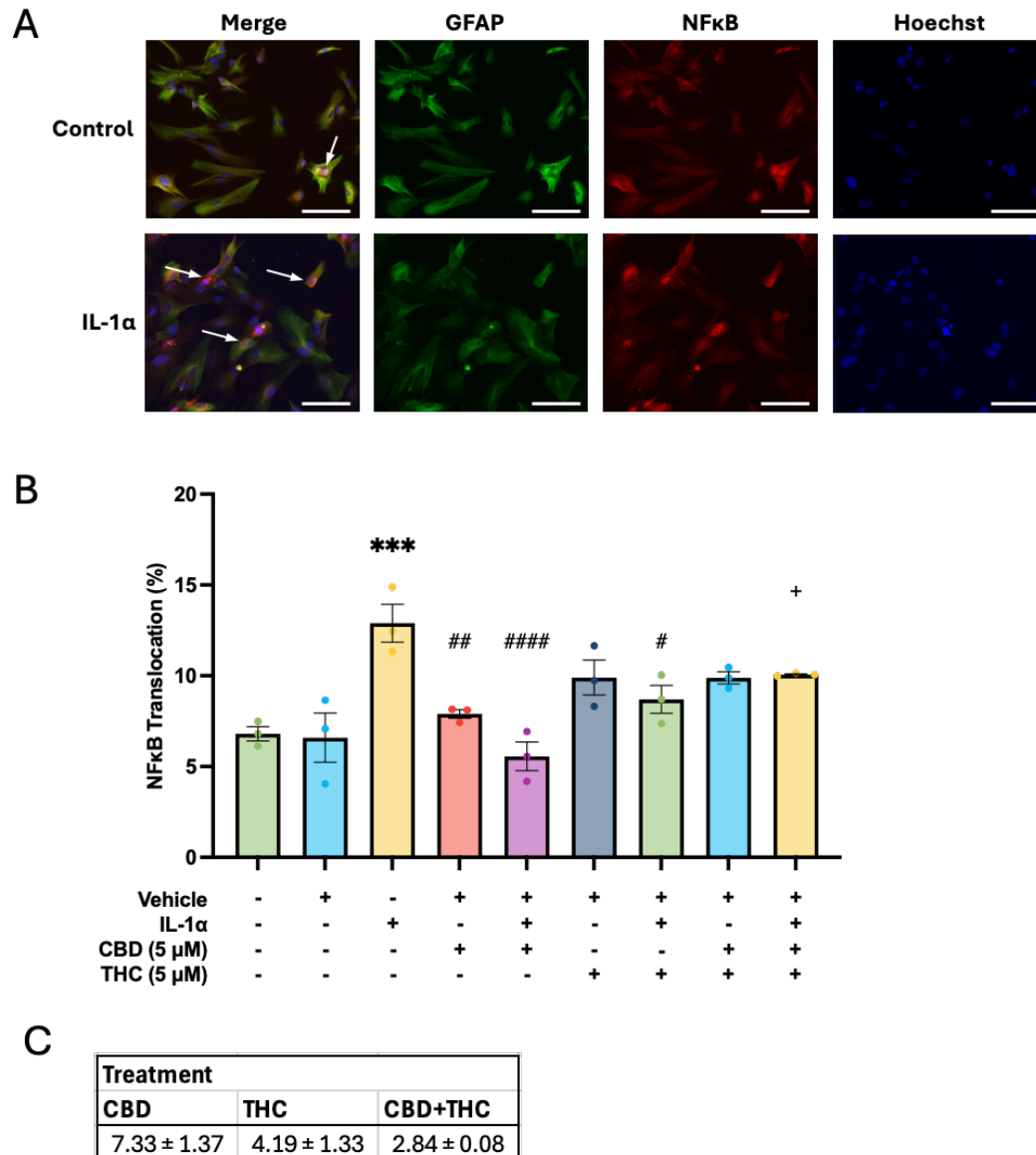


Figure 5.3.6 NFκB translocation into the nucleus is rescued by cannabinoid treatment.

(A) Representative images of control and IL-1α-stimulated CTX-Astros immunostained for GFAP (green), NFκB (red) and Hoechst (blue). IL-1α-stimulated CTX-Astros display a stronger presence of NFκB staining localised to the nucleus, compared to the control, where NFκB is localised to the cytoplasm. White arrows indicate examples of cells with nuclear NFκB translocation into the nucleus. (B) Stimulation with IL-1α significantly increased NFκB translocation into the nucleus, compared to the control, whereas treatment with CBD and THC significantly reduced the translocation compared to IL-1α stimulation. However, the combination of CBD+THC was not as effective at preventing NFκB translocation as treatment with either CBD or THC alone. (C) Quantification of the percentage decrease in IL-1α induced NFκB translocation with CBD alone, THC alone or both in combination. Scale bars: 100 μm. Magnification: x20. Data expressed as mean ± SEM (N=3; 100 cells per N). **p<0.01, ****p<0.0001 vs control; #p<0.05, #####p<0.0001 vs IL-1α stimulation. +p<0.05 vs CBD+IL-1α. One-way ANOVA with Tukey's post hoc.

The expression of genes associated with astrocyte reactivity and inflammation was also assessed in CTX-Astros stimulated with IL-1 α and treated with CBD, THC or CBD+THC (**Fig. 5.3.7**). As expected, the addition of cannabinoids to unstimulated CTX-Astros did not upregulate the mRNA expression of *IL6*, *CXCL10*, *CCL5* or *C3*. In contrast, stimulation with IL-1 α significantly increased the expression of *IL6* (**** $p < 0.0001$), *CXCL10* (**** $p < 0.0001$), *CCL5* (**** $p < 0.0001$) and *C3* (**** $p < 0.0001$) compared to the control.

Treatment with CBD significantly decreased the expression of *CXCL10* ($\#p = 0.0363$) and *CCL5* (#### $p < 0.0001$) mRNA, when compared to IL-1 α stimulation alone (**Fig. 5.3.7.B-C**). Although a trend toward a decrease in *C3* mRNA (**Fig. 5.3.7.D**) expression was also observed post-CBD treatment, this was non-significant. A non-significant trend toward an increase in *IL6* mRNA expression was also observed post-CBD treatment (**Fig. 5.3.7.A**).

Stimulated CTX-Astros treated with THC also exhibited a significant decrease in mRNA expression of *CXCL10* ($\#p = 0.0065$) and *CCL5* ($\#p = 0.0005$), when compared to IL-1 α stimulation alone (see **Fig. 5.3.7.B and C** below). Although a trend toward a decrease in *C3* mRNA expression and a trend toward an increase in *IL6* mRNA expression were also observed post-treatment with THC, this did not reach statistical significance (**Fig. 5.3.7 D and A**, respectively).

Treating IL-1 α -stimulated CTX-Astros with CBD+THC was the most effective across all treatment groups. A significant decrease in *CXCL10* ($\#p = 0.0013$) and *CCL5* (#### $p < 0.0001$) mRNA expression was observed in stimulated CTX-Astros post-CBD+THC treatment, compared to IL-1 α stimulation alone (**Fig. 5.3.7.B-C**). Moreover, the *CCL5* mRNA expression post-CBD+THC treatment was significantly lower compared to the treatment with THC only ($^{\wedge}p = 0.0118$). Again, while a trend toward a decrease in *C3* mRNA expression and a trend toward a decrease in *IL6* mRNA expression were observed post-treatment with CBD+THC, although this was not significant.

The same cells were also assessed for the expression of cannabinoid-associated receptor genes, *PPARA*, *PPARG*, *CNR1* (**Fig. 5.3.8**) and *CNR2*, which encode for the PPAR α , PPAR γ , CB $_1$ and CB $_2$ receptors, respectively. *PPARG* mRNA expression was significantly decreased post-stimulation with IL-1 α (*p=0.0402), when compared to the control (**Fig. 5.3.8.B**). A similar result was observed when CTX-Astros were treated with THC (*p=0.0401) or CBD+THC (*p=0.0452), but not CBD, compared to the control. However, the unstimulated CTX-Astros subjected to treatment with CBD (##p=0.0011) or THC (#p=0.0251) or CBD+THC (##p=0.0011) expressed significantly more *PPARG* mRNA compared to the IL-1 α -stimulated group, thus suggesting that IL-1 α stimulation inhibited the *PPARG* mRNA expression, regardless of treatment. Although a very similar trend across all treatment groups was observed in *CNR1* mRNA expression, none of these results were significant (**Fig. 5.3.8.C**). Stimulation with IL-1 α or cannabinoid treatment had no effect on *PPARA* mRNA expression (**Fig. 5.3.8.A**). The assessment of CTX-Astros for *CNR2* mRNA expression (not shown) was undetermined by the software, thus confirming that human astrocytes do not express the CB $_2$ receptor.

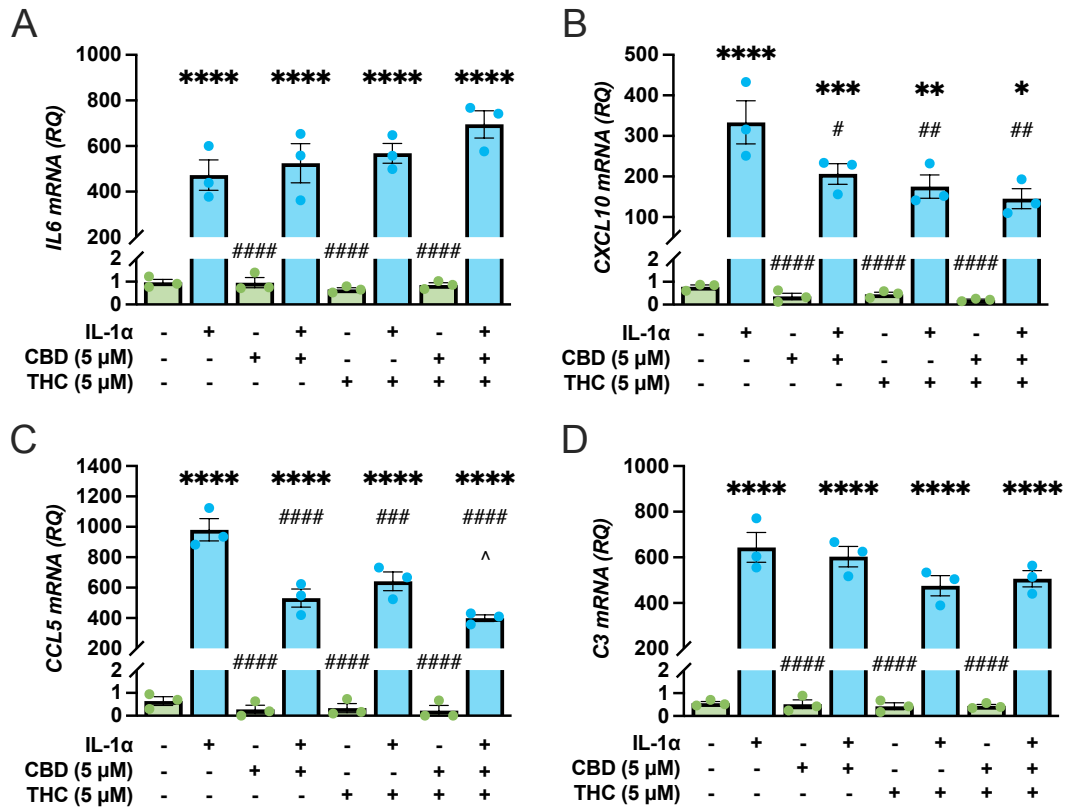


Figure 5.3.7 Treatment with cannabinoids effectively downregulates the expression of CXCL10 and CCL5 mRNA.

Treatment with CBD, THC and CBD+THC contributes to a higher expression of IL6 mRNA (**A**) compared to IL-1α stimulation alone. However, the same treatment significantly downregulates the expression of CXCL10 (**B**) and CCL5 (**C**) mRNA compared to the IL-1α stimulation. Although a trend towards a downregulation of C3 expression post-treatment was also observed, this was not significant. Data expressed as mean ± SEM (N=3, r=3). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs control; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$ vs IL-1α stimulation; ^ $p < 0.05$ vs treatment with THC. Two-way ANOVA with Tukey's post hoc.

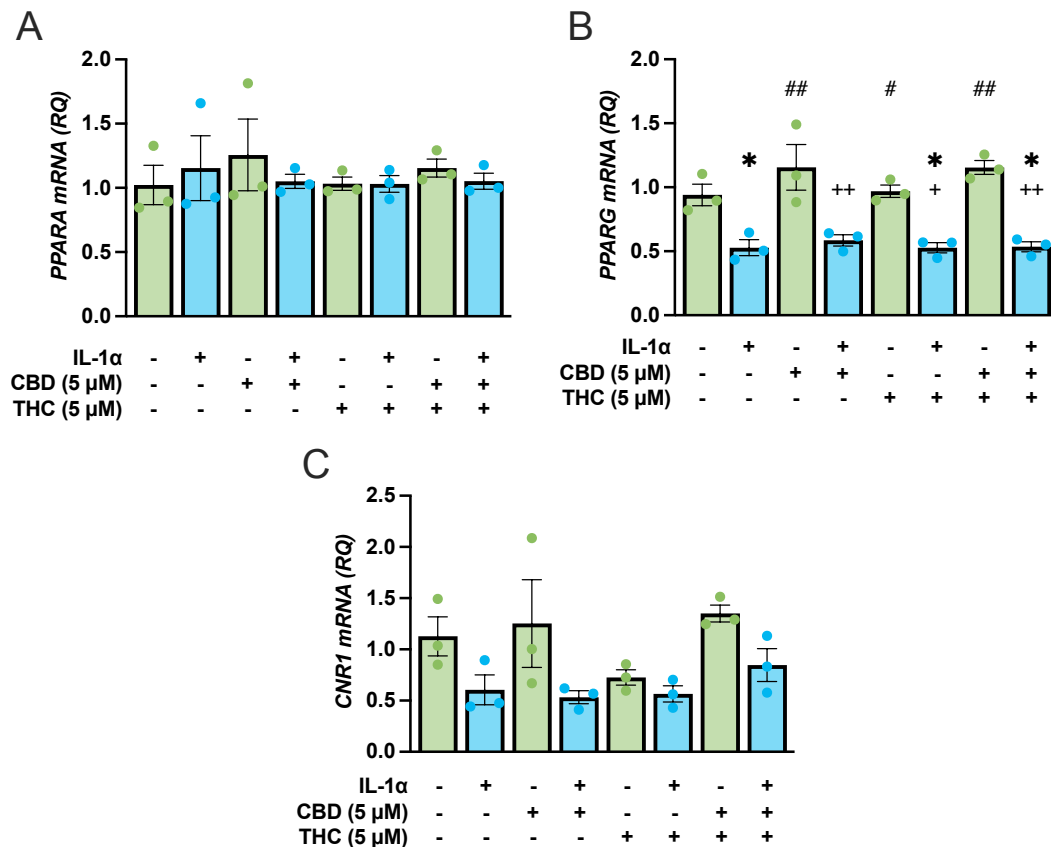


Figure 5.3.8 The expression of CB_1 and PPAR γ receptor genes is downregulated in reactive CTX-Astros.

Control and stimulated CTX-Astros treated with cannabinoids were assessed for their expression of PPAR α , PPAR γ and CNR1 mRNA. Although the stimulation or the treatment did not have an effect on PPAR α (A) mRNA expression, stimulation with IL-1 α significantly downregulated the expression of PPAR γ mRNA (B) in CTX-Astros treated with cannabinoids compared to the control and treatment alone. A similar trend was observed in CNR1 mRNA (C) expression, but this was non-significant. Data expressed as mean $\pm$ SEM (N=3, r=3). * p <0.05 vs control; # p <0.05, ## p <0.01 vs IL-1 α stimulation; + p <0.05, ++ p <0.01 vs treatment alone. Two-way ANOVA with Tukey's post hoc.

Together, these experiments demonstrate that cannabinoids consistently reduce several markers of IL-1 α -induced reactivity in CTX-Astros. CBD, THC, and the CBD+THC combination decreased the release of multiple pro-inflammatory cytokines and chemokines, reduced NF κ B nuclear translocation, and downregulated CXCL10 and CCL5 mRNA expression. Although the magnitude of the effect varied between treatments, all three cannabinoids attenuated at least part of the IL-1 α -induced reactive response. IL-1 α stimulation also altered the

expression of the PPARG and CNR1 genes, while PPARA remained unchanged and CNR2 was undetected. These findings establish the overall anti-inflammatory profile of cannabinoids across multiple assays in CTX-Astros.

5.3.3. PPAR γ receptor agonist and antagonist as well as CB $_1$ receptor antagonist effectively modulate inflammatory responses in reactive astrocytes

To determine whether pharmacological targeting of PPAR γ and CB $_1$ receptors could modulate astrocyte reactivity independently of cannabinoids, IL-1 α -stimulated CTX-Astros were treated with Pioglitazone, T007 and SR14 without the addition of cannabinoids. We first quantified the effects of these compounds on cytokine and chemokine release and then assessed their impact on the expression of inflammatory and receptor-associated genes. This allowed us to evaluate whether direct modulation of cannabinoid-related receptors is sufficient to alter the reactive profile of CTX-Astros. Prior to IL-1 α stimulation, the effect of treatment with Pioglitazone, T007 and SR14 alone was assessed. Treatment with Pioglitazone, T007 or SR14 alone did not significantly affect cell viability, cytokine/chemokine release or inflammatory gene expression (**Appendix 1, Supplemental Fig.1-6**).

IL-1 α -stimulated CTX-Astros released significantly higher levels of CXCL10, IL-6, RANTES and GM-CSF, when compared to the control (**** $p < 0.0001$ for all). However, once treated with Pioglitazone, a significant reduction in CXCL10 (## $p = 0.0040$), RANTES (### $p = 0.0004$) and GM-CSF (# $p = 0.0338$) production was observed when compared to IL-1 α stimulation (**** $p < 0.0001$ for CXCL10, ** $p = 0.0011$ for RANTES and *** $p = 0.0007$ for GM-CSF; **Fig. 5.3.9.A,C-D**). Treatment with Pioglitazone had no effect on IL-6 production in IL-1 α -stimulated CTX-Astros (**Fig. 5.3.9.B**).

Treatment with T0070907 alone also had a significant effect on cytokine and chemokine release by IL-1 α -stimulated CTX-Astros. A significant decrease in the release of CXCL10 (#### $p < 0.0001$), IL-6 (#### $p < 0.0001$) and GM-CSF (## $p = 0.0051$)

was observed when compared to IL-1 α alone, and although a trend towards a decrease in RANTES release was also seen, this result was not significant (**Fig. 5.3.9.E-H**). Nevertheless, stimulated CTX-Astros treated with T007 still expressed significantly higher levels of CXCL10 (****p<0.0001), IL-6 (****p<0.0001), RANTES (***p=0.0009) and GM-CSF (***p=0.0009), when compared to the control.

A similar type of a response was also observed when stimulated CTX-Astros were treated with SR141716. Once treated with SR14, CTX-Astros released significantly lower levels of CXCL10 (####p<0.0001), RANTES (#p=0.0374) and GM-CSF (#p=0.0138) compared to cells treated with IL-1 α alone (**Fig. 5.3.9.I,K-L**). In parallel with other treatments, this decrease in cytokine and chemokine release was still significantly higher compared to the control (****p<0.0001 for CXCL10, IL-6 and RANTES and **p=0.0035 for GM-CSF).

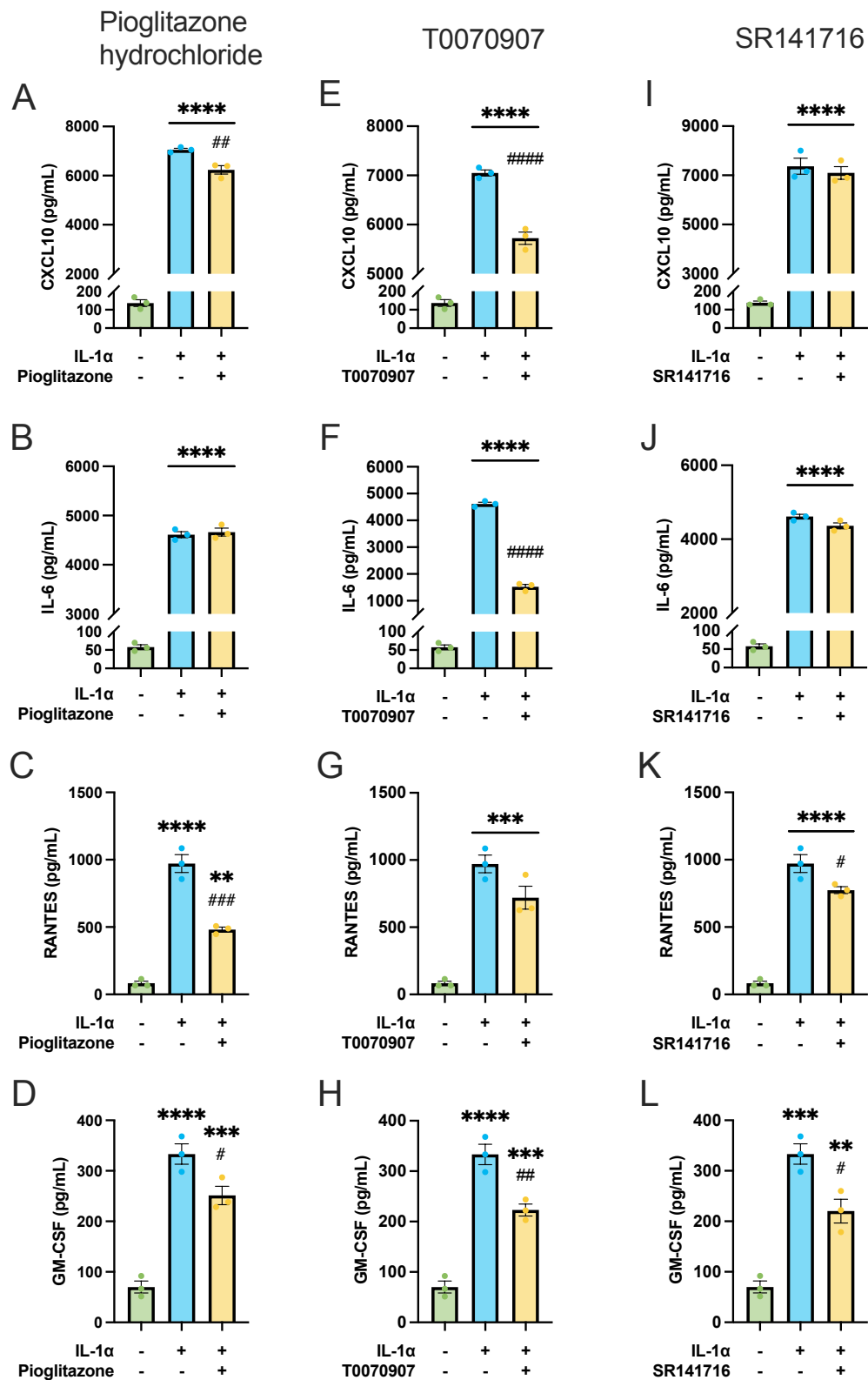


Figure 5.3.9 Cannabinoid-related receptor (ant)agonists effectively reduce pro-inflammatory cytokine release in CTX-Astros.

Stimulation with IL-1α significantly increased the release of pro-inflammatory cytokines and chemokines by CTX-Astros (A-L). Treatment with Pioglitazone hydrochloride significantly reduced

the release of CXCL10 (**A**), RANTES (**C**) and GM-CSF (**D**), but had no effect on IL-6 release in IL-1 α -stimulated CTX-Astros. Treatment of stimulated CTX-Astros with T007 significantly reduced the release of CXCL10 (**E**), IL-6 (**F**) and GM-CSF (**H**). Although a trend toward a decrease of RANTES release was also observed post-T007 treatment, this result was not significant. Similar to Pio, the treatment with SR14 also significantly reduced the release of CXCL10 (**I**), RANTES (**K**) and GM-CSF (**L**) by CTX-Astros stimulated with IL-1 α . (**J**) A trend toward lower IL-6 release was also observed post-SR14 treatment, but this result was not significant. Data expressed as mean $\pm$ SEM (N=3, r=3). **p<0.01, ***p<0.001, ****p<0.0001 vs control; #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001 vs IL-1 α stimulation. One-way ANOVA with Tukey's post hoc.

The treatment with Pioglitazone of IL-1 α -stimulated CTX-Astros resulted in a varied response in terms of inflammatory gene expression. The expression of CCL5 mRNA was the only inflammatory gene where a significant downregulation post-Pio treatment was observed, when compared to IL-1 α stimulation (####p<0.0001; **Fig. 5.3.10.C**). A trend towards reduced CXCL10, IL6, CSF2 and TLR4 mRNA expression was observed in Pioglitazone-treated groups, but these results were not significant (**Fig. 5.3.10.A-B, D and F**). A trend towards an increase of C3 mRNA expression in Pio-treated group was also observed, but this result was also not statistically significant (**Fig. 5.3.10.E**). Overall, the inflammatory gene expression was still significantly higher compared to the control regardless of treatment (**p=0.0036 for CXCL10, *p=0.0220 for IL6, ****p<0.0001 for CCL5 and C3, ***p=0.0005 for CSF2 and TLR4).

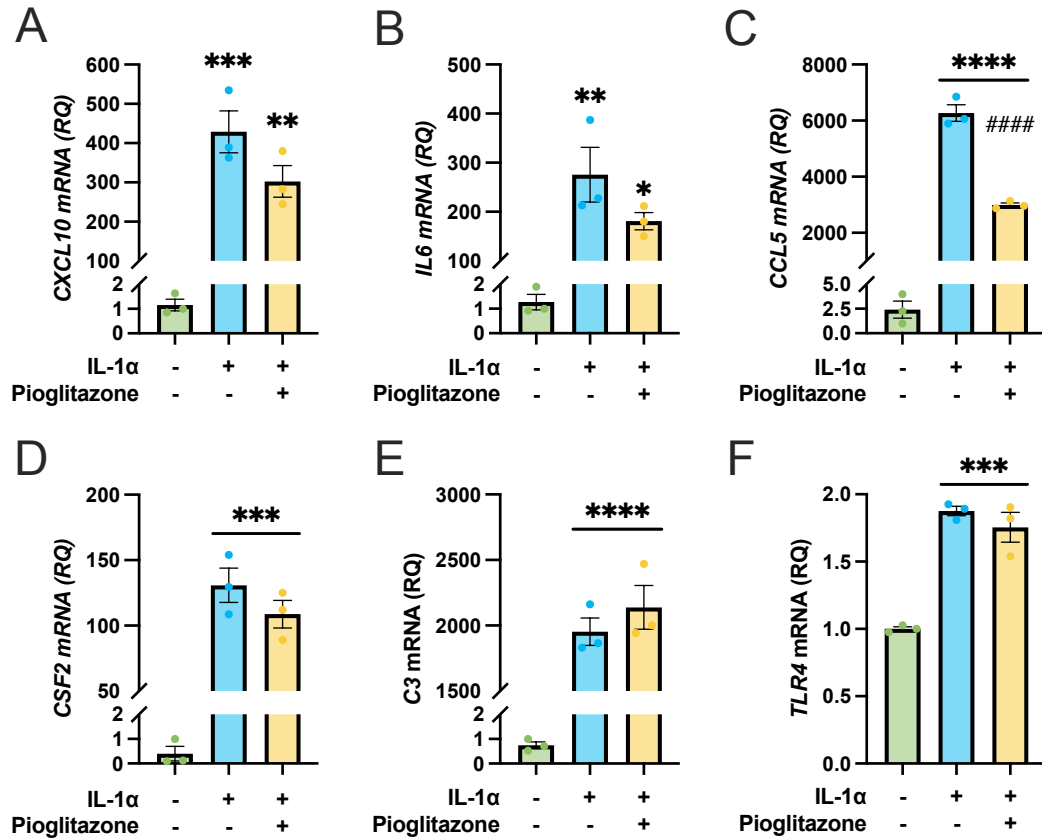


Figure 5.3.10 Treatment with Pioglitazone hydrochloride had a varied effect on inflammatory gene expression in CTX-Astros.

Stimulation of CTX-Astros with IL-1 α significantly increased the expression of inflammatory genes (A-F). However, when the same astrocytes were treated with Pioglitazone, this was somewhat reversed. (C) CCL5 mRNA expression was significantly reduced in CTX-Astros treated with Pioglitazone compared to stimulation alone. The expression of CXCL10 (A), IL6 (B), CSF2 (D) and TLR4 (F) by stimulated CTX-Astros also trended towards a decrease once treated with Pioglitazone, although this was not significant. (E) A trend towards an upregulation of C3 mRNA was also observed post-pioglitazone treatment, but this was not significant. Data expressed as mean $\pm$ SEM (N=3, r=3). * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001 vs control; #### p <0.0001 vs IL-1 α stimulation. One-way ANOVA with Tukey's post hoc.

CTX-Astros that were treated with T007 were also assessed for cellular expression of inflammatory genes. Data presented herein indicate a significant decrease in the mRNA expression of CXCL 10 (## p =0.0068), IL6 (## p =0.0096), CCL5 (## p =0.0059), CSF2 (### p =0.0001) and TLR4 (#### p <0.0001) in stimulated CTX-Astros post-T007 treatment, when compared to the stimulation alone (Fig. 5.3.11.A-D and F). A slight

decrease in C3 mRNA expression was also determined post-T007 treatment, but this was not significant (**Fig. 5.3.11.E**). Despite significant downregulation of mRNA compared to IL-1 α stimulation, the expression of *CXCL10* (** $p=0.0090$), *CCL5* (**** $p<0.0001$) and *TLR4* (* $p=0.0132$) was still significantly higher in T007-treated CTX-Astros when compared to the control cells.

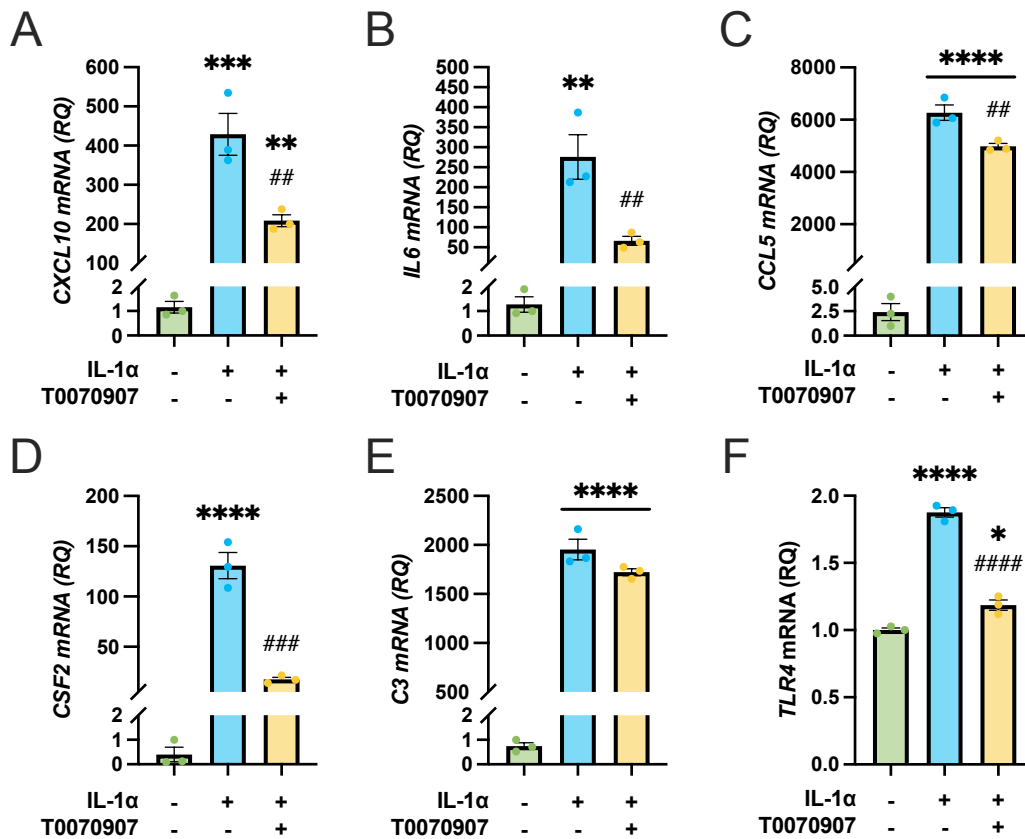


Figure 5.3.11 Treatment with T0070907 effectively downregulates the expression of inflammatory genes in CTX-Astros.

Stimulation of CTX-Astros with IL-1 α significantly increased the expression of inflammatory genes (**A-F**). However, the treatment of the same astrocytes with T007 significantly downregulated the expression of the same genes. Stimulated CTX-Astros treated with T007 exhibited a significantly reduced mRNA expression of *CXCL10* (**A**), *IL6* (**B**), *CCL5* (**C**), *CSF2* (**D**) and *TLR4* (**F**), compared to IL-1 α stimulation alone. (**E**) Although a trend towards a decrease in *C3* mRNA expression was also observed post-T007 treatment, this result was not significant. Data expressed as mean $\pm$ SEM ($N=3$, $r=3$). * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ vs control; ## $p<0.01$, ### $p<0.001$, #### $p<0.0001$ vs IL-1 α stimulation. One-way ANOVA with Tukey's post hoc.

Unlike the treatment with Pioglitazone and T007, treatment of IL-1 α stimulated CTX-Astros with SR14 alone did not have any significant effect on inflammatory gene expression when compared to IL-1 α stimulation alone, with the exception of the expression of the C3 gene, where a significant upregulation of mRNA was observed post-SR14 treatment (##p=0.0090) compared to IL-1 α -stimulated group (**Fig. 5.3.12.E**). Although a small and insignificant trend towards downregulation of the other inflammatory genes was observed, the mRNA expression of CXCL10 (**p=0.0021), IL6 (**p=0.0057), CCL5 (****p<0.0001), CSF2 (**p=0.0028), C3 (**p=0.0090) and TLR4 (**p=0.0003) was still significantly higher in SR14-treated groups, when compared to the control (**Fig. 5.3.12.A-F**). Overall, the treatment with T0070907 alone was more successful at modulating the expression of inflammatory genes post-IL-1 α stimulation than the treatments with Pioglitazone or SR14.

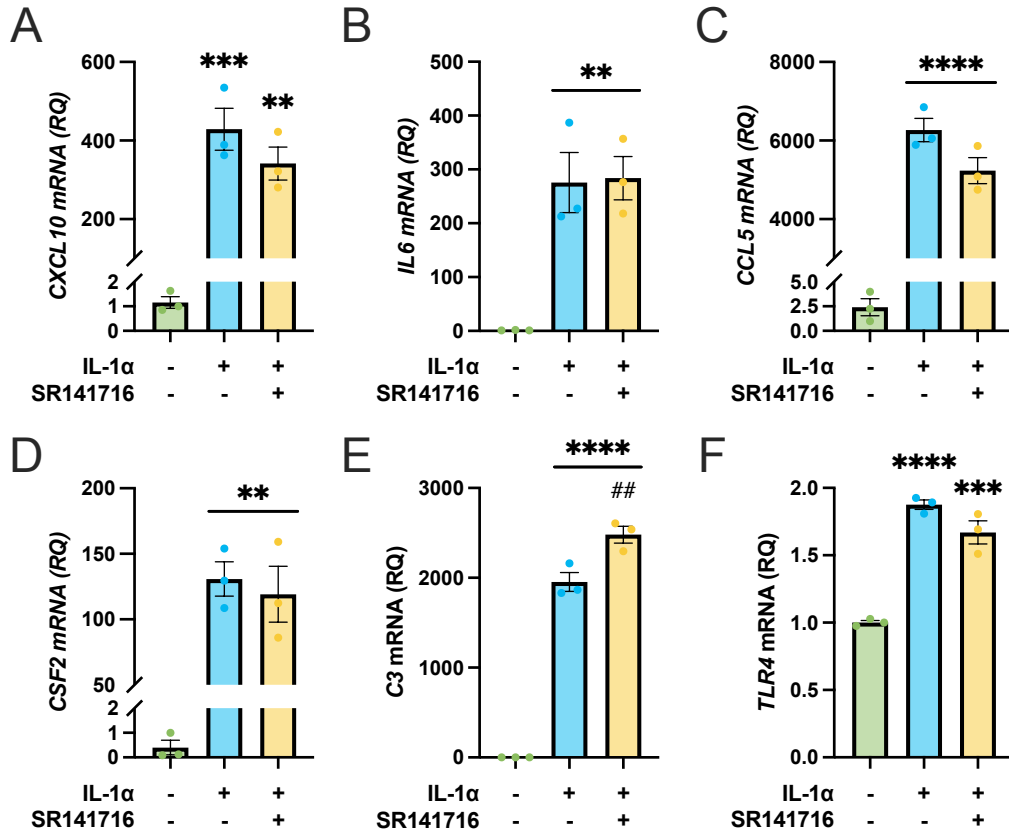


Figure 5.3.12 Treatment with SR14 does not downregulate the inflammatory gene expression in CTX-Astros.

Stimulation of CTX-Astros with IL-1 α significantly increased the expression of inflammatory genes (A-F). Although there was a trend towards downregulation of CXCL10 (A), CCL5 (C), CSF2 (D) and TLR4 (F) mRNA expression post-treatment with SR14, this was not significant. (E) Moreover, the treatment with SR14 further increased the expression of C3 mRNA in stimulated CTX-Astros. (B) There were no differences observed in IL6 mRNA expression between the IL-1 α stimulated group and the group treated with SR14. Data expressed as mean $\pm$ SEM (N=3, r=3). **p<0.01, ***p<0.001, ****p<0.0001 vs control; ##p<0.01 vs IL-1 α stimulation. One-way ANOVA with Tukey's post hoc.

Stimulation with IL-1 α significantly downregulated the mRNA expression of *CNR1* (*p=0.0427) and *PPARG* (**p=0.0046) but not *PPARA*, compared to the control. The treatments with Pio, T007 and SR14 alone in IL-1 α -stimulated CTX-Astros were also assessed for their ability to modulate the mRNA expression of *CNR1*, *PPARG* and *PPARA* (see **Fig.5.3.13** below). Indeed, the treatment with Pio significantly increased the expression of *PPARG* mRNA (##p=0.0067) compared to IL-1 α (**Fig. 5.3.13.B**), but it did not have any effect on *CNR1* and *PPARA* mRNA expression. In contrast, treatment with T007 significantly downregulated the expression of *CNR1* mRNA and significantly increased the *PPARA* mRNA expression compared to IL-1 α (**Fig. 5.3.13.D and F**). Additionally, a trend in upregulation of *PPARG* mRNA was observed in T007-treated CTX-Astros, although this was non-significant (**Fig. 5.3.13.E**). Treatment with SR14 did not have any significant effect on *CNR1*, *PPARG* or *PPARA* mRNA expression compared to IL-1 α (**Fig. 5.3.13.G-I**).

Overall, these data show that PPAR γ - and CB $_1$ -targeting compounds can partially modulate inflammatory responses in IL-1 α -stimulated CTX-Astros, even in the absence of cannabinoids. Pioglitazone produced modest reduction in selected cytokines and a clear downregulation of *CCL5* mRNA, but its effects on other inflammatory genes were limited. In contrast, T0070907 exerted a more robust anti-inflammatory profile, thus significantly reducing multiple cytokines and consistently downregulating *CXCL10*, *IL6*, *CCL5*, *CSF2* and *TLR4* expression, while also increasing *PPARA* and *PPARG* mRNA. SR141716 decreased cytokine release at the protein level, but had minimal impact on inflammatory gene expression and was associated with increased *C3* mRNA. Together, these findings indicate that direct modulation of PPAR signalling, particularly via T0070907, is more effective at dampening astrocyte reactivity than CB $_1$ antagonism alone.

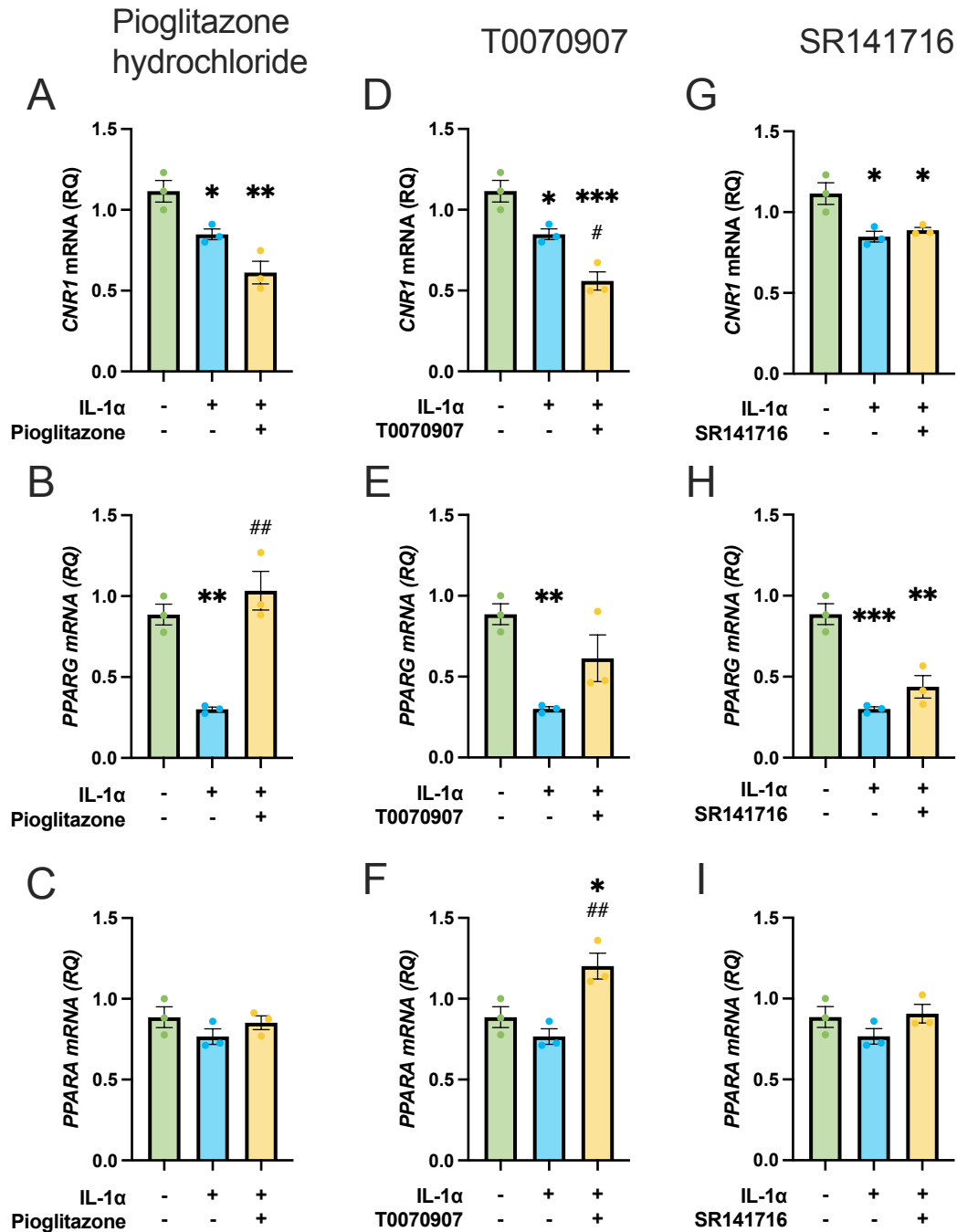


Figure 5.3.13 Treatment with cannabinoid-related receptor (ant)agonists had a varied effect on the expression of the receptor genes in CTX-Astros.

Stimulation with IL-1 α significantly reduced the expression of CNR1 (A, D, G) and PPARG (B, E, H) mRNA, but not PPARA (C, F, I) mRNA. (A) Treatment with Pioglitazone further downregulated the CNR1 mRNA expression compared to the control. However, the expression of PPARG mRNA (B) in Pioglitazone-treated group was significantly upregulated compared to stimulation alone. (C) No changes were observed in PPARA mRNA expression across all groups. (D) Treatment with T007 also significantly downregulated CNR1 mRNA expression, compared to control and IL-1 α -stimulated

groups. **(E)** The partial inhibition of PPARG mRNA expression induced by IL-1 α stimulation was reversed by the treatment with T007. **(F)** T007 also significantly upregulated the expression of PPARA mRNA, compared to the control and IL-1 α stimulation. In contrast, SR14 treatment did not have any effect on CNR1 **(G)**, PPARG **(H)** or PPARA **(I)** mRNA expression compared to IL-1 α -stimulated group. However, CNR1 and PPARG mRNA expression were significantly lower in SR14-treated groups compared to the control. Data expressed as mean $\pm$ SEM (N=3, r=3). ** p <0.01, *** p <0.001 vs control; # p <0.05, ## p <0.01 vs IL-1 α stimulation. One-way ANOVA with Tukey's post hoc.

5.3.4. Utilisation of cannabinoid-related receptor (ant)agonists further enhances the anti-inflammatory potential of cannabinoids

In order to further elucidate the involvement of the PPAR γ and CB $_1$ receptors in the anti-inflammatory potential of cannabinoids, PPAR γ agonist (Pioglitazone hydrochloride; Pio), PPAR γ antagonist (T0070907; T007) and CB $_1$ receptor antagonist (SR141716; SR14) were utilised in combination with CBD or THC or CBD+THC, and the cytokine/chemokine levels as well as inflammatory gene expression were measured to determine how pharmacological modulation of PPAR γ and CB $_1$ signalling influences cannabinoid responses. The treatment with cannabinoids and (ant)agonists without IL-1 α stimulation were first tested and the combined treatment did not have a significant effect on cell viability, cytokine/chemokine release or gene expression compared to the control (**Appendix 1, Supplemental Fig.1-6**). The same treatment regime was then used along IL-1 α stimulation of CTX-Astros. As in previous assays, stimulating CTX-Astros with 0.3 ng/mL of IL-1 α resulted in a significantly increased expression of CXCL10 (**** p <0.0001), IL-6 (**** p <0.0001), RANTES (**** p <0.0001) and GM-CSF (**** p <0.0001) compared to the control (**Fig. 5.3.14**).

Treating these stimulated CTX-Astros with CBD alone significantly lowered the release of CXCL10 (# p =0.0120), IL-6 (### p =0.0002) and RANTES (#### p <0.0001), but not GM-CSF (p =0.0561), compared to the stimulation alone. The treatment with THC significantly lowered the release of CXCL10 (## p =0.0016), IL-6 (# p =0.0217), RANTES (#### p <0.0001) and GM-CSF (# p =0.0218). Treating the stimulated CTX-

Astros with the combination of CBD+THC resulted in an even more significant decrease in CXCL10 (####p<0.0001), IL-6 (#p=0.0143) and RANTES (####p<0.0001) but not the GM-CSF, compared to the stimulation alone (**Fig. 5.3.14.A-D**).

When the IL-1 α -stimulated CTX-Astros were treated with a combination of cannabinoids and Pio, a significant decrease of CXCL10 (**Fig. 5.3.14.A**) and RANTES (**Fig. 5.3.14.C**), but not IL-6 (**Fig. 5.3.14.B**) or GM-CSF (**Fig. 5.3.14.D**) was observed. Treatment with CBD+Pio (#p=0.0120), THC+Pio (##p=0.0044) and CBD+THC+Pio (####p<0.0001) significantly reduced the CXCL10 release compared to IL-1 α -stimulated group, with the CBD+THC+Pio treatment being most effective, where CXCL10 release was significantly lower compared to CBD+Pio (+++p=0.0003) and THC+Pio (^^p=0.0008) treated cells, as seen in **Fig. 5.3.14.A** below. The treatment with CBD+THC+Pio was not as effective at reducing the CXCL10 release as the treatment with CBD+THC alone (&&p=0.0010).

A very similar effect was observed in RANTES release when stimulated CTX-Astros were treated with CBD+Pio (####p<0.0001), THC+Pio (####p<0.0001) and CBD+THC+Pio (####p<0.0001) where the release was significantly reduced compared to IL-1 α stimulation alone (**Fig. 5.3.14.C**). The combination of CBD+THC+Pio was also the most effective at reducing the levels of RANTES in stimulated CTX-Astros compared to CBD+Pio (+p=0.0268) and THC+Pio (^p=0.0073).

The release of GM-CSF by stimulated CTX-Astros remained unaffected when the cannabinoids were combined with Pioglitazone (**Fig. 5.3.14.D**), despite trending towards a lower GM-CSF release compared to stimulation alone.

The combined treatments with cannabinoids+Pio were also unsuccessful at lowering the release of IL-6 (**Fig. 5.3.14.B**). Moreover, release of IL-6 post THC+Pio (+p=0.0189) treatment was also significantly higher compared to the treatment with CBD+Pio. Overall, the addition of Pioglitazone to the cannabinoids resulted in a significantly higher IL-6 release compared to the treatment with

cannabinoids alone ($p=0.0021$ for CBD, $p=0.0001$ for THC and $p=0.0009$ for CBD+THC).

Combining cannabinoids with T007 also successfully downregulated the release of pro-inflammatory cytokines and chemokines in stimulated CTX-Astros (**Fig. 5.3.14.E-H**). A significant reduction in CXCL10 release was observed post-treatment with CBD+T007 ($p<0.0001$), THC+T007 ($p<0.0001$) and CBD+THC+T007 ($p<0.0001$) compared to IL-1 α -stimulated group, with the CBD+THC+T007 treatment resulting in a lower CXCL10 release compared to CBD+T007 ($p=0.0003$) and THC+T007 ($p<0.0001$), as seen in **Fig. 5.3.14.E**. Moreover, the addition of T007 to cannabinoid treatment further enhanced their anti-inflammatory properties, with a significantly lower CXCL10 release compared to CBD alone ($p<0.0001$), THC alone ($p<0.0001$) and CBD+THC ($p=0.0024$).

Unlike in the case of combining cannabinoids with Pio, the addition of T007 to CBD ($p<0.0001$), THC ($p<0.0001$) and CBD+THC ($p<0.0001$) resulted in a significant decrease in IL-6 release compared to stimulated CTX-Astros (**Fig. 5.3.14.F**). No significant differences were observed between these treatment groups, but the overall use of cannabinoids and T007 resulted in a significantly lower IL-6 release compared to the cannabinoids alone ($p<0.0001$ for all three).

Treatment with CBD+T007 ($p<0.0001$), THC+T007 ($p<0.0001$) and CBD+THC+T007 ($p<0.0001$) also significantly decreased the release of RANTES by CTX-Astros, compared to stimulation alone. Moreover, a more potent decrease was observed post-treatment with CBD+THC+T007 compared to CBD+T007 ($p=0.0393$) but not THC+T007. The addition of T007 to THC resulted in a significantly decreased RANTES release compared to THC alone ($p=0.0125$).

A very similar effect was observed in GM-CSF release where all three cannabinoids combined with T007 significantly reduced the release compared to stimulation alone ($p=0.0002$ for CBD+T007, $p=0.0001$ for THC+T007 and $p<0.0001$ for CBD+THC+T007). Again, a trend toward the most potent decrease was observed

in the CBD+THC+T007 treatment, but this was non-significant. Although the combination of cannabinoids and T007 trended towards a more significant GM-CSF release compared to cannabinoids alone, this was significant only in CBD+THC+T007 ($p=0.0123$).

Treating stimulated CTX-Astros with cannabinoids and SR14 successfully downregulated the release of CXCL10, IL-6, RANTES and GM-CSF, as seen in **Fig. 5.3.14.I-L** below. CTX-Astros treated with CBD+SR14 ($####p<0.0001$), THC+SR14 ($###p=0.0006$) and CBD+THC+SR14 ($####p<0.0001$) released significantly less CXCL10 compared to the stimulation alone (**Fig. 5.3.14.I**). The treatment with CBD+THC+SR14 also had the most potent anti-inflammatory effect on CXCL10 release compared to CBD+SR14 ($+++p=0.0201$) and THC+SR14 ($^^^^p<0.0001$). The addition of SR14 to CBD ($&p=0.0349$) and CBD+THC ($&&p=0.0023$) also further enhanced their CXCL10 inhibition compared to the treatment with CBD or CBD+THC alone.

The release of IL-6 by CTX-Astros (**Fig. 5.3.14.J**) was also significantly reduced post-treatment with CBD+SR14 ($####p=0.0008$), THC+SR14 ($\#p=0.0194$) and CBD+THC+SR14 ($###p=0.0022$) compared to stimulation alone. However, no significant differences were observed between the different treatment groups. The addition of SR14 to CBD+THC further decreased the IL-6 release compared to the treatment with CBD+THC alone ($&p=0.0487$).

Stimulated CTX-Astros also released significantly less RANTES post-treatment with CBD+SR14, THC+SR14 and CBD+THC+SR14 ($####p<0.0001$ for all) compared to IL-1 α stimulation, with the CBD+THC+SR14 treatment being most effective compared to CBD+SR14 ($+p=0.0201$) and THC+SR14 ($^^^p=0.0003$; **Fig. 5.3.14.K**). No significant differences were observed between the treatment with cannabinoids alone or their combination with SR14.

A very similar effect is also observed in terms of GM-CSF release where the treatment with CBD+SR14 ($\#p=0.0133$), THC+SR14 ($\#p=0.0066$) and CBD+THC+SR14 ($###p=0.0009$) significantly reduces GM-CSF levels in CTX-Astros compared to the stimulation alone. Although a trend towards a more potent effect

of the CBD+THC+SR14 treatment compared to the CBD+SR14 or THC+SR14 treatments was observed, this result was non-significant. No significant differences were observed either between the treatment with cannabinoids alone and their combination with SR14.

Overall, the combination of cannabinoids and T007 had the strongest anti-inflammatory effect, as seen in **Table 5.3.1** below listing the percentage of inhibition of the cytokine/chemokine release post-treatment. The only exception observed was the combination of cannabinoids with SR14, which resulted in a stronger decrease in GM-CSF release than any other treatment combination. Amongst all the treatments, the combination of CBD+THC paired with one of the (ant)agonists tended to have a stronger effect than when these two cannabinoids were separated.

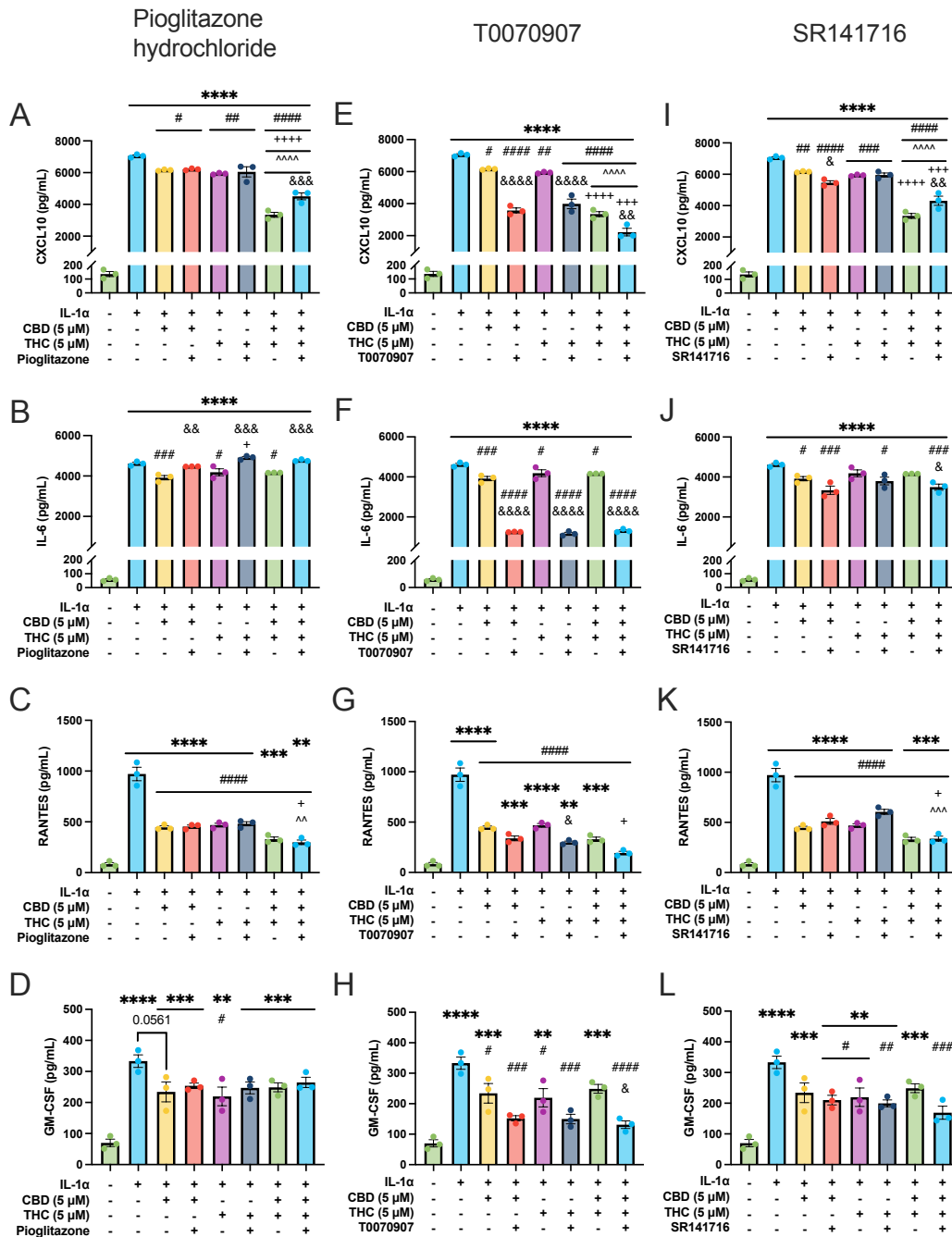


Figure 5.3.14 Combination of cannabinoids and receptor (ant)agonists effectively reduces pro-inflammatory cytokine release in stimulated CTX-Astros (previous page)

Stimulating CTX-Astros with IL-1α significantly increased the release of pro-inflammatory cytokines and chemokines. Treatment with CBD, THC and CBD+THC successfully ameliorated this pro-inflammatory cytokine release, especially in the case of CXCL10, IL-6 and RANTES (A-L). The combination of cannabinoids with Pioglitazone hydrochloride, a PPARγ agonist, significantly reduced the release of CXCL10 (A), RANTES (C) in IL-1α stimulated CTX-Astros. However, the treatment did not have an effect on the release of IL-6 (B) in the same cells. Replacing Pioglitazone with T0070907, a PPARγ antagonist, resulted in a significantly lower release of CXCL10 (E), IL-6 (F), RANTES (G) and

GM-CSF (**H**) when combined with cannabinoids before IL-1 α stimulation. Similarly, the combination of cannabinoids with SR141716, a CB₁ receptor antagonist, significantly reduced the release of CXCL10 (**I**), IL-6 (**J**), RANTES (**K**) and GM-CSF (**L**) in CTX-Astros stimulated with IL-1 α . Overall, the strongest effect was observed when CBD+THC were combined with receptor (ant)agonists. Data expressed as mean $\pm$ SEM (N=3, r=3). ** p <0.01, *** p <0.001, **** p <0.0001 vs control; # p <0.05, ## p <0.01, ### p <0.001, #### p <0.0001 vs IL-1 α stimulation; + p <0.05, +++ p <0.001, ++++ p <0.0001 vs CBD treatment; ^^ p <0.01, ^^ p <0.001, ^^ p <0.0001 vs THC treatment; & p <0.05, && p <0.01, &&& p <0.001, &&&& p <0.0001 vs the same cannabinoid with no (ant)agonist. One-way ANOVA with Tukey's post hoc.

Table 5.3.1 Percentage inhibition (Mean $\pm$ SEM) of IL-1 α -induced cytokine and chemokine production by cannabinoids and (ant)agonists in CTX-Astros

Treatment	Percentage inhibition of cytokine/chemokine release			
	CXCL10	IL-6	RANTES	GM-CSF
CBD	12.63 $\pm$ 0.66	15.10 $\pm$ 4.97	53.76 $\pm$ 3.56	29.52 $\pm$ 16.33
THC	15.78 $\pm$ 0.54	9.27 $\pm$ 5.59	51.16 $\pm$ 7.80	34.26 $\pm$ 12.13
CBD+THC	52.52 $\pm$ 4.47	9.73 $\pm$ 2.03	65.99 $\pm$ 1.29	24.91 $\pm$ 9.34
CBD+Pioglitazone	11.90 $\pm$ 2.19	2.99 $\pm$ 2.38	52.99 $\pm$ 3.37	23.62 $\pm$ 3.92
THC+Pioglitazone	14.18 $\pm$ 8.01	0	50.24 $\pm$ 2.64	25.50 $\pm$ 10.87
CBD+THC+Pioglitazone	36.06 $\pm$ 4.58	0	69.19 $\pm$ 2.43	20.46 $\pm$ 4.27
CBD+T0070907	49.28 $\pm$ 4.14	72.88 $\pm$ 0.83	64.59 $\pm$ 8.00	54.02 $\pm$ 6.49
THC+T0070907	43.45 $\pm$ 7.65	74.59 $\pm$ 2.95	69.10 $\pm$ 1.60	55.05 $\pm$ 6.56
CBD+THC+T0070907	68.47 $\pm$ 5.85	71.53 $\pm$ 2.07	80.07 $\pm$ 2.48	60.55 $\pm$ 6.27
CBD+SR141716	22.52 $\pm$ 2.73	29.06 $\pm$ 6.44	47.35 $\pm$ 1.90	78.37 $\pm$ 0.27
THC+SR141716	15.56 $\pm$ 1.80	19.12 $\pm$ 5.95	37.30 $\pm$ 6.77	79.36 $\pm$ 1.50
CBD+THC+SR141716	38.92 $\pm$ 6.79	25.86 $\pm$ 4.86	65.24 $\pm$ 2.91	82.66 $\pm$ 2.37

Having established that these combined treatments modulated cytokine and chemokine release at the protein level, we next examined whether similar effects were reflected at gene level. When CTX-Astros were stimulated with IL-1 α , a significant upregulation was observed in the expression of CXCL10 (*** p =0.0004), CCL5 (**** p <0.0001), C3 (**** p <0.0001) and TLR4 (* p =0.0105) mRNA compared to

the control (**Fig. 5.3.15.A-F**). Although an upregulation of *IL6* and *CSF2* mRNA was also observed, this was non-significant.

Treatment with CBD alone significantly downregulated the mRNA expression of *CXCL10* ($##p=0.0053$) compared to IL-1 α stimulation. Although a downregulation was observed in terms of expression of *IL6*, *CCL5*, *CSF2*, *C3* and *TLR4* post-CBD treatment, this was not significant. Treatment with THC alone also significantly downregulated the *CXCL10* ($##p=0.0013$) mRNA, as well as *C3* ($##p=0.0024$), compared to IL-1 α . Similarly, a trend towards downregulation of *IL6*, *CCL5*, *CSF2* and *TLR4* mRNA were also observed, but these results were not significant. The combination of CBD+THC, however, had a somewhat different effect to the treatment with each cannabinoid separately. Namely, an upregulation in *IL6* ($\#p=0.0101$), *CSF2* ($##p=0.0049$) and *TLR4* ($\#p=0.0444$) mRNA expression was observed compared to IL-1 α alone. A trend towards *CXCL10* and *CCL5* mRNA upregulation were also observed, without significance. The only gene showing signs of the downregulation of mRNA expression compared to stimulation alone was *C3*, also without significance. The mRNA expression of *CXCL10* ($+++p=0.002$ vs CBD, $^^^p<0.0001$ vs THC), *IL6* ($++p=0.0049$ vs CBD, $^p=0.0023$ vs THC), *CSF2* ($++p=0.0012$ vs CBD, $^p=0.0011$ vs THC) and *TLR4* ($++p=0.0026$ vs CBD, $^^p=0.0009$ vs THC) post-CBD+THC treatment were also significantly higher compared to CBD and THC treatments.

Treatment with THC+Pio ($\#p=0.0389$) and CBD+THC+Pio ($\#p=0.0244$) significantly downregulated the *CXCL10* mRNA expression compared to IL-1 α , and while CBD+Pio treatment also did trend toward a downregulation of *CXCL10* mRNA expression, this result was non-significant (**Fig. 5.3.15.A**). Stimulation with CBD+THC+Pio also resulted in a significantly lower *CXCL10* mRNA expression compared to the treatment with CBD+THC ($\&\&p=0.0010$).

Similar to the protein level, stimulated CTX-Astros treated with CBD+THC+Pio exhibited a significantly upregulated *IL6* mRNA expression compared to the control ($***p=0.0001$), stimulation alone ($##p=0.0071$), and the treatment with CBD+Pio ($+p=0.0435$) and THC+Pio ($^p=0.0155$; **Fig. 5.3.15.B**). While a trend

towards upregulation of *IL6* mRNA was also observed in CBD+Pio and THC+Pio-treated groups, this was non-significant.

The treatment of CTX-Astros with CBD+Pio (## $p=0.0011$), THC+Pio (### $p=0.0007$) and CBD+THC+Pio (### $p=0.0003$) significantly downregulated the expression of *CCL5* mRNA compared to the stimulation alone (**Fig. 5.3.15.C**). Moreover, the *CCL5* mRNA expression post-CBD+THC+Pio treatment was significantly lower compared to the treatment with CBD+THC ($p=0.0203$).

Similar to the *IL6* mRNA expression, a significant upregulation in *CSF2* mRNA expression was observed in the treated CTX-Astros. While the treatment with CBD+Pio (** $p=0.0064$) was only significantly upregulated compared to the control, the combined treatment with CBD+THC+Pio resulted in an upregulation of *CSF2* mRNA compared to the control (**** $p<0.0001$), IL-1 α stimulation (#### $p<0.0001$), CBD+Pio treatment (++ $p=0.0047$), as well as the treatment with THC+Pio (^^^ $p<0.0001$; **Fig. 5.3.15.D**). There was no effect of the treatment with THC or THC+Pio observed on the *CSF2* mRNA expression.

Although the expression of *C3* mRNA was quite similar across all stimulated groups regardless of treatment (**** $p<0.0001$ for all), the treatment with CBD+Pio upregulated the *C3* mRNA expression even further compared to IL-1 α stimulation alone (# $p=0.0347$) or the treatment with CBD only (& $p=0.0030$; **Fig. 5.3.15.E**). Treatment with THC+Pio (&& $p=0.0003$) and CBD+THC+Pio (& $p=0.0026$) also resulted in a significantly higher *C3* mRNA release compared to THC or CBD+THC without the addition of Pioglitazone.

The expression of *TLR4* mRNA post-treatment with CBD+Pio (* $p=0.0113$), CBD+THC (** $p=0.0001$) and CBD+THC+Pio (* $p=0.0217$) was significantly upregulated compared to the control only. Treatment with CBD+THC alone significantly upregulated the *TLR4* mRNA expression compared to IL-1 α stimulation (# $p=0.0444$), as well as the treatments with CBD (++ $p=0.0026$) and THC (^^ $p=0.0009$; **Fig. 5.3.15.F**).

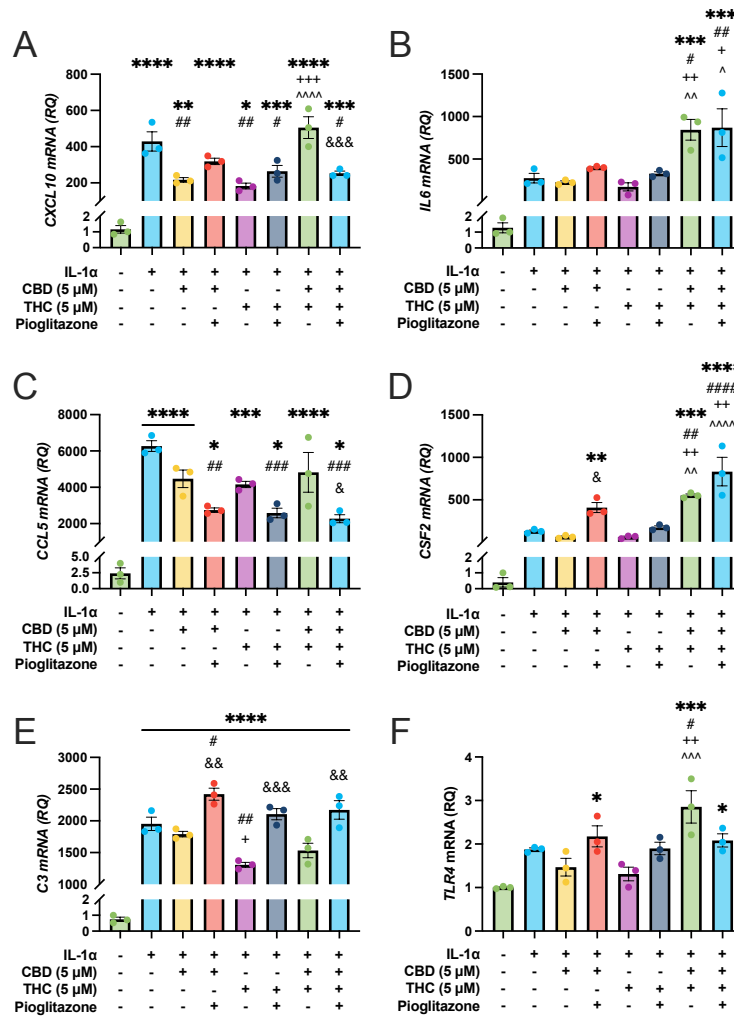


Figure 5.3.15 Treatment with cannabinoids and Pioglitazone hydrochloride has a varied effect on inflammatory gene expression in CTX-Astros

Stimulating CTX-Astros with IL-1α significantly increased the expression of inflammatory genes (A-F). Treatment with CBD and THC alone and the combined treatment of THC+Pio and CBD+THC+Pio reduced the CXCL10 mRNA (A) expression. Treatment with CBD+THC with and without Pio significantly increased the expression of IL6 (B) and CSF2 (D) mRNA in stimulated CTX-Astros. (C) CCL5 mRNA expression was significantly downregulated in CTX-Astros post-treatment with cannabinoids+Pio, but not cannabinoids alone. (E) Treatment with cannabinoids+Pio significantly upregulated the C3 mRNA expression in stimulated CTX-Astros, while THC alone downregulated the C3 mRNA expression. (F) Treating CTX-Astros with cannabinoids+Pio did not have an effect on the TLR4 mRNA expression, but the treatment with CBD+THC alone did significantly upregulate TLR4 mRNA. Data expressed as mean ± SEM (N=3, r=3). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs control; #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001 vs IL-1α stimulation; +p<0.05, ++p<0.01, +++p<0.001 vs CBD treatment; ^p<0.05, ^^p<0.01, ^^p<0.001 vs THC treatment; &p<0.05, &&p<0.01, &&&p<0.001 vs the same cannabinoid with no (ant)agonist. One-way ANOVA with Tukey's post hoc.

CTX-Astros stimulated with IL-1 α were also subjected to the treatment combining cannabinoids with T007. Unlike Pioglitazone, the additional treatment with T007 further enhanced the anti-inflammatory potential of cannabinoids, as seen in **Fig. 5.3.16.A-F** below.

Treating stimulated CTX-Astros with CBD+T007, THC+T007 and CBD+THC+T007 has significantly downregulated the expression of *CXCL10* (#### $p < 0.0001$ for all). Moreover, the addition of T007 resulted in a lower *CXCL10* mRNA expression compared to the treatment with cannabinoids alone, especially in the case of the CBD+THC+T007 combination where *CXCL10* mRNA levels observed were significantly lower compared to CBD+THC alone (&&& $p < 0.0001$; **Fig. 5.3.16.A**).

The mRNA expression of *IL6* was not significantly reduced by the combination of cannabinoids and T007, despite an observed trend towards a downregulation. The treatment with CBD+THC+T007 (&&& $p < 0.0001$) did however significantly downregulate the *IL6* mRNA expression compared to the previously observed upregulation in the CBD+THC-treated cells (**Fig. 5.3.16.B**).

Treatment with CBD+T007 (### $p = 0.0003$), THC+T007 (### $p = 0.0001$), and CBD+THC+T007 (#### $p < 0.0001$) significantly decreased the expression of *CCL5* mRNA compared to IL-1 α stimulated group (**Fig. 5.3.16.C**), with the combined treatment of CBD+THC+T007 being the most effective at downregulating *CCL5* mRNA expression, especially compared to the treatment with CBD+THC (&& $p = 0.0006$).

The *CSF2* mRNA was significantly downregulated by the treatment with CBD (## $p = 0.0030$), CBD+T007 (#### $p < 0.0001$), THC (## $p = 0.0021$), THC+T007 (#### $p < 0.0001$) and CBD+THC+T007 (#### $p < 0.0001$), compared to the stimulation alone (**Fig. 5.3.16.D**). Treatment with CBD+THC alone, however, resulted in a significant upregulation of *CSF2*, compared to the control (**** $p < 0.0001$), IL-1 α stimulation (#### $p < 0.0001$), CBD (++++ $p < 0.0001$) and THC (^^^^ $p < 0.0001$). The addition of T007 to THC (& $p = 0.0172$) and

CBD+THC (&&&p<0.0001) resulted in a significantly lower *CSF2* mRNA expression compared to THC and CBD+THC without T007.

A similar effect was observed in terms of *C3* mRNA expression where CBD+T007 (###p=0.0004), THC (####p<0.0001), THC+T007 (####p<0.0001), CBD+THC (##p=0.0063) and CBD+THC+T007 (####p<0.0001) significantly downregulated *C3* mRNA levels compared to IL-1 α stimulation (**Fig. 5.3.16.E**). The treatment with THC (++p=0.0017) resulted in a significantly lower *C3* mRNA expression compared to treatment with CBD. The treatment with CBD+T007 (&p=0.0113) and CBD+THC+T007 (&&p=0.0032) also significantly downregulated the expression of *C3* mRNA compared to CBD and CBD+THC, respectively.

The expression of *TLR4* mRNA was significantly reduced by the treatment with THC+T007 (#p=0.0114) compared to IL-1 α -stimulated CTX-Astros, but not any other treatment (**Fig. 5.3.16.F**). In this case, the treatment with THC+T007 was not only the most effective but also has brought the *TLR4* mRNA expression back to baseline. The treatment with CBD+THC, however, significantly upregulated the *TLR4* mRNA expression compared to the control (****p<0.0001), IL-1 α stimulation (#p=0.0138), as well as the treatments with CBD (+++p=0.0005) and THC (^^^p=0.0002). The treatment with CBD+THC+T007 resulted in a significant downregulation of *TLR4* mRNA compared to CBD+THC (&&&p<0.0001). These effects on the inflammatory gene expression have further reaffirmed the potent effectiveness of using T007 alongside cannabinoids to enhance their anti-inflammatory potential.

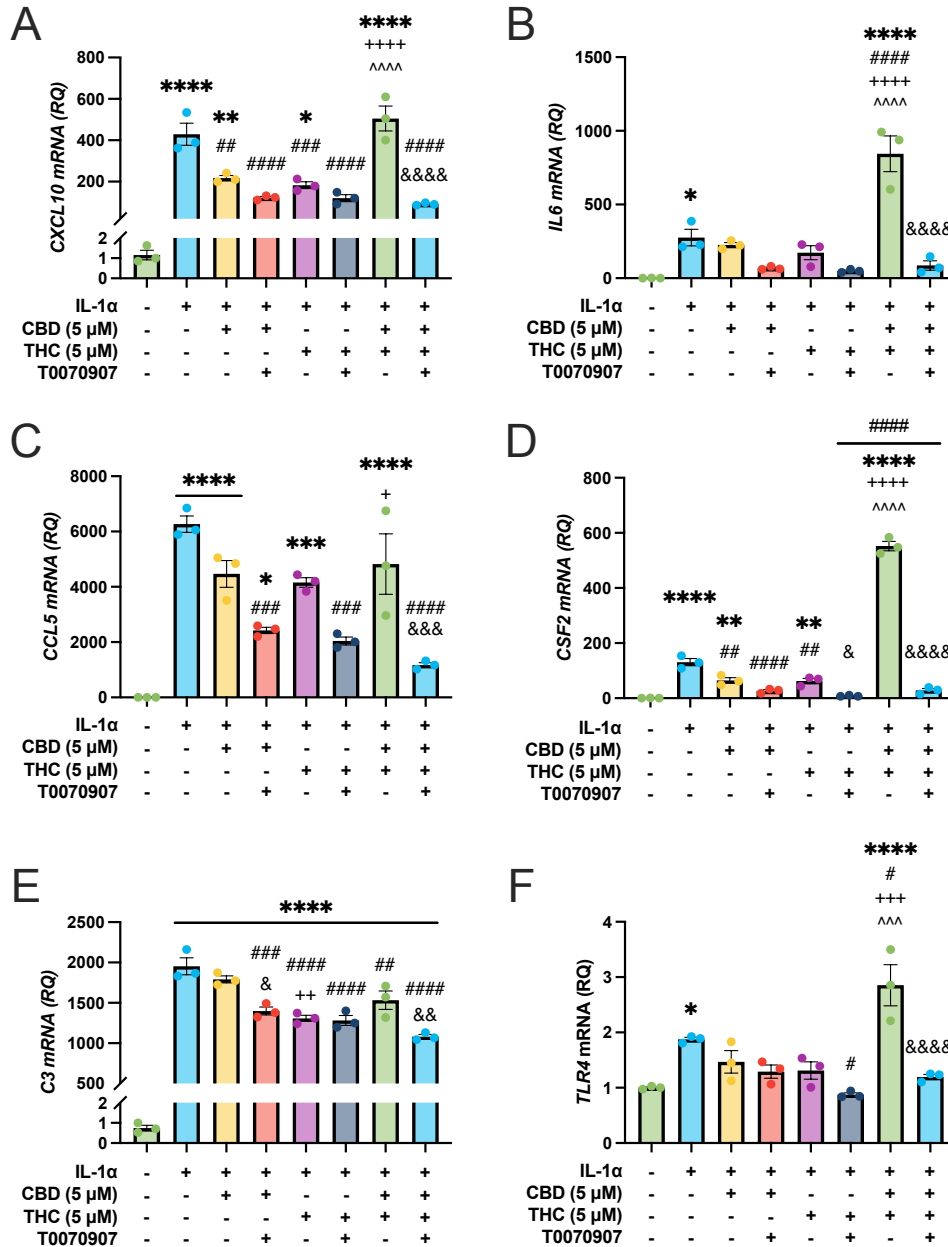


Figure 5.3.16 Treatment with cannabinoids and T0070907 significantly downregulates inflammatory gene expression in CTX-Astros

Stimulating CTX-Astros with IL-1α significantly increased the expression of inflammatory genes (A-F). However, the combined treatment of stimulated CTX-Astros with cannabinoids and T0070907 significantly downregulated the mRNA expression of CXCL10 (A), CCL5 (C), CSF2 (D) and C3 (E). However, a treatment with CBD+THC significantly upregulated the expression of IL6 (B), CSF2 (D) and TLR4 (F) mRNA. Data expressed as mean ± SEM (N=3, r=3). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs control; #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001 vs IL-1α stimulation; +p<0.05, ++p<0.01, +++p<0.001, ++++p<0.0001 vs CBD treatment; ^p<0.01, ^^p<0.001, ^^p<0.0001 vs THC treatment; &p<0.05, &&p<0.01, &&&p<0.0001 vs the same cannabinoid with no (ant)agonist. One-way ANOVA with Tukey's post hoc.

Similar to the combined treatment with Pioglitazone, the use of SR14 alongside cannabinoids also had a varying effect on the inflammatory gene expression. Stimulated CTX-Astros treated with CBD ($##p=0.0044$), CBD+SR14 ($##p=0.0031$), THC ($##p=0.0010$), THC+SR14 ($##p=0.0021$) and CBD+THC+SR14 ($###p=0.0008$) expressed significantly less *CXCL10* mRNA compared to IL-1 α stimulation alone (**Fig. 5.3.17.A**). Treatment with CBD+THC+SR14 ($****p<0.0001$) also exhibited a significantly lower *CXCL10* mRNA expression compared to the treatment with CBD+THC, which trended towards an upregulation compared to IL-1 α stimulation and was significantly higher compared to the control ($****p<0.0001$), CBD ($+++p=0.0002$) and THC ($^^^^p<0.0001$).

In contrast, the combined cannabinoid treatment with SR14 failed to alter the *IL6* mRNA expression compared to IL-1 α . Moreover, an upregulation of *IL6* mRNA was observed post-CBD+THC treatment compared to the control ($****p<0.0001$), IL-1 α stimulation ($####p<0.0001$), CBD treatment ($++++p<0.0001$), THC treatment ($^^^^p<0.0001$), as well as the treatment with CBD+THC+SR14 ($***p=0.0005$; **Fig. 5.3.17.B**). The treatment with CBD+THC+SR14 also significantly upregulated the *IL6* mRNA expression compared to the control ($**p=0.0055$).

The *CCL5* mRNA expression in stimulated CTX-Astros was significantly downregulated by the treatments with CBD+SR14 ($##p=0.0074$), THC+SR14 ($#p=0.0421$) and CBD+THC+SR14 ($###p=0.0004$) compared to IL-1 α stimulation. Moreover, without the addition of SR14, the *CCL5* mRNA expression post-CBD, THC and CBD+THC treatment was not significantly downregulated compared to the stimulation alone. The combined treatment of CBD+THC+SR14 was more effective at reducing *CCL5* mRNA expression compared to CBD+THC ($&p=0.0325$; **Fig. 5.3.17.C**).

The expression of *CSF2* mRNA, although slightly reduced by CBD+SR14 and THC+SR14 treatment, did not have a significant effect on *CSF2* mRNA levels compared to IL-1 α (**Fig. 5.3.17.D**). Additionally, the treatment with CBD+THC significantly increased *CSF2* mRNA expression compared to the control ($****p<0.0001$), IL-1 α stimulation ($####p<0.0001$),

CBD+SR14 (++++ $p<0.0001$) and THC+SR14 (^^^ $p<0.0001$). Treating CTX-Astros with CBD+THC+SR14 significantly downregulated the CSF2 mRNA expression compared to CBD+THC (&&& $p<0.0001$) but was still significantly higher compared to the control (**** $p<0.0001$) and the treatment with THC+SR14 ($p=0.0322$).

The expression of C3 mRNA remained unchanged post-CBD+SR14 and THC+SR14 treatment but was significantly downregulated by the treatment with THC (### $p=0.0003$), CBD+THC ($p=0.0164$) and CBD+THC+SR14 (## $p=0.0074$) compared to IL-1 α stimulated group (**Fig. 5.3.17.E**). Both, the treatment with THC (++ $p=0.0050$) and CBD+THC+SR14 (++ $p=0.0034$) resulted in a significantly lower C3 mRNA expression compared to the treatment with CBD and CBD+SR14, respectively. The treatment with THC+SR14 (&& $p=0.0005$) also exhibited a higher C3 mRNA expression compared to the treatment with THC alone.

The combined treatment with cannabinoids and SR14, although trending towards a downregulation, did not have any significant effect on the expression of *TLR4* mRNA, as seen in **Fig. 5.3.17.F** below. Treatment with CBD+THC without the addition of SR14, however, led to a significantly higher *TLR4* mRNA expression compared to the control (*** $p=0.0003$), CBD (++ $p=0.0059$), THC ($p=0.0021$) and CBD+THC+SR14 (& $p=0.0176$).

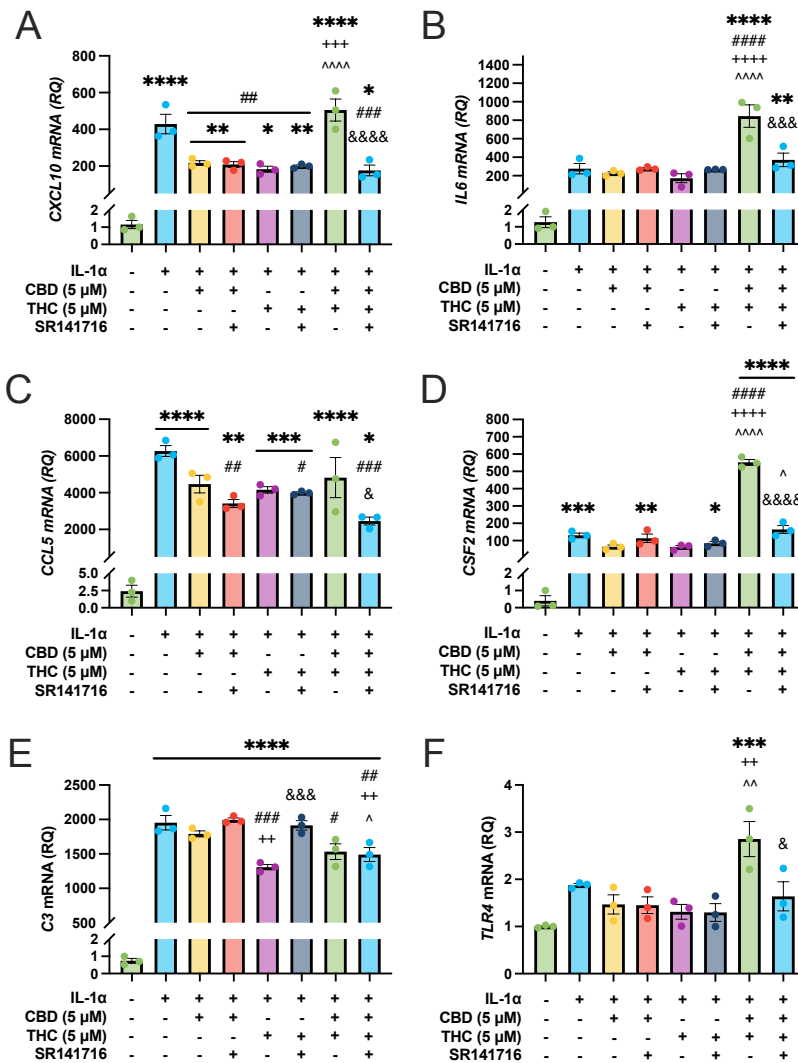


Figure 5.3.17 Treatment with cannabinoids and SR141716 has a varied effect on inflammatory gene expression in CTX-Astros

Stimulating CTX-Astros with IL-1 α significantly increased the expression of inflammatory genes (A-F). However, the treatment with cannabinoids and SR141716 somewhat rescued that effect. CXCL10 (A) and CCL5 (B) mRNA expression were significantly downregulated when stimulated CTX-Astros were treated with cannabinoids+SR14. (E) C3 mRNA expression was also downregulated once stimulated CTX-Astros were treated with CBD+THC+SR14. Although there was a trend towards a decreased CSF2 (D) and TLR4 (F) mRNA expression post cannabinoid+SR14 treatment, these results were non-significant. (B) There were no significant effects of the cannabinoid+SR14 treatment on IL6 mRNA expression in IL-1 α -stimulated CTX-Astros. Data expressed as mean $\pm$ SEM (N=3, r=3). * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001 vs control; # p <0.05, ## p <0.01, ### p <0.001, #### p <0.0001 vs IL-1 α stimulation; ++ p <0.01, +++ p <0.001, ++++ p <0.0001 vs CBD treatment; ^ p <0.05, ^^ p <0.01, ^^ p <0.001 vs THC treatment; & p <0.05, && p <0.01, &&& p <0.001 vs the same cannabinoid with no (ant)agonist. One-way ANOVA with Tukey's post hoc.

The expression of CB₁, PPAR α and PPAR γ receptor genes (*CNR1*, *PPARA* and *PPARG*, respectively) was also assessed post-treatment with cannabinoids with and without Pio, T007 or SR14. Treatment with cannabinoids+Pio, although non-significant, did upregulate the expression of *CNR1*, *PPARG* and *PPARA* compared to IL-1 α stimulation (**Fig. 5.3.18.A-C**). The treatment with CBD+THC, however, significantly upregulated the *PPARA* mRNA expression compared to the control (*p=0.0484), IL-1 α stimulation (#p=0.0105), as well as the treatments with CBD (+p=0.0116) and THC (^p=0.0065; **Fig. 5.3.18.C**).

The treatment with cannabinoids and T007 did not have any significant effect on the expression of *CNR1* mRNA compared to IL-1 α stimulation alone, although a small trend toward an upregulation was observed (**Fig. 5.3.18.D**). Treating CTX-Astros with THC+T007, however, significantly downregulated the *CNR1* mRNA expression compared to the control (*p=0.0490). A very similar trend was observed with *PPARG* mRNA expression post-CBD+T007, THC+T007 and CBD+THC+T007 treatment of CTX-Astros, but this was also non-significant compared to IL-1 α -stimulated group (**Fig. 5.3.18.E**). The treatment with CBD+THC alone (#p=0.0398) did significantly upregulate the expression of *PPARG* mRNA compared to IL-1 α stimulation. A significant upregulation of *PPARA* mRNA was observed when stimulated CTX-Astros were treated with CBD+THC, compared to the control (*p=0.0487), IL-1 α stimulation (#p=0.0106), as well as the treatments with CBD (+p=0.0117) and THC (^p=0.0066; **Fig. 5.3.18.F**), although other treatments such as CBD+T007, THC+T007 and CBD+THC+T007 also trended towards *PPARA* mRNA upregulation.

The treatment of stimulated CTX-Astros with cannabinoids and SR14 did not have any effect on the mRNA expression of *CNR1*, *PPARA* or *PPARG* compared to IL-1 α stimulation alone (**Fig. 5.3.18.G-I**). The *CNR1* mRNA expression was significantly lower post-CBD (**p=0.0074), CBD+SR14 (*p=0.0439) and THC (*p=0.0136) treatment compared to the control (**Fig. 5.3.18.G**). The *PPARG* mRNA expression of the CBD+THC group was significantly higher compared to the IL-1 α (#p=0.0178), CBD (+p=0.0299) and THC-treated (^p=0.0430) groups. Although a trend towards upregulation of *PPARG* mRNA was observed across the three treatment groups, this

was also non-significant (**Fig. 5.3.18.H**). Similarly, the *PPARA* mRNA expression post-CBD+THC treatment was also significantly higher compared to the IL-1 α -stimulated (#p=0.0428) and the CBD (+p=0.0463) and THC-treated (^p=0.0291) groups. Although a trend towards upregulation of *PPARA* mRNA was observed across the three treatment groups, this was also non-significant (**Fig. 5.3.18.I**).

Taken together, these experiments show that combining cannabinoids with PPAR γ - and CB $_1$ -targeting (ant)agonists further modulates IL-1 α -induced astrocyte reactivity at both protein and gene expression level. Overall, T007 produced the most consistent and pronounced reductions in CXCL10, IL-6, RANTES, GM-CSF and multiple inflammatory transcripts, whereas SR14 and Pio had more selective or mixed effects. In general, treatments containing CBD+THC tended to exert stronger effects on chemokine and cytokine suppression than either cannabinoid alone when combined with receptor (ant)agonists. Finally, modulation of *PPARA* and *PPARG* expression by these treatments indicates that PPAR signalling is dynamically regulated in reactive CTX-Astros and can be at least partially restored by specific cannabinoid–(ant)agonist combinations. These findings extend the previous section by demonstrating that pharmacological targeting of cannabinoid-related receptors can shape the magnitude and profile of the astrocyte response to cannabinoids.

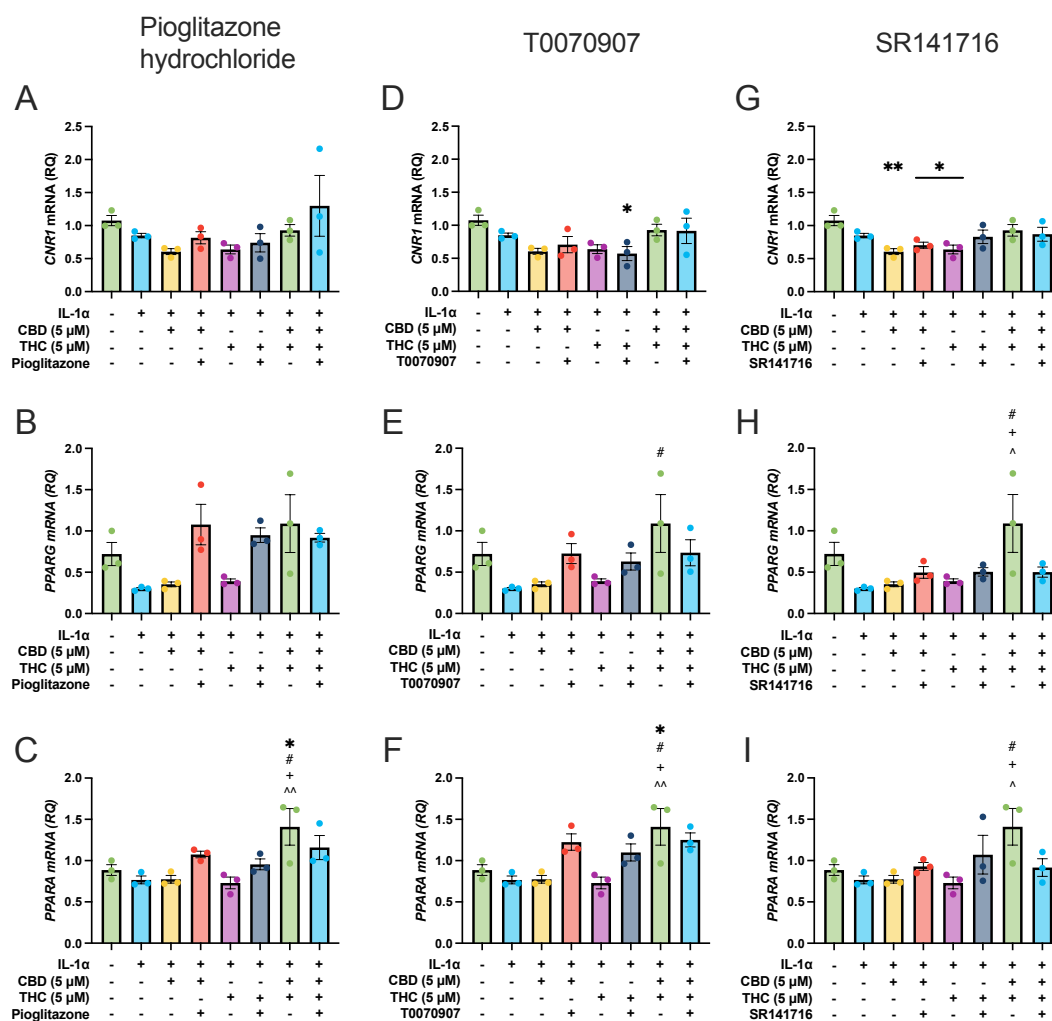


Figure 5.3.18 Treatment with cannabinoids and (ant)agonists reverse the IL-1 α -induced inhibition of PPAR γ receptor gene

IL-1 α stimulation of CTX-Astros trended toward a downregulation of the CNR1, PPARG and PPARA mRNA expression (A-I). (B) Treatment with cannabinoids+Pio reversed the IL-1 α -induced downregulation of PPARG mRNA expression. (C) The expression of PPARA mRNA was upregulated in stimulated CTX-Astros treated with CBD+THC compared to stimulation alone. (D) Treatment with THC+T007 significantly downregulated CNR1 mRNA expression in CTX-Astros compared to the control. (E) Treating stimulated CTX-Astros with cannabinoids+T007 also reversed the IL-1 α -induced downregulation of PPARG mRNA. (F) CBD+THC treatment significantly upregulated PPARA mRNA expression compared to the control, stimulation alone and CBD and THC treatments. (G) Treatment with CBD, CBD+SR14 and THC significantly reduced the expression of CNR1 mRNA compared to the control. (H) Treatment with CBD+THC significantly upregulated the PPARG mRNA expression compared to IL-1 α stimulation as well as CBD and THC treatments. (I) Treatment with CBD+THC significantly upregulated the PPARA mRNA expression compared to IL-1 α stimulation as well as CBD and THC treatments. Data expressed as mean $\pm$ SEM (N=3, r=3). * p <0.05, ** p <0.01, *** p <0.001 vs control; # p <0.05, ## p <0.01 vs IL-1 α stimulation. One-way ANOVA with Tukey's post hoc.

5.3.5. MCC950 has a very subdued anti-inflammatory effect on reactive astrocytes

To evaluate whether direct inhibition of the NLRP3 inflammasome could modulate astrocyte reactivity, CTX-Astros were treated with a range of concentrations of MCC950 in the absence or presence of IL-1 α stimulation. The 10 μ M, 5 μ M and 0.5 μ M concentrations of MCC950 were investigated for any potential effects on cell viability and cytokine release in unstimulated CTX-Astros. Although no changes were observed in the alamarBlue metabolic activity of CTX-Astros post-MCC950 treatment, a significant increase in LDH activity was observed when cells were treated with MCC950 at 10 μ M (**p=0.0013) and 0.5 μ M (*p=0.0242; **Fig. 5.3.19.A-B**). However, this did not promote any significant changes in cytokine or chemokine release post-treatment (**Fig. 5.3.19.C-F**).

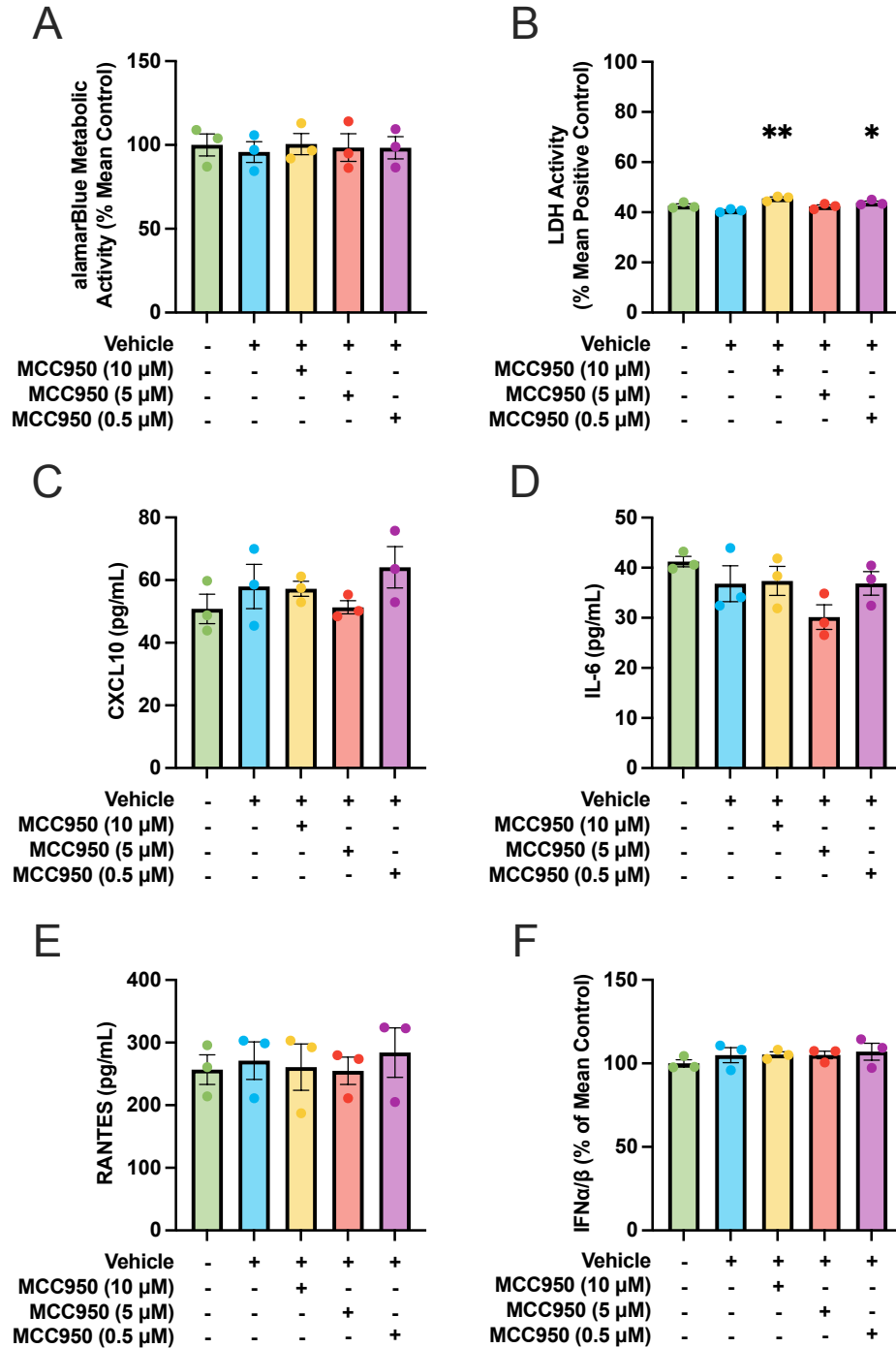


Figure 5.3.19 Potential therapeutic doses of MCC950 did not increase the release of pro-inflammatory cytokines and chemokines despite slight increase in cell cytotoxicity.

Treatment with 0.5, 5 and 10 μ M concentrations of MCC950 did not have an effect on alamarBlue metabolic activity in CTX-Astros (**A**), although a slight, albeit significant, increase in LDH activity (**B**) was detected in cells treated with the 0.5 and 10 μ M of MCC950. Despite this, no increase in the release of CXCL10 (**C**), IL-6 (**D**), RANTES (**E**) or IFN α / β (**F**) was observed post-treatment. Data expressed as mean $\pm$ SEM (N=3, r=3). * p <0.05, ** p <0.01 vs control. One-way ANOVA with Tukey's post hoc.

When the same concentrations of MCC950 were used in conjunction with IL-1 α stimulation, no significant changes were observed in alamarBlue metabolic activity of CTX-Astros either (**Fig. 5.3.20.A**). However, the significant increase in LDH activity was still present in stimulated CTX-Astros post-10 μ M (*p=0.0138) and 0.5 μ M (**p=0.0079) MCC950 treatment (**Fig. 5.3.20.B**). Despite this low-grade level of potential cell cytotoxicity, the stimulated CTX-Astros treated with MCC950 still showed limited signs of potential anti-inflammatory properties of the treatment. Namely, the CXCL10 release was significantly decreased compared to IL-1 α stimulation when CTX-Astros were treated with 10 μ M (##p=0.0034), 5 μ M (#p=0.0177) or 0.5 μ M (#p=0.0222) of MCC950 (**Fig. 5.3.20.C**). A significant decrease in IFN α / β release was also observed post-treatment with 0.5 μ M MCC950 compared to IL-1 α (#p=0.0107) alone, as well as the 10 μ M (+p=0.0120) and 5 μ M (^p=0.0047) MCC950 concentrations (**Fig. 5.3.20.F**). However, no changes were observed between IL-1 α stimulation alone and MCC950 treatment in terms of IL-6 and RANTES release. Furthermore, a time-course gene expression analysis revealed that human iPSC-derived CTX-Astros only express low levels of CD14 by the time they reach D90, which is the earliest point at which these cells can be first treated or stimulated. The highest CD14 mRNA expression was observed between D20 and D35 at which point these cells are still NPC cultured in neuronal media or NPC that were just transitioned into astrocyte media.

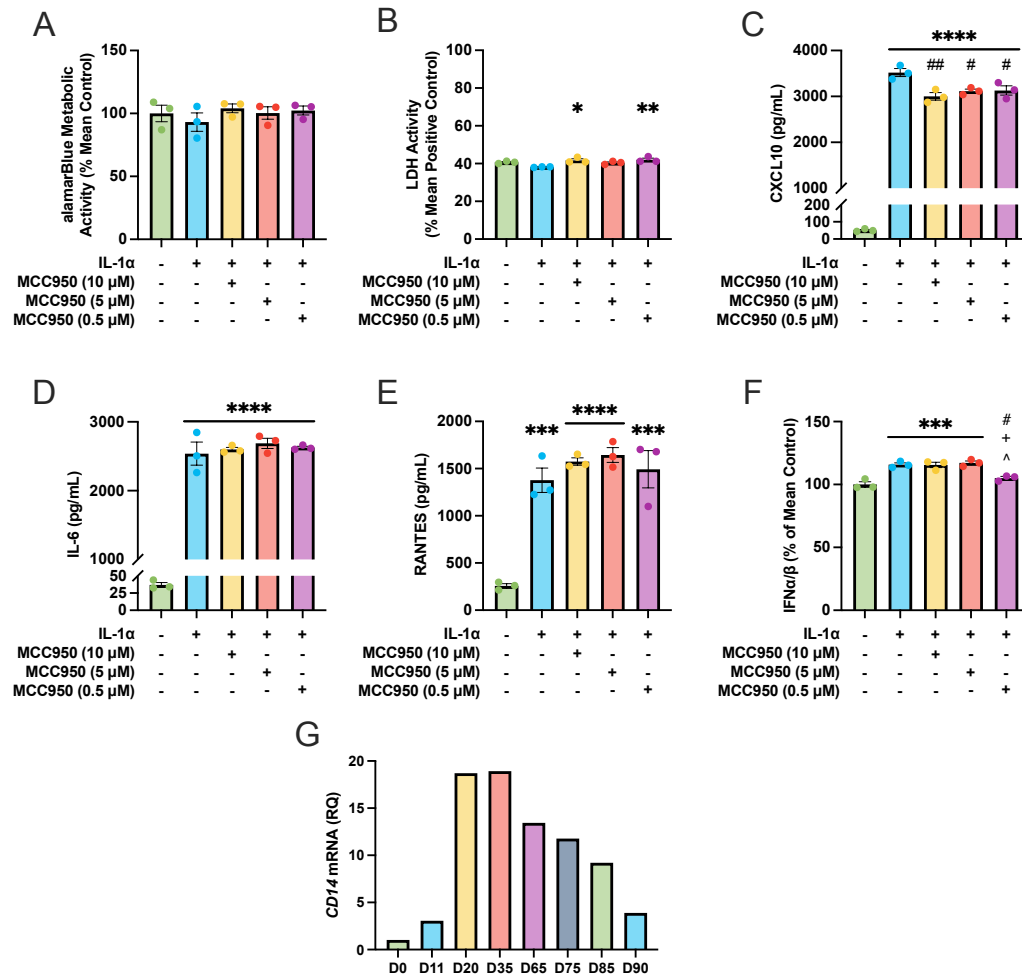


Figure 5.3.20 Treatment with MCC950 has a very subdued anti-inflammatory effect on stimulated CTX-Astros.

CTX-Astros stimulated with IL-1 α released significant amounts of pro-inflammatory cytokines and chemokines without affecting the cells' viability (**A-F**). Stimulated CTX-Astros treated with MCC950 did not display any changes in metabolic activity (**A**), but the 10 μ M and 0.5 μ M treatment significantly increased the LDH activity (**B**). (**C**) Treatment with MCC950 significantly decreased the CXCL10 release compared to IL-1 α stimulation across all concentrations. (**F**) A significant decrease in IFN α/β release was also observed with 0.5 μ M MCC950 treatment compared to stimulated CTX-Astros. No effect of MCC950 treatment was observed in terms of IL-6 (**D**) and RANTES (**E**) release. (**G**) qPCR analysis of cells across differentiation timeline (D0-90) revealed that CD14 mRNA expression is highest in CTX-NPC and young CTX-APC and decreases severely over time. Data expressed as mean $\pm$ SEM (N=3, r=3). * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001 vs control; # p <0.05, ## p <0.01 vs IL-1 α stimulation; + p <0.05 vs 10 μ M MCC950+IL-1 α ; ^ p <0.05 vs 5 μ M MCC950+IL-1 α . One-way ANOVA with Tukey's post hoc.

Taken together, MCC950 showed only limited anti-inflammatory activity in IL-1 α -stimulated CTX-Astros. Although all concentrations reduced CXCL10 release, and the lowest dose also decreased IFN α/β secretion, MCC950 did not alter IL-6 or RANTES levels despite producing a mild increase in LDH activity. Overall, MCC950 exerted a more subdued effect on reactive CTX-Astros compared with the cannabinoid-based treatments and receptor-modulating compounds described in previous sections.

5.4. Discussion

The present study examined the capacity of cannabinoids and cannabinoid-related modulators to attenuate inflammatory responses in human iPSC-derived cortical astrocytes. Using IL-1 α as a pro-inflammatory stimulus, we assessed a range of functional, molecular and signalling readouts to evaluate how CBD, THC and their 1:1 combination, influence astrocyte reactivity. Between viability assays, cytokine and chemokine secretion, inflammatory gene expression and NF κ B nuclear translocation, cannabinoids consistently reduced several hallmarks of IL-1 α -induced activation, with CBD+THC and small-molecule receptor modulators further shaping the magnitude of these responses. Collectively, these findings provide a comprehensive analysis of the anti-inflammatory potential of cannabinoids in human astrocytes and support the involvement of PPAR γ - and CB $_1$ -associated pathways in modulating astrocyte reactivity.

5.4.1. Overview of Main Findings

This study investigated the ability of cannabinoids and related receptor modulators to alter inflammatory responses in human iPSC-derived cortical astrocytes. Using IL-1 α as a stimulant, CTX-Astros exhibited a robust reactive profile characterised by increased secretion of CXCL10, IL-6, RANTES and GM-CSF, upregulation of inflammatory genes and enhanced NF κ B nuclear translocation. Treatment with CBD, THC or a 1:1 CBD+THC combination reduced several components of this reactive phenotype. CBD consistently lowered IL-6 and CXCL10 release and CBD+THC exerted the strongest effects on chemokine suppression. Cannabinoids also attenuated NF κ B nuclear translocation and decreased the expression of key inflammatory genes, including *CXCL10* and *CCL5*.

Combining cannabinoids with PPAR γ - and CB $_1$ -related receptor antagonists and agonists further modulated astrocyte reactivity. The PPAR γ antagonist, T0070907, produced the strongest effects, markedly reducing cytokine secretion and downregulating multiple inflammatory genes. Pioglitazone and SR141716 each

reduced selected cytokines but had more variable effects at the transcriptional level. Treatments containing CBD+THC tended to achieve greater reductions than either cannabinoid alone when paired with receptor modulators.

In contrast, the NLRP3 inhibitor, MCC950, showed only a modest anti-inflammatory effect, decreasing CXCL10 and IFN α / β release but not IL-6 or RANTES. These findings, together with low *CD14* expression in fully-differentiated CTX-Astros, suggest limited involvement of inflammasome-associated signalling under the conditions tested. Collectively, the results demonstrate that cannabinoids and pharmacological modulators of cannabinoid-related receptors can reshape multiple aspects of IL-1 α -induced astrocyte reactivity in human iPSC-derived astrocytes.

5.4.2. Cannabinoid Modulation of Astrocyte Reactivity

The present findings align with a growing body of evidence demonstrating that cannabinoids exert anti-inflammatory effects in glial cells, including astrocytes (Kozela, Juknat and Vogel, 2017; Tomaszewska-Zaremba, Gajewska and Misztal, 2025). Previous studies have shown that CBD reduces the expression of pro-inflammatory mediators such as IL-6, TNF α and CXCL10, and attenuates NF κ B signalling in both rodent and human astrocytes (Kozela, Juknat and Vogel, 2017; Nichols and Kaplan, 2020). Similarly, THC has been reported to modulate inflammatory pathways in glia, although its effects are often more variable and context-dependent (Cabral and Griffin-Thomas, 2009; Cutando *et al.*, 2013). In the current study, both cannabinoids reduced aspects of IL-1 α -induced reactivity, with CBD showing consistent effects on IL-6 and CXCL10 and the CBD+THC combination producing the strongest suppression of chemokine release. This pattern supports earlier observations of synergistic or additive interactions between phytocannabinoids, often referred to as the “entourage effect,” particularly in the context of inflammatory regulation (Russo, 2011).

The reduction in NF κ B nuclear translocation observed following CBD or THC treatment provides a mechanistic anchor for these anti-inflammatory effects. NF κ B

is a central transcriptional regulator of many cytokines and chemokines released by reactive astrocytes, and its inhibition has previously been associated with decreased CXCL10 and CCL5 production in glial cells (Yeruva, Ramadori and Raddatz, 2008; Chi *et al.*, 2024). Consistent with this, cannabinoids in the present study reduced NFκB activation and downregulated *CXCL10* and *CCL5* gene expression, indicating that modulation of this pathway contributes to the attenuation of astrocyte reactivity. The partial dissociation between *IL6* mRNA and IL-6 protein levels following cannabinoid treatment is also consistent with prior observations that IL-6 production is influenced by additional post-transcriptional and autocrine regulatory mechanisms (Sanchis *et al.*, 2020).

The enhancement of cannabinoid effects by the PPARγ antagonist, T0070907, further supports a role for PPAR signalling in regulating astrocyte inflammatory responses. Although PPARγ activation is typically associated with anti-inflammatory activity in peripheral tissues, several studies have reported context-dependent roles in the CNS, including dynamic regulation during astrocyte activation (Daynes and Jones, 2002; Drew *et al.*, 2006; Bernardo and Minghetti, 2007). The strong suppression of cytokine release and inflammatory gene expression following T007 treatment suggests that endogenous PPARγ activity may also affect certain anti-inflammatory pathways engaged by cannabinoids, thus contributing to their synergistic anti-inflammatory effect. In contrast, Pioglitazone, a PPARγ agonist, exerted a narrower effect, selectively reducing CCL5 and a subset of cytokines, which aligns with previous reports of limited efficacy of PPARγ agonism in some models of astrocyte-mediated inflammation (Bernardo and Minghetti, 2007, 2008).

CB₁ signalling has also been implicated in the regulation of astrocyte function, particularly in controlling intracellular calcium dynamics, metabolic activity and cytokine release (Navarrete and Araque, 2008). In the present study, the CB₁ antagonist, SR141716, reduced several cytokines but produced minimal changes at the transcriptional level, suggesting that CB₁-linked pathways may influence secretion mechanisms more than upstream gene expression. This is consistent with reports that CB₁ activity in astrocytes can modulate vesicular release and

synaptic-associated signalling independently of transcriptional control (Busquets-Garcia, Bains and Marsicano, 2018; Covelo *et al.*, 2021; Eraso-Pichot *et al.*, 2023).

Finally, the limited anti-inflammatory effect of the NLRP3 inhibitor MCC950 is consistent with prior evidence that human iPSC-derived astrocytes express low levels of inflammasome-associated genes, including *CD14* and *NLRP3*, particularly at later stages of maturation (Zhang *et al.*, 2014). While microglia robustly engage the NLRP3–IL-1 β axis, astrocytes exhibit a more restricted and context-specific inflammasome response. The mild reduction of CXCL10 and IFN α/β release observed here suggests partial engagement of inflammasome-linked pathways but reinforces that IL-1 α -induced astrocyte reactivity is predominantly driven by NF κ B, IRF and JAK/STAT-associated mechanisms rather than canonical inflammasome activation (Liddel *et al.*, 2017).

5.4.3. Differential Sensitivity of Inflammatory Cytokines and Genes

The present study revealed that different inflammatory mediators released by CTX-Astros exhibit distinct sensitivities to cannabinoid treatment. CXCL10 consistently showed the greatest reduction across conditions, both at the protein and mRNA levels, which aligns with its well-characterised dependence on NF κ B and STAT1 signalling pathways (Yeruva, Ramadori and Raddatz, 2008; Liddel *et al.*, 2017; Chi *et al.*, 2024). Because cannabinoids potently reduced NF κ B nuclear translocation in our study, the strong suppression of CXCL10 is consistent with its reliance on this pathway. CCL5 displayed a similar pattern, with reductions at both transcript and protein levels in most cannabinoid-treated groups, particularly when CBD and THC were used in combination and paired with T007.

In contrast, IL-6 proved more resistant to cannabinoid-mediated suppression. Although CBD reduced IL-6 secretion in some conditions, transcriptional responses were variable and often showed partial or no reduction. This discrepancy likely reflects the multilayered regulation of IL-6, which is influenced not only by NF κ B but also by AP-1, C/EBP and autocrine feedback loops that stabilise IL-6 production

even when upstream activation is dampened (Van Wagoner and Benveniste, 1999; Spooren *et al.*, 2011). These mechanisms may explain why *IL6* mRNA did not always mirror the changes observed in *CXCL10* or *CCL5* expression, and thus further emphasise the differential effects of cannabinoids on NFκB and IRF signalling.

GM-CSF also exhibited treatment-dependent modulation, with THC and cannabinoid–agonist/antagonist combinations reducing its release more effectively than CBD alone. The variable responsiveness of GM-CSF may relate to its regulation by multiple transcription factors, including NFκB and IRF family members, making it more sensitive to context-dependent shifts in signalling (Schreck and Baeuerle, 1990; Fleetwood *et al.*, 2013). Similarly, C3 expression showed limited responsiveness to cannabinoid treatment, suggesting that complement-associated pathways are less directly influenced by the receptor systems targeted in this study (Liddelow *et al.*, 2017; Clarke *et al.*, 2018).

Together, these findings highlight that cannabinoid-mediated modulation of astrocyte reactivity is not uniform across inflammatory mediators. Cytokines and chemokines with strong NFκB regulation (e.g., *CXCL10*, *CCL5*) were most effectively suppressed, while those controlled by additional transcriptional or post-transcriptional pathways (e.g., *IL-6*, *C3*) exhibited more modest or inconsistent changes. This differential sensitivity underscores the complexity of astrocyte inflammatory signalling and suggests that cannabinoid effects may preferentially target specific branches of the reactive astrocyte repertoire (Sofroniew, 2020; Santiago-Balmaseda *et al.*, 2024).

5.4.4. Receptor Pathways Implicated

The present findings highlight a coordinated involvement of several receptor-associated pathways in the modulation of astrocyte reactivity by cannabinoids. Cannabinoid treatment consistently reduced NFκB nuclear translocation, indicating that suppression of this central inflammatory signalling hub is a key mechanism underlying the observed decreases in *CXCL10*, *CCL5* and other cytokines (Kozela, Juknat and Vogel, 2017; Nichols and Kaplan, 2020). NFκB is a

major transcriptional regulator of astrocyte inflammatory responses (Yeruva, Ramadori and Raddatz, 2008; Kaltschmidt and Kaltschmidt, 2009; Chi *et al.*, 2024), and its inhibition is consistent with the marked reduction of chemokine expression observed in this study.

In addition to direct or indirect modulation of NFκB, the data implicate components of the PPARγ pathway in cannabinoid-mediated inflammatory regulation (Drew *et al.*, 2006; Eraso-Pichot *et al.*, 2023; Şerban, Toader and Covache-Busuioc, 2025). CBD and THC partially restored *PPARG* mRNA levels that were suppressed by IL-1α stimulation, suggesting that cannabinoids can counteract inflammation-induced downregulation of this nuclear receptor. Notably, inhibition of PPARγ with T0070907 enhanced the anti-inflammatory effects of cannabinoids across both cytokine release and gene expression profiles, a much stronger response than that observed with the PPARγ agonist Pioglitazone. These findings suggest that endogenous PPARγ activity may constrain certain anti-inflammatory pathways in reactive astrocytes, and that cannabinoids interact with PPARγ-linked mechanisms in a context-dependent manner (Drew *et al.*, 2006; Bernardo and Minghetti, 2007; O'Sullivan, 2007).

CB₁-associated pathways also appear to contribute to the modulation of astrocyte reactivity. Treatment with the CB₁ antagonist, SR141716, reduced CXCL10, RANTES and GM-CSF release in IL-1α-stimulated astrocytes, although changes at the transcriptional level were limited. This pattern aligns with current understanding of astrocytic CB₁ signalling, which can influence intracellular calcium dynamics, vesicular release and metabolic processes independently of transcriptional control (Navarrete and Araque, 2008; Busquets-Garcia, Bains and Marsicano, 2018; Eraso-Pichot *et al.*, 2023). The fact that both cannabinoids and CB₁ antagonism reduced cytokine secretion suggests that CB₁-linked pathways interact with other signalling cascades, including NFκB and PPAR-associated mechanisms, to regulate astrocyte inflammatory output.

The differential effects of Pioglitazone, T0070907 and SR141716 also emphasise that the anti-inflammatory properties of cannabinoids are not mediated through a single receptor system, but rather through the convergence of multiple pathways.

The strong synergy observed between cannabinoids and T007, along with the partial restoration of *PPARG* expression by cannabinoid treatment, points to a dynamic interplay between cannabinoid signalling and PPAR γ regulatory networks. Conversely, the limited transcriptional impact of SR141716 suggests that CB $_1$ modulation primarily affects downstream secretion mechanisms (Fernández-Moncada and Marsicano, 2023). Together, these findings indicate that cannabinoids influence astrocyte reactivity through a multifaceted mechanism involving NF κ B suppression, modulation of PPAR γ -related nuclear signalling, and regulation of CB $_1$ -linked functional pathways (Baker, Jackson and Pryce, 2007).

5.4.5. Comparison with MCC950 Findings

In contrast to the broad anti-inflammatory effects observed with cannabinoids and receptor modulators, MCC950 produced only a modest reduction in astrocyte reactivity. Although all tested concentrations of MCC950 decreased CXCL10 release, and the lowest dose reduced IFN α/β , the treatment had no effect on IL-6 or RANTES production and did not alter inflammatory gene expression. These findings suggest that inhibiting the NLRP3 inflammasome only minimally impacts the IL-1 α -induced reactive phenotype in CTX-Astros.

The subdued response likely reflects the limited engagement of canonical inflammasome pathways in human iPSC-derived astrocytes under IL-1 α stimulation. Unlike microglia, which robustly activate NLRP3 in response to inflammatory cues, astrocytes express low levels of *NLRP3*, *ASC* and upstream pattern recognition receptors such as *CD14* (Zhang *et al.*, 2014; Anderson *et al.*, 2023). Indeed, the developmental timeline in the present study revealed that *CD14* expression is high during early neural progenitor stages but declines significantly by D90, when CTX-Astros can be first used for stimulation. This low *CD14/NLRP3* expression profile aligns with recent transcriptomic studies showing that mature human astrocytes have limited capacity for inflammasome activation (Honda, Ku and Anders, 2023; Koller *et al.*, 2024).

These observations indicate that the inflammatory pathways predominantly driving IL-1 α -induced astrocyte reactivity rely on NF κ B and JAK/STAT signalling rather than NLRP3-dependent mechanisms (Liddel *et al.*, 2017; Sofroniew, 2020). Consequently, targeting the inflammasome with MCC950 provides only restricted benefit in this cellular context. The contrast with the broader effects of cannabinoids and receptor modulators reinforces the conclusion that astrocyte inflammatory signalling is pathway-specific, and that effective modulation requires targeting the transcriptional and signalling hubs most relevant to IL-1 α -driven reactivity.

5.4.6. Study Limitations

Although the present study provides valuable insight into how cannabinoids and receptor-associated pathways modulate astrocyte reactivity, several limitations should be acknowledged. First, the analysis was restricted to acute IL-1 α stimulation and short-term cannabinoid exposure. Astrocytes respond dynamically to the duration and intensity of inflammatory cues, and chronic or repeated stimulation can induce distinct transcriptional states that may respond differently to cannabinoid modulation (Reid and Kuipers, 2021; Verkhatsky *et al.*, 2023; Saliu and Zhao, 2024; Derevyanko, Tao and Allen, 2025). Long-term exposure would therefore be valuable to determine whether the observed anti-inflammatory effects persist, attenuate or become maladaptive over time.

This study focuses on astrocytes in isolation. Although monoculture systems are essential for mechanistic clarity, astrocyte behaviour *in vivo* is strongly shaped by interactions with microglia, neurons and endothelial cells. In particular, microglia are major producers of IL-1 α and strongly influence astrocyte reactivity (Jha, Lee and Suk, 2016; Liddel *et al.*, 2017; Bernaus, Blanco and Sevilla, 2020). Co-culture or organoid-based models would therefore be needed to determine how cannabinoids influence astrocyte-microglia crosstalk and whether these interactions amplify or constrain their anti-inflammatory potential.

Finally, while the study identified changes in mRNA expression of cannabinoid-related and inflammatory genes, receptor protein levels and downstream signalling events were not assessed. Protein-level validation, particularly for PPAR γ and CB $_1$, would strengthen mechanistic interpretations and help determine whether the observed transcriptional changes translate into functional consequences. Together, these limitations highlight opportunities for future work but do not detract from the overall conclusion that cannabinoids modulate multiple aspects of IL-1 α -induced astrocyte reactivity.

5.4.7. Future Directions

Building on the findings of this study, several avenues for future research should be pursued to further elucidate how cannabinoids influence astrocyte reactivity and to evaluate their therapeutic potential in neuroinflammatory contexts. First, extending the study to include chronic or repeated inflammatory stimulation would help determine whether the anti-inflammatory effects of cannabinoids persist over time. Astrocytes undergo substantial transcriptional and functional remodelling during prolonged inflammation, and it remains unclear whether cannabinoid-mediated suppression of NF κ B and chemokine release is sustained or diminishes under long-term conditions.

Second, evaluating these effects across multiple iPSC-derived astrocyte lines, including those harbouring neurodegeneration-associated mutations, would help assess the generalisability of the observed responses. Genetic background and disease-related variants can meaningfully alter inflammatory signalling, cytokine responsiveness and cannabinoid sensitivity. Incorporating astrocytes derived from PD, ALS or APOE-variant lines would therefore offer important insight into the translational relevance of cannabinoid modulation.

Third, future work should incorporate models that capture glial–glial and glial–neuronal interactions. Co-culture systems, organoids or microfluidic platforms involving microglia and neurons would be particularly valuable, as these cells provide critical cues that shape astrocyte state transitions. Understanding how

cannabinoids influence astrocyte-microglia signalling, including the IL-1 α /TNF α /C1q axis, would be especially informative given its central role in driving neurotoxic astrocyte states.

Fourth, broadening the scope of assays to include functional outcomes beyond cytokine release would help establish the full extent of cannabinoid effects on astrocyte biology. Calcium imaging, metabolic profiling, glutamate uptake assays and assessments of morphological complexity could reveal whether cannabinoids restore astrocyte homeostatic functions alongside reducing inflammation. Evaluating mitochondrial activity and oxidative stress responses would provide further mechanistic insight.

Finally, deeper molecular interrogation is warranted to clarify the specific signalling pathways engaged by cannabinoids and receptor modulators. This could include phospho-NF κ B, STAT1/3 and IRF analyses, proteomic profiling, receptor protein quantification (particularly PPAR γ and CB $_1$), and potential involvement of additional receptors such as TRPV1 or GPR55 (Navarro *et al.*, 2016; Yang, Zhou and Lehmann, 2016). Integrating these data with time-resolved transcriptomic analyses would help define the temporal hierarchy of signalling events and identify key molecular nodes that mediate cannabinoid responsiveness. Together, such studies would significantly advance our understanding of cannabinoid-astrocyte interactions and support the development of targeted therapeutic strategies for neuroinflammatory and neurodegenerative diseases.

5.4.8. Summary of Key Findings

Table 5.4.1 Summary of cannabinoid-, receptor-mediated modulation of reactive astrocytes

Experimental area	Main findings	Key markers/features affected	Overall interpretation
IL-1 α -induced astrocyte reactivity	IL-1 α induced a robust reactive astrocyte phenotype in CTX-Astros	$\uparrow$ IL-6, CXCL10, RANTES, GM-CSF; $\uparrow$ <i>IL6</i> , <i>CXCL10</i> , <i>CCL5</i> , <i>CSF2</i> ;	Established a reproducible inflammatory astrocyte model
CBD treatment	CBD reduced inflammatory cytokine release and inflammatory gene expression in reactive CTX-Astros	$\downarrow$ IL-6, CXCL10, RANTES; CXCL10; (strongest effect at protein level) $\downarrow$ <i>CCL5</i> ; $\downarrow$ NF κ B translocation	CBD exerted anti-inflammatory effects in reactive astrocytes
THC treatment	THC attenuated inflammatory responses, although effects varied between markers	$\downarrow$ CXCL10, GM-CSF; $\downarrow$ <i>CXCL10</i> , <i>CCL5</i> ; $\downarrow$ NF κ B translocation; (overall moderate effect)	THC modulated astrocyte inflammatory signalling
Combined CBD+THC treatment	Combined cannabinoid treatment produced broader anti-inflammatory effects than either compound alone	$\downarrow$ CXCL10, RANTES, CXCL10; $\downarrow$ <i>CCL5</i> (most effective at gene level)	Combined cannabinoid signalling enhanced immunomodulation
PPAR γ agonism (Pioglitazone)	Pioglitazone modestly reduced inflammatory responses in IL-1 α -stimulated astrocytes	$\downarrow$ CXCL10, RANTES, GM-CSF; $\downarrow$ <i>CCL5</i> (moderate effect overall)	PPAR γ activation partially suppresses astrocyte reactivity
PPAR γ antagonism (T0070907)	T0070907 altered inflammatory signalling and modified cannabinoid responses	$\downarrow$ CXCL10, IL-6, GM-CSF; $\downarrow$ <i>CXCL10</i> , <i>IL6</i> , <i>CCL5</i> , <i>CSF2</i> , <i>TLR4</i> (most effective overall)	PPAR γ signalling contributes to cannabinoid-mediated astrocyte regulation
CB1 antagonism (SR141617)	SR141617 modulated inflammatory responses and altered cannabinoid efficacy	$\downarrow$ RANTES, GM-CSF; $\uparrow$ C3 (least effective overall)	CB1 signalling contributes to regulation of astrocyte inflammatory activity
Cannabinoids combined with receptor modulators	Combined treatments produced differential effects depending on receptor pathway targeted	Cannabinoids combined with T007 most anti-inflammatory potential at gene and protein level	Cannabinoid effects are partly mediated through PPAR γ and CB1-associated pathways
NLRP3 inhibition (MCC950)	MCC950 produced limited effects in IL-1 α -stimulated CTX-Astros	Minor decrease in CXCL10 and IFN α / β	NLRP3 signalling may play a limited role in this astrocyte model
Baseline treatment controls	Treatment with cannabinoids or receptor modulators alone did not significantly alter astrocyte viability or inflammatory profile	No changes in viability, cytokine release or inflammatory gene expression	Cannabinoids and receptor modulators show potential in their modulation of neuroinflammation

5.4.9. Concluding Remarks

In summary, this chapter demonstrates that cannabinoids exert measurable anti-inflammatory effects in human iPSC-derived cortical astrocytes and modulate multiple components of the IL-1 α -induced reactive astrocyte phenotype. CBD, THC and their combination reduced key inflammatory mediators, including CXCL10, IL-6, RANTES and GM-CSF, with CBD+THC often producing the strongest chemokine suppression. These effects were accompanied by reduced NF κ B nuclear translocation and downregulation of inflammatory gene expression, highlighting suppression of central transcriptional pathways as a major mechanism of action.

Pharmacological manipulation of cannabinoid-related receptor systems further clarified the signalling pathways responsible for these effects. Inhibition of PPAR γ with T0070907 markedly enhanced cannabinoid-mediated suppression of inflammatory outputs, whereas PPAR γ agonism with Pioglitazone had a more selective and modest impact. CB $_1$ antagonism similarly reduced cytokine release but produced limited transcriptional changes, suggesting a stronger influence on secretion or downstream effector processes. These findings together indicate that cannabinoids modulate astrocyte reactivity through the combined engagement of NF κ B, PPAR γ -associated and CB $_1$ -linked pathways, rather than a single receptor mechanism.

In contrast, MCC950 showed only a subdued anti-inflammatory effect, consistent with low expression of CD14 and limited inflammasome competence in mature human astrocytes. This comparison reinforces that IL-1 α -driven astrocyte reactivity is mediated primarily by NF κ B- and JAK/STAT-associated cascades, and that targeting the NLRP3 inflammasome provides minimal benefit in this context.

Collectively, the data presented in this chapter provide a comprehensive characterisation of cannabinoid effects on human astrocyte inflammatory states and identify specific receptor-associated pathways that shape their responsiveness. These findings not only extend current understanding of how

cannabinoids regulate astrocyte biology but also highlight their potential as modulators of neuroinflammation in disease contexts where astrocyte reactivity contributes to pathology. The mechanistic insight gained here lays the foundation for future work exploring cannabinoid-based or cannabinoid-adjacent therapeutic strategies in more complex cellular and disease-relevant models.

Chapter 6. Overall Discussion

6.1. Overall Synthesis of Key Findings

Astrocytes are increasingly recognised as central regulators of neuroinflammation, metabolic homeostasis and synaptic integrity in neurodegenerative disease. The work presented in this thesis aimed to dissect how three major axes of astrocyte vulnerability, regional identity, APOE genotype and exposure to A β and inflammatory cytokines, shape astrocyte reactivity in human induced pluripotent stem cell-derived models. Together, the findings show that astrocyte dysfunction arises from the convergence of intrinsic developmental programming, genetic susceptibility and pathological stimuli, and that this dysfunction is mechanistically linked to metabolic instability and inflammatory amplification.

The first major outcome of this thesis is the demonstration that human astrocytes retain region-specific properties following directed differentiation. CTX and VM-Astors differed in morphology, protein expression and baseline cytokine profiles, and exhibited distinct metabolic characteristics. CTX-Astros displayed a more reactive phenotype, with enhanced cytokine release and a greater shift toward glycolysis following stimulation. In contrast, VM-Astros showed comparatively muted inflammatory responses and more stable glycolytic respiration. These findings align with observations in rodent and human tissue showing region-specific astrocyte transcriptional signatures and immune responsiveness (Hasel *et al.*, 2017; Batiuk *et al.*, 2020; Bayraktar *et al.*, 2020), suggesting that intrinsic regional identity may contribute to selective vulnerability in neurodegenerative disease.

The second major finding concerns the profound influence of APOE genotype on astrocyte phenotype. Using isogenic APOE2/2, APOE3/3 and APOE4/4 iPSC-derived astrocytes, the data show that APOE4 establishes a baseline state of molecular and metabolic priming characterised by elevated inflammatory gene expression, reduced glutamate homeostatic markers and impaired glycolysis. This priming effect manifested in exaggerated cytokine release and metabolic deficits upon inflammatory stimulation. APOE2 astrocytes exhibited the most resilient profile, consistent with the protective effects observed in clinical and genetic studies (Liu *et al.*, 2013; Yamazaki *et al.*, 2019). These results reinforce the concept that APOE4

is not merely a risk factor but a driver of altered glial physiology that predisposes the brain to early dysfunction.

The third major line of investigation demonstrated that A β oligomers induce metabolic stress and moderate inflammatory activation, with A β_{1-42} producing stronger effects than A β_{1-40} . Importantly, A β exposure alone did not recapitulate the full inflammatory phenotype observed in vivo, but co-stimulation with IL-1 α produced synergistic inflammatory and metabolic disturbances, particularly in APOE4 astrocytes. These results support a “two-hit” model in which A β primes astrocytes metabolically, while cytokines from microglial activation drive the transition into a highly reactive state (Benilova, Karran and De Strooper, 2012; Liddelow *et al.*, 2017; Perez-Nievas and Serrano-Pozo, 2018).

A unifying theme emerging from these results is the centrality of astrocyte metabolism. Across all experimental paradigms, a shift toward glycolysis correlated strongly with inflammatory activation. CTX-Astros stimulated with TIC and A β_{1-40} exhibited increased oxidative phosphorylation and increased spare respiratory capacity. These findings are consistent with growing evidence that astrocyte metabolic reprogramming is an early driver of neuronal vulnerability in AD (Allaman, Bélanger and Magistretti, 2011; Demetrius and Simon, 2012).

Finally, the therapeutic section of this thesis demonstrated that astrocyte reactivity is mechanistically targetable. Cannabinoids (CBD and THC), PPAR γ agonist and antagonist, CB1 antagonist and inflammasome inhibition (MCC950) all modulated inflammatory and metabolic phenotypes, with CBD's effects shown to be partially PPAR γ -independent (Carrier, Auchampach and Hillard, 2006; Kozela *et al.*, 2010; Ruiz-Valdepeñas *et al.*, 2011). These findings highlight actionable molecular pathways that may mitigate early astrocyte dysfunction.

Together, these results support a conceptual model in which neuroinflammation acts as a Trojan Horse, silently destabilising astrocyte homeostasis across multiple vulnerability axes, regional identity, APOE genotype and A β exposure, long before overt neurodegeneration occurs.

6.2. The Role of Astrocytes in Neuroinflammation

Astrocytes are now recognised as dynamic regulators of inflammation, metabolism and synaptic homeostasis. The findings of this thesis align with and extend this evolving perspective by demonstrating how regional identity, APOE genotype, amyloid exposure and metabolic state collectively define astrocyte vulnerability. Across all experimental chapters, human iPSC-derived astrocytes emerge not as passive responders to neuronal injury but as early and crucial participants in the trajectory of neurodegenerative stress.

6.2.1. Astrocyte Heterogeneity and Regional Identity

Astrocytes exhibit substantial regional heterogeneity in morphology, transcriptional identity and functional specialisation (Zhang *et al.*, 2016; Batiuk *et al.*, 2020; Bayraktar *et al.*, 2020). The present work demonstrates that CTX- and VM-Astros retain these intrinsic differences, with CTX-Astros showing stronger inflammatory responses and greater metabolic disruption following stimulation. This observation closely parallels transcriptomic studies reporting region-specific enrichment of genes linked to cytokine signalling, glutamate metabolism and oxidative stress regulation (Batiuk *et al.*, 2020; Bayraktar *et al.*, 2020). *In vivo* evidence similarly indicates that cortical astrocytes are preferentially prone to NFkB-mediated activation during neurodegeneration, whereas ventral midbrain astrocytes exhibit enhanced antioxidant and mitochondrial resilience consistent with the metabolic demands of dopaminergic circuits (Booth, Hirst and Wade-Martins, 2017). The persistence of regional identity *in vitro* reinforces the view that neurodegenerative diseases exploit pre-existing astrocytic susceptibilities, rather than affecting all astrocytes uniformly.

6.2.2. APOE Genotype and Astrocyte Reactivity

APOE genotype remains the strongest genetic determinant of late-onset Alzheimer's disease risk, and extensive evidence implicates astrocytes as central mediators of APOE-dependent vulnerability (Liu *et al.*, 2013; Huang and Mahley, 2014; Yamazaki *et al.*, 2019). The heightened inflammatory reactivity of APOE4 astrocytes in this thesis is consistent with prior studies demonstrating increased

cytokine production, complement activation and oxidative stress in APOE4 backgrounds (Tcw *et al.*, 2017; Lin *et al.*, 2018).

The behaviour of APOE2 astrocytes further clarifies genotype-dependent vulnerability. Although APOE2 is associated with reduced AD risk, APOE2 astrocytes displayed increased IL-6 and GM-CSF secretion under inflammatory stimulation. This is consistent with earlier findings that IL-6 signalling can have both neuroprotective and neurotoxic properties, depending on context (Maezawa *et al.*, 2006; Li *et al.*, 2020). Meanwhile, APOE3 astrocytes showed comparatively attenuated cytokine responses. Together, these patterns support a model in which APOE genotype establishes astrocyte-intrinsic thresholds for reactivity, metabolic efficiency and resilience to perturbation.

6.2.3. Amyloid–Astrocyte Interactions in Human Cells

Human iPSC-derived astrocytes provide a powerful system for studying amyloid responses due to species differences in A β aggregation and toxicity (Benilova, Karran and De Strooper, 2012). While some literature indicates that A β_{1-42} exerts stronger bioenergetic and inflammatory effects than A β_{1-40} , the present work found more nuanced differences, with A β_{1-40} producing greater glycolytic induction in some contexts. Nevertheless, A β exposure impaired mitochondrial function and provoked modest cytokine release. Notably, A β synergised strongly with IL-1 α to produce amplified inflammatory responses, echoing the mechanism described by Liddelow *et al.* (2017), in which microglia-derived cytokines drive astrocytes toward reactive phenotypes.

6.2.4. Metabolic Reprogramming as a Central Feature of Astrocyte Reactivity

Across regional identity, APOE genotype and amyloid stimulation, astrocyte metabolism consistently emerged as a central axis of vulnerability. Healthy astrocytes rely on coordinated glycolysis, oxidative phosphorylation and antioxidant defence to support neuronal function (Allaman, Bélanger and Magistretti, 2011). CTX-Astros displayed higher glycolytic rate and glycolytic capacity than VM-Astros. Stimulation with A β_{1-40} also impaired mitochondrial

function. These findings align with work demonstrating early metabolic impairment in astrocytes in AD models (Zhang, Alshakhshir and Zhao, 2021), that neuroinflammatory signalling promotes glycolytic reprogramming linked to NFκB and STAT3 activation (Kaltschmidt *et al.*, 1997; Liu *et al.*, 2017) and that Aβ disrupts metabolic homeostasis (Yamazaki *et al.*, 2019). Metabolic fragility thus appears to act as an integrative failure point, lowering the threshold for astrocyte reactivity and diminishing neuronal support capacity.

6.2.5. IL-6 as a Dual-Role Cytokine in Astrocyte Reactivity

The high levels of IL-6 transcription and secretion observed in this thesis should not be interpreted exclusively as evidence of detrimental or pathogenic astrocyte activation. IL-6 is well recognised as a cytokine with fundamentally context-dependent functions in the CNS. Although traditionally associated with pro-inflammatory responses, IL-6 can also exert neuroprotective, regenerative and anti-inflammatory effects, depending on the cellular environment, receptor availability and stage of disease. Several studies have shown that IL-6 promotes neuronal survival, astrocyte-mediated metabolic support and protection against excitotoxicity under moderate or acute stress conditions (Scheller *et al.*, 2011; Erta, Quintana and Hidalgo, 2012; Rose-John, 2012). IL-6 classical signalling, which depends on the membrane-bound IL-6 receptor, is particularly associated with neurotrophic and repair-promoting outcomes, including enhancement of glucose metabolism, support of synaptic function and facilitation of injury resolution. In contrast, IL-6 trans-signalling, mediated through soluble IL-6 receptor, tends to dominate during chronic inflammation and contributes more directly to sustained immune activation and tissue dysfunction (Jones, Scheller and Rose-John, 2011; Rose-John, 2012; Rose-John *et al.*, 2023).

This duality means that elevated IL-6 release from astrocytes is not inherently pathological; rather, its impact depends on whether it participates in supportive classical signalling or maladaptive trans-signalling pathways. Notably, IL-6 upregulation in APOE2 astrocytes, observed here and in previous reports (Maezawa *et al.*, 2006), may reflect a compensatory or protective response, as APOE2 is associated with reduced Alzheimer's disease risk and enhanced resilience.

Therefore, while IL-6 is a key marker of astrocyte reactivity, its functional significance cannot be inferred solely from its magnitude. Instead, IL-6 should be viewed as a multifunctional cytokine whose biological effects depend on the broader inflammatory milieu, the balance of receptor signalling modes and the metabolic status of the responding cells.

6.2.6. Therapeutic Modulation of Astrocyte Reactivity

The therapeutic findings, discussed at large in Chapter 5 Discussion, demonstrate that astrocyte reactivity is mechanistically tractable. Cannabinoids and T0070907 suppressed NF κ B-linked cytokine production, consistent with earlier descriptions of cannabinoid-mediated anti-inflammatory effects (Esposito *et al.*, 2011) and PPAR γ -dependent modulation of glial activation (Heneka *et al.*, 2015). The anti-inflammatory effects of pioglitazone hydrochloride and SR141716 were much more subdued than those of cannabinoids on their own, or T0070907 administration. Collectively, these findings suggest that cannabinoid- and receptor-associated signalling pathways may converge on shared inflammatory transcriptional mechanisms within reactive astrocytes. A proposed schematic summarising these potential signalling interactions is presented in **Figure 6.2.1** below.

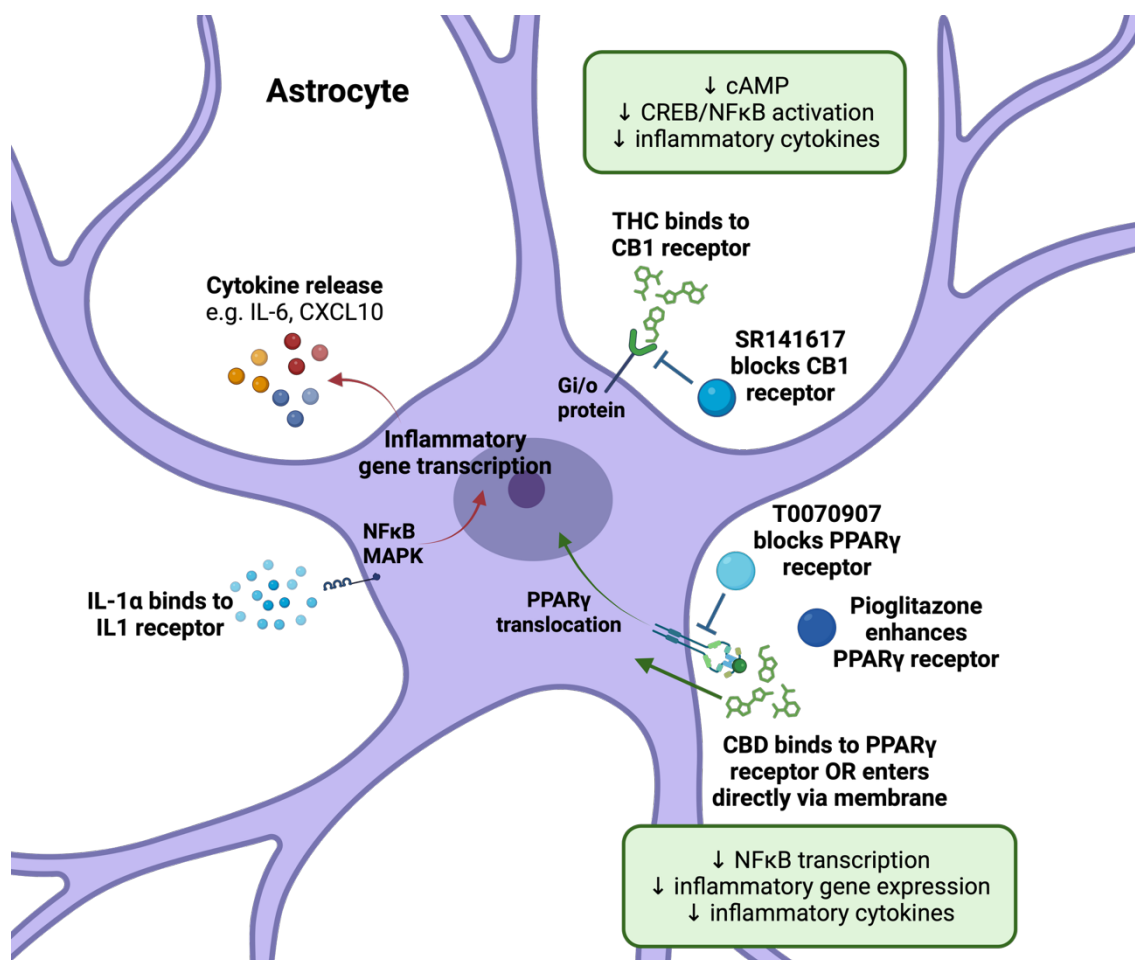


Figure 6.2.1 Proposed mechanisms of cannabinoid-, CB_1 - and $PPAR_\gamma$ -mediated modulation of astrocyte inflammatory signalling.

Stimulation with IL-1 α activates NF κ B- and MAPK-associated inflammatory signalling pathways in reactive astrocytes, promoting inflammatory gene transcription and cytokine release. THC may exert anti-inflammatory effects through activation of the Gi/o-coupled CB_1 receptor, resulting in decreased cAMP signalling and modulation of CREB/NF κ B activation. CBD may additionally modulate astrocyte inflammatory responses through activation and nuclear translocation of $PPAR_\gamma$, leading to suppression of NF κ B-mediated inflammatory gene expression. Pharmacological modulation of these pathways using the $PPAR_\gamma$ agonist Pioglitazone hydrochloride, $PPAR_\gamma$ antagonist T0070907 and CB_1 antagonist SR141617 was used to investigate the contribution of receptor-associated signalling to cannabinoid-mediated regulation of astrocyte reactivity, with T0070907 exerting further anti-inflammatory properties when used alone or in combination with cannabinoids. Schematic created with BioRender.com.

6.2.7. Neuroinflammation as a Trojan Horse in Neurodegeneration

Together, these findings support a conceptual framework in which neuroinflammation operates as a Trojan Horse in Alzheimer's disease. Latent vulnerabilities are embedded in astrocytes through regional identity, APOE genotype and metabolic configuration long before pathological stimuli emerge. These intrinsic features create a primed yet clinically silent state in which modest inflammatory or amyloidogenic cues provoke disproportionately large reactions. A β alone produced limited activation, but in combination with IL-1 α , CTX-Astros transitioned into reactive states marked by increased glycolytic rate and amplified cytokine output. This reflects a progression in which initial perturbations enter the cellular system almost unnoticed, destabilising metabolic and inflammatory balance from within and rendering astrocytes increasingly susceptible to subsequent stress.

Metabolic fragility appears central to this infiltration process. Declining mitochondrial function and increased glycolysis reduce the energetic capacity required for neuronal support and lowers the threshold for reactive state induction. As this instability accumulates, astrocytes become less able to maintain synaptic and metabolic homeostasis, contributing to a silent erosion of neuronal resilience well before clinical symptoms arise. Crucially, the therapeutic interventions in this thesis demonstrate that these early infiltrative processes are reversible, highlighting astrocyte metabolism–inflammation cross-talk as a promising entry point for disease modification. In reframing neuroinflammation as an early covert infiltrator rather than a secondary consequence of neuronal injury, this thesis positions astrocytes as primary coordinators of neurodegenerative vulnerability and identifies new mechanistically tractable opportunities for early intervention.

6.3. Strengths and Limitations

The experimental framework of this thesis brings together human iPSC-derived astrocyte models, isogenic genetic systems, amyloidogenic and inflammatory stimuli, and targeted pharmacological perturbation. This integrative strategy provides a powerful platform for examining how regional identity, APOE genotype and A β exposure shape astrocyte reactivity. However, as with all *in vitro* approaches, there are inherent strengths and limitations that influence the interpretation and translational relevance of the findings.

A major strength of this work is its exclusive use of human iPSC-derived astrocytes, which offer greater physiological relevance than rodent glia. Human astrocytes differ from rodent astrocytes in morphology, transcriptional complexity and immune responsiveness (Zhang *et al.*, 2016). Rodent A β also differs by three amino acids, making it less aggregation-prone and less toxic than human A β (Benilova, Karran and De Strooper, 2012). By using human cells, this thesis bypasses several species-specific inconsistencies that have historically contributed to translational failures in AD research. The ability to study A β and cytokine responses in cells that more faithfully model human glial biology therefore greatly strengthens the validity of the mechanistic insights generated.

Another key advantage is the use of regionally patterned astrocytes derived from cortical and ventral midbrain progenitors. Few studies have systematically examined how intrinsic regional identity shapes human astrocyte behaviour. The finding that these region-specific properties persist *in vitro*, affecting baseline morphology, inflammatory thresholds and metabolic resilience, would not have been possible without this dual-lineage approach. It provides a rare human-cell perspective on the longstanding observation that different brain regions exhibit unique vulnerabilities in neurodegenerative disease.

The inclusion of isogenic APOE2/2, APOE3/3 and APOE4/4 astrocytes represents another important methodological strength. Because all three variants were generated within the same genetic background, observed differences in inflammation, metabolism or gene expression can confidently be attributed to

APOE genotype rather than inter-donor variability. This level of genetic control is essential for interpreting APOE-driven phenotypes, especially given the subtle but widespread transcriptional and metabolic disturbances associated with APOE4 (Huang and Mahley, 2014). The inclusion of APOE2, a protective variant often overlooked in mechanistic studies, further enhances the interpretive power of the dataset by providing a full allele spectrum.

A further strength is the integration of inflammatory, amyloidogenic and metabolic paradigms, which reflects the multifactorial nature of AD pathology. Many previous studies examine only a single stimulus, making it difficult to understand how pathogenic factors interact *in vivo*. By systematically combining A β exposure with cytokine stimulation and assessing outcomes across regional and genetic backgrounds, this thesis provides a more holistic view of astrocyte vulnerability. The addition of Seahorse metabolic profiling adds another layer of mechanistic insight by revealing how mitochondrial and glycolytic pathways are affected by region, genotype and pathology. This is valuable because astrocyte bioenergetics are increasingly recognised as central to neuroinflammatory and neurodegenerative processes (Allaman, Bélanger and Magistretti, 2011; Zhang, Alshakhshir and Zhao, 2021).

Mechanistic pharmacology further strengthens the study. The use of a PPAR γ agonist (pioglitazone), a PPAR γ antagonist (T0070907), a CB $_1$ antagonist (SR141716A) and an NLRP3 inhibitor (MCC950) allows direct testing of the pathways through which cannabinoids and inflammatory stimuli operate. This approach provides causal evidence, showing, for example, that CBD's anti-inflammatory effects are only partially PPAR γ -dependent, which adds a high degree of mechanistic clarity quite rarely achieved in human astrocyte studies to date.

Despite these strengths, several limitations must also be acknowledged. Foremost is the absence of microglia and neurons. Astrocytes *in vivo* operate within complex signalling networks involving multiple cell types, particularly microglia, which produce many of the cytokines used in this thesis. Although TIC stimulation was employed to approximate microglial cues, co-culture systems or organoids would

provide richer insight into reciprocal glial interactions, synaptic consequences and disease propagation. Still, the monoculture design was intentional and appropriate for isolating astrocyte-intrinsic mechanisms without confounding cellular influences.

The limitations of 2D culture systems must also be acknowledged. Astrocytes grown on plastic cannot fully recapitulate 3D structural organisation, vasculature interactions, perivascular end-feet or synaptic territory mapping. However, 2D cultures remain the gold standard for tightly controlled mechanistic studies and are compatible with quantitative methods such as Seahorse assays, qPCR and ELISA.

Another limitation relates to the use of synthetic A β oligomers, which, although widely used, differ structurally from patient-derived aggregates and represent acute exposures rather than chronic deposition. This means the model captures early A β -induced stress rather than long-term plaque-associated pathology. However, oligomeric A β is considered the most toxic species in early AD (Benilova, Karran and De Strooper, 2012), and the controlled preparation used here ensures reproducibility across experiments.

Finally, the time scale of *in vitro* stimulation does not capture the chronicity of AD pathology, which unfolds over years. Acute perturbations nevertheless provide critical mechanistic insight into the initial steps of astrocyte dysfunction, which is consistent with the early pathological focus of this thesis.

In summary, while limitations in cellular complexity and chronicity temper the degree to which these findings can be generalised to the human brain, none undermine the central conclusions. The experimental strengths, including the use of human, isogenic and region-specific astrocytes; combined inflammatory and amyloidogenic perturbation; integrated metabolic profiling; and mechanistic pharmacology, provide a rigorous and translationally relevant foundation for interpreting astrocyte behaviour in AD. These strengths also create a springboard for future research, which can build upon the mechanistic insights presented here by moving into more complex multicellular and long-term systems.

6.4. Future Directions

The findings presented in this thesis illuminate several mechanisms by which regional identity, APOE genotype and amyloidogenic or inflammatory stimuli shape astrocyte behaviour in AD. At the same time, they reveal important gaps that future research must address to develop a comprehensive, physiologically grounded model of astrocyte function in neurodegeneration. Building on the structures developed here, subsequent work should incorporate increasingly complex human models, the assessment of A β stimulation in terms of cytokine release, gene expression and metabolic function on APOE isogenic lines, multi-omic approaches and long-term paradigms to deepen our understanding of early astrocyte dysfunction and translate mechanistic insight into therapeutic strategies.

A priority for future studies is the development of more physiologically relevant experimental systems that capture the intercellular signalling networks and three-dimensional architecture of the human brain. Although monoculture systems were essential in this thesis for isolating astrocyte-intrinsic responses, they necessarily omit the influence of microglia, neurons and vascular cells. Incorporating iPSC-derived microglia into co-culture systems would enable analyses of bidirectional glial signalling, reciprocal cytokine loops and the dynamic role of microglia in inducing and sustaining astrocyte reactivity. This is particularly relevant because microglia-derived cytokines such as IL-1 α , TNF α and C1q drive A1-like phenotypes *in vivo*, and understanding how APOE4 astrocytes influence or respond to microglial activation will be critical for clarifying early AD pathogenesis. Similarly, incorporating neurons into astrocyte–neuron co-cultures or assembloids would allow examination of metabolic coupling, glutamate cycling and lactate shuttling, all of which are processes that are tightly linked to astrocyte metabolic resilience and are likely affected by APOE genotype and A β exposure.

The expansion into three-dimensional (3D) organoids and region-specific assembloids represents another promising direction. Organoid technologies now permit the generation of cortical, hippocampal or midbrain-like structures, while assembloids allow fusion of distinct regional modules. Applying the frameworks

developed here in 3D could determine whether the region-specific vulnerabilities observed in 2D cultures persist in a tissue-like environment containing gradients, extracellular matrix components and endogenous signalling niches. Moreover, 3D systems support long-term culture, enabling modelling of chronic A β exposure, progressive inflammatory adaptation and the emergence of plaque-like aggregates, all of which are processes that cannot be recreated in short-term 2D systems.

Future studies should also incorporate single-cell and spatial transcriptomic approaches to resolve astrocyte subpopulations that may exhibit differential susceptibility to inflammatory or amyloidogenic stress. Although the D80–D100 astrocyte cultures used in this thesis were relatively homogeneous, even subtle transcriptional heterogeneity may prove biologically meaningful. Single-cell RNA sequencing could identify early reactive states, APOE4-driven priming signatures or A β -responsive clusters that are masked by bulk assays. Spatial transcriptomics applied to organoids would further enable mapping of astrocytic transcriptional states onto microenvironments, revealing whether APOE genotype or A β exposure drives specific astrocyte phenotypes to occupy particular niches associated with metabolic stress or inflammatory gradients.

Another important direction is the incorporation of chronic stimulation paradigms that better reflect the temporal dynamics of AD. While the acute exposures used in this thesis provide mechanistic clarity, AD pathology develops over decades. Long-term exposure to low concentrations of A β , microglial-derived cytokines or oxidative stress could reveal adaptive or maladaptive astrocyte trajectories not captured in short-term experiments. Sequential stimulation paradigms, for example, A β followed by cytokines, or vice versa, may reveal order-of-exposure effects, providing insight into how early amyloid stress sensitises astrocytes to subsequent inflammatory activation. These models may also clarify the tipping point at which metabolic compensation fails, allowing identification of the earliest irreversible steps in astrocyte dysfunction.

Future research should further explore therapeutic and mechanistic interventions, particularly those targeting PPAR γ , NLRP3, cannabinoid signalling and mitochondrial resilience. The clear, PPAR γ -independent anti-inflammatory effects

of CBD observed in this thesis raise the question of whether cannabinoids depend on any receptors to manifest their anti-inflammatory action, or if they do so via non-receptor mediated mechanisms, such as passive diffusion, similar to many other highly lipophilic drugs. Expanding the pharmacological toolkit to include a broader range of cannabinoids (such as CBG, CBC or β -caryophyllene), synthetic agonists or allosteric modulators could help delineate the relative contributions of PPAR γ -, CB1-, CB2- or TRPV1-mediated pathways.

Given the central role of APOE genotype in determining astrocyte behaviour, A β -stimulation should be included across the APOE2/2, APOE3/3 and APOE4/4 isogenic lines to further elucidate the complex relationship between APOE4 and A β aggregation. Cytokine release, transcriptomic analysis as well as the metabolic stress tests, such as the Seahorse Glycolysis Stress Test should form the basis of the future studies. These future studies should also apply lipidomics, proteomics and receptor signalling analyses to elucidate how APOE4 alters cholesterol trafficking, membrane composition, mitochondrial function and receptor-mediated A β uptake. Mass spectrometry-based lipidomics could reveal shifts in phospholipid species or lipid droplet dynamics, while mitochondrial proteomics could identify deficits in oxidative phosphorylation complexes or antioxidant enzymes. Such approaches would help determine whether APOE4-driven metabolic fragility originates in lipid homeostasis or mitochondrial dysfunction, or whether these processes interact in a mutually reinforcing cycle.

In summary, the next phase of research should leverage more complex human models, long-term paradigms and multi-omics tools to capture the full spectrum of astrocyte responses under physiological and pathological conditions. By extending the mechanistic foundations established in this thesis, future work can advance toward astrocyte-targeted interventions that mitigate early inflammatory and metabolic vulnerabilities, ultimately contributing to more effective therapeutic strategies for Alzheimer's disease.

6.5. Final Conclusion

Astrocytes are increasingly recognised as central regulators of CNS homeostasis and as early, active contributors to neurodegenerative disease. Through their roles in metabolism, synaptic regulation, inflammatory signalling and glial–neuronal communication, astrocytes integrate a broad array of environmental and genetic cues that shape regional vulnerability in AD. This thesis provides a comprehensive examination of how regional identity, APOE genotype and amyloidogenic or inflammatory stimuli converge to determine astrocyte behaviour in human iPSC-derived systems. Together, the findings offer mechanistic insight into early astrocyte dysfunction and reveal opportunities for therapeutic intervention that extend beyond neuron-centric models of AD.

Across three main experimental chapters, this thesis demonstrates that astrocyte reactivity is not a uniform or secondary response. Rather, it emerges from distinct intrinsic properties that set the stage for how astrocytes respond to pathological stress. Cortical astrocytes displayed heightened inflammatory reactivity and metabolic fragility compared with ventral midbrain astrocytes, providing a potential cellular explanation for the selective vulnerability of cortical regions in AD. These findings reinforce growing evidence that astrocytes retain regional identity *in vitro* and that this identity imposes fundamental constraints on their stress responses.

APOE genotype further shaped astrocyte function, with APOE4 conferring a primed inflammatory phenotype and altered gene expression. APOE4 astrocytes responded more strongly to inflammatory cytokines and produced higher levels of pro-inflammatory mediators. The inclusion of APOE2 allowed demonstration of a protective gradient, with APOE2 astrocytes maintaining more stable inflammatory signatures.

A β_{1-40} and A β_{1-42} exerted modest transcriptional effects in isolation but co-stimulation with IL-1 α revealed a synergistic interaction whereby amyloidogenic and inflammatory stimuli jointly amplified cytokine release and metabolic impairment. This supports a “two-hit” model in which A β initiates metabolic vulnerability, while inflammatory cues drive the full transition into reactive states.

Such synergy may help explain why early microglial activation is detectable in preclinical AD prior to extensive plaque formation.

A recurring theme across the thesis is that astrocyte metabolism serves as a central integrator of regional, genetic and pathological influences. Glycolytic dependence and reduced oxidative phosphorylation consistently aligned with increased inflammatory reactivity and vulnerability to A β . These findings position energy metabolism not merely as a downstream consequence of inflammation, but as a driver of astrocyte state transitions, and therefore, a promising therapeutic target.

The final experimental arm demonstrated that astrocyte reactivity is modifiable. CBD and THC suppressed cytokine release and partially restored metabolic deficits; CBDs actions were shown to be independent of PPAR γ , while THC engaged cannabinoid receptors to exhibit its anti-inflammatory function. T007, a PPAR γ antagonist, reproduced many of CBDs anti-inflammatory effects, and MCC950, an NLRP3 inhibitor, only slightly attenuated IL-1 α -driven reactivity. These observations highlight the therapeutic potential of targeting upstream inflammatory and metabolic pathways in astrocytes. Importantly, they also show that early pathological changes in astrocyte function are not fixed and can be redirected toward more homeostatic states.

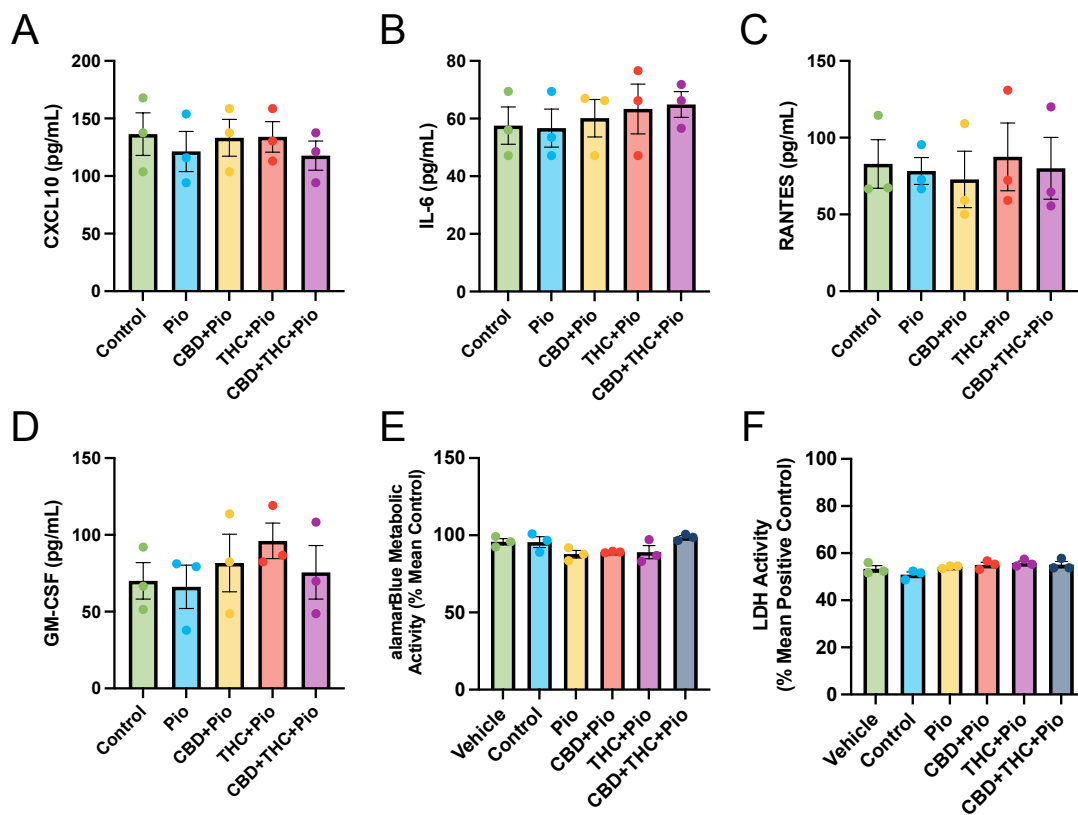
Synthesised together, the findings of this thesis support a conceptual shift: neuroinflammation operates as a Trojan Horse in neurodegeneration. It arrives early, subtly alters astrocyte metabolism and signalling, and primes the CNS for exaggerated responses to amyloid and other stressors. Regional identity, APOE genotype and A β exposure each open different “doors” through which this Trojan Horse enters, but the downstream consequences converge on inflammatory amplification, metabolic collapse and impaired neuronal support. Crucially, the data presented here show that these early processes are mechanistically tractable and therapeutically targetable.

While the work has several limitations, it also establishes a strong foundation for future research. More complex systems such as organoids, tri-cultures, chronic stimulation models and multi-omics approaches will be essential for building on

the mechanistic insights developed here. Ultimately, a more complete understanding of astrocyte biology will be critical for developing interventions that halt or reverse early pathological changes in AD.

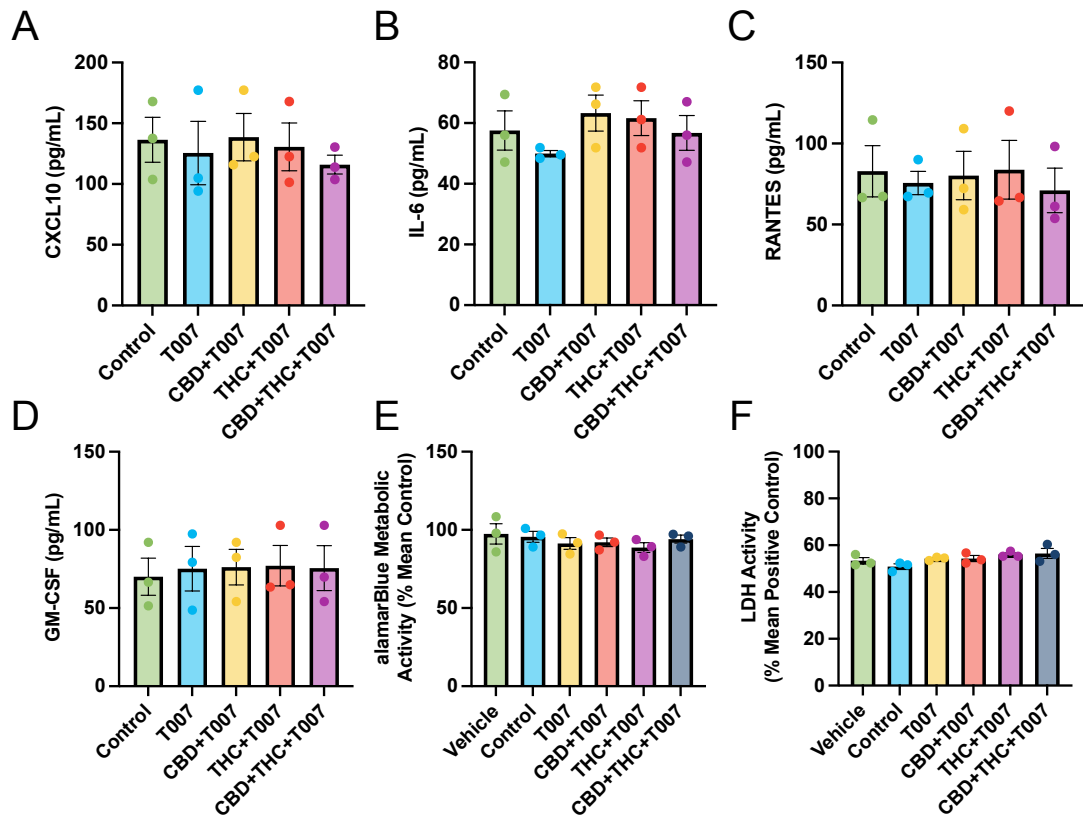
In conclusion, this thesis provides new mechanistic evidence that astrocytes are early, active drivers of neuroinflammation and metabolic dysfunction in Alzheimer's disease. It identifies regional identity, APOE genotype and A β exposure as key determinants of astrocyte vulnerability and demonstrates that therapeutic modulation of inflammatory and metabolic pathways is feasible. By integrating these insights within a unified conceptual framework, this work advances our understanding of early AD pathogenesis and points toward astrocyte-targeted therapeutic strategies that may help reshape the course of neurodegenerative disease.

Appendix 1.



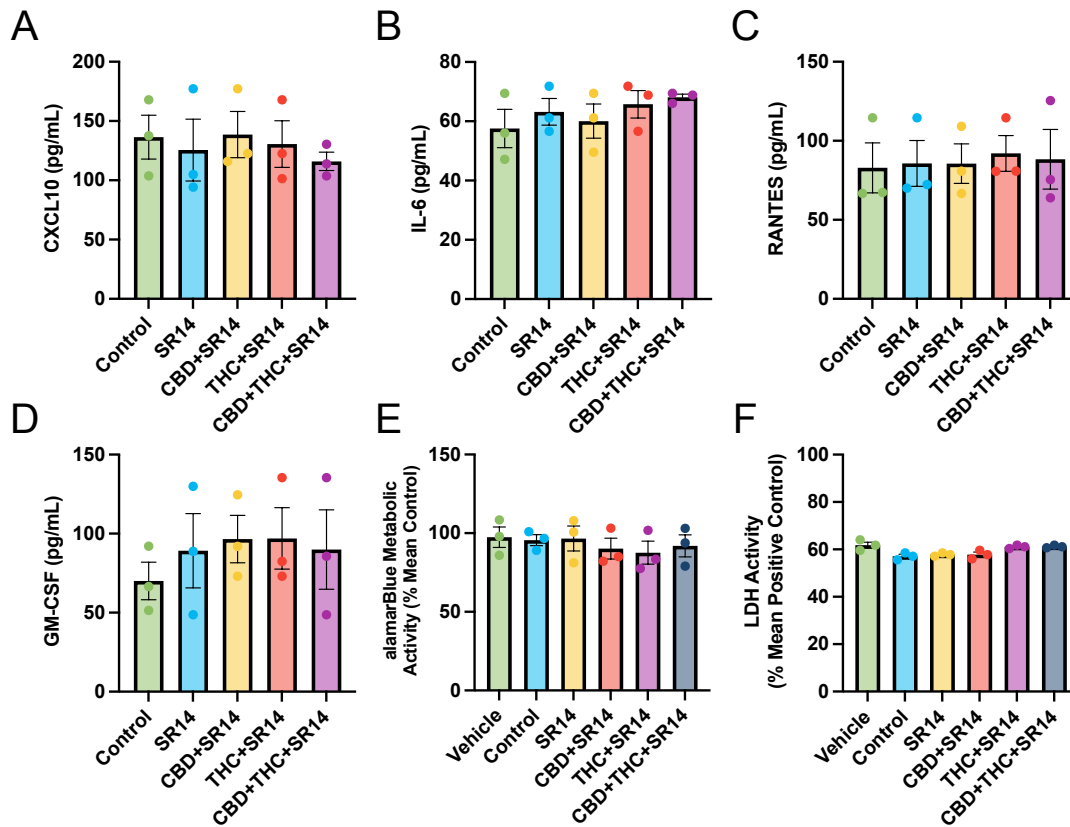
Supplemental Figure 1 Treatment with Pioglitazone hydrochloride does not have a negative effect on cytokine release or cell viability in CTX-Astros.

CTX-Astros treated with Pioglitazone hydrochloride (1 μ M) alone, or in combination with cannabinoids, did not release significantly higher levels of CXCL10 (A), IL-6 (B), RANTES (C), or GM-CSF (D). Treatment with Pioglitazone, with or without cannabinoids, also did not alter the metabolic activity (E) or LDH release (F) of CTX-Astros. Data expressed as mean $\pm$ SEM (N=3, r=3). One-way ANOVA.



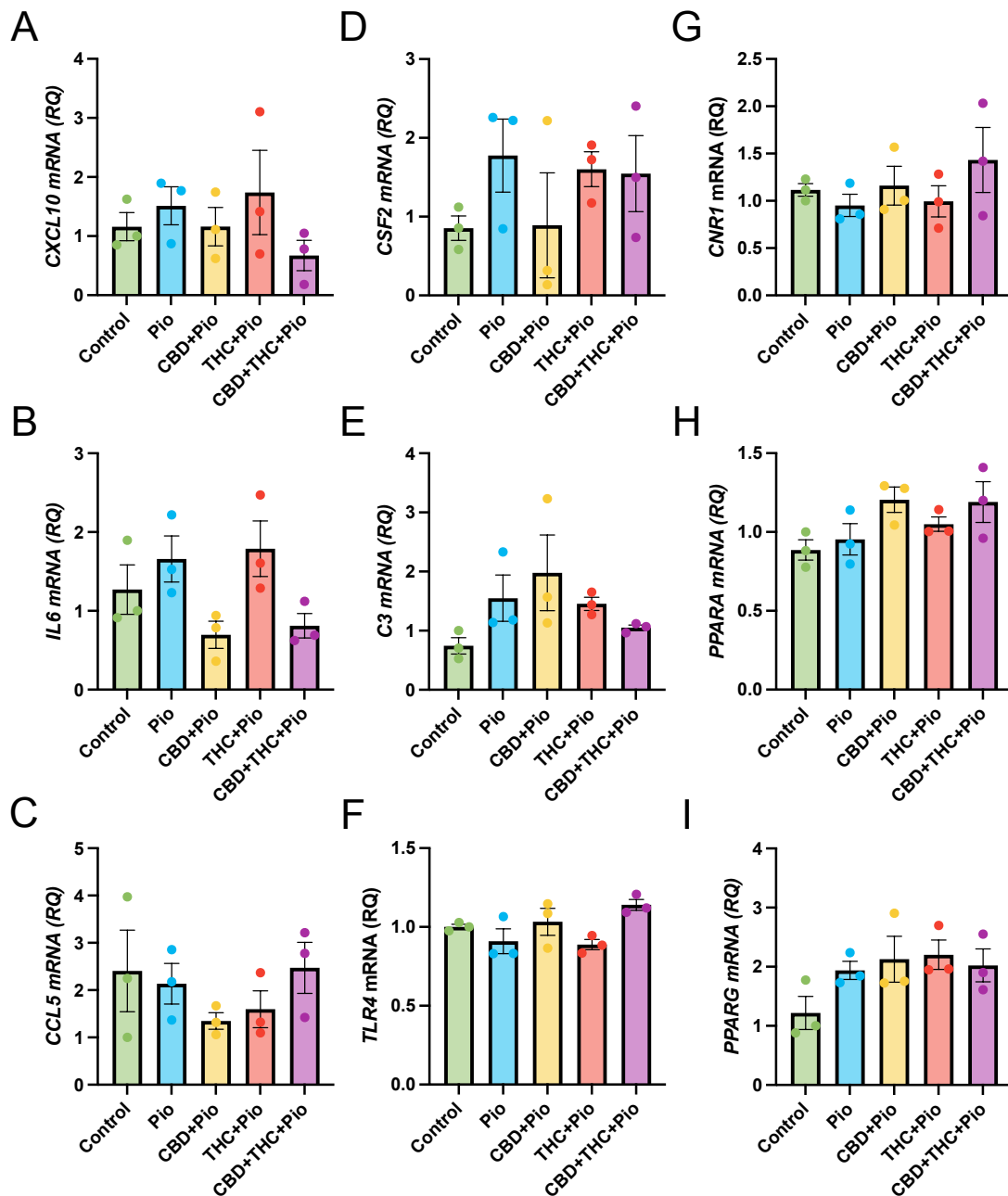
Supplemental Figure 2 Treatment with T0070907 does not have a negative effect on cytokine release or cell viability in CTX-Astros.

CTX-Astros treated with T0070907 (1 μ M) alone, or in combination with cannabinoids, did not release significantly higher levels of CXCL10 (A), IL-6 (B), RANTES (C), or GM-CSF (D). Treatment with T0070907, with or without cannabinoids, also did not alter the metabolic activity (E) or LDH release (F) of CTX-Astros. Data expressed as mean $\pm$ SEM (N=3, r=3). One-way ANOVA.



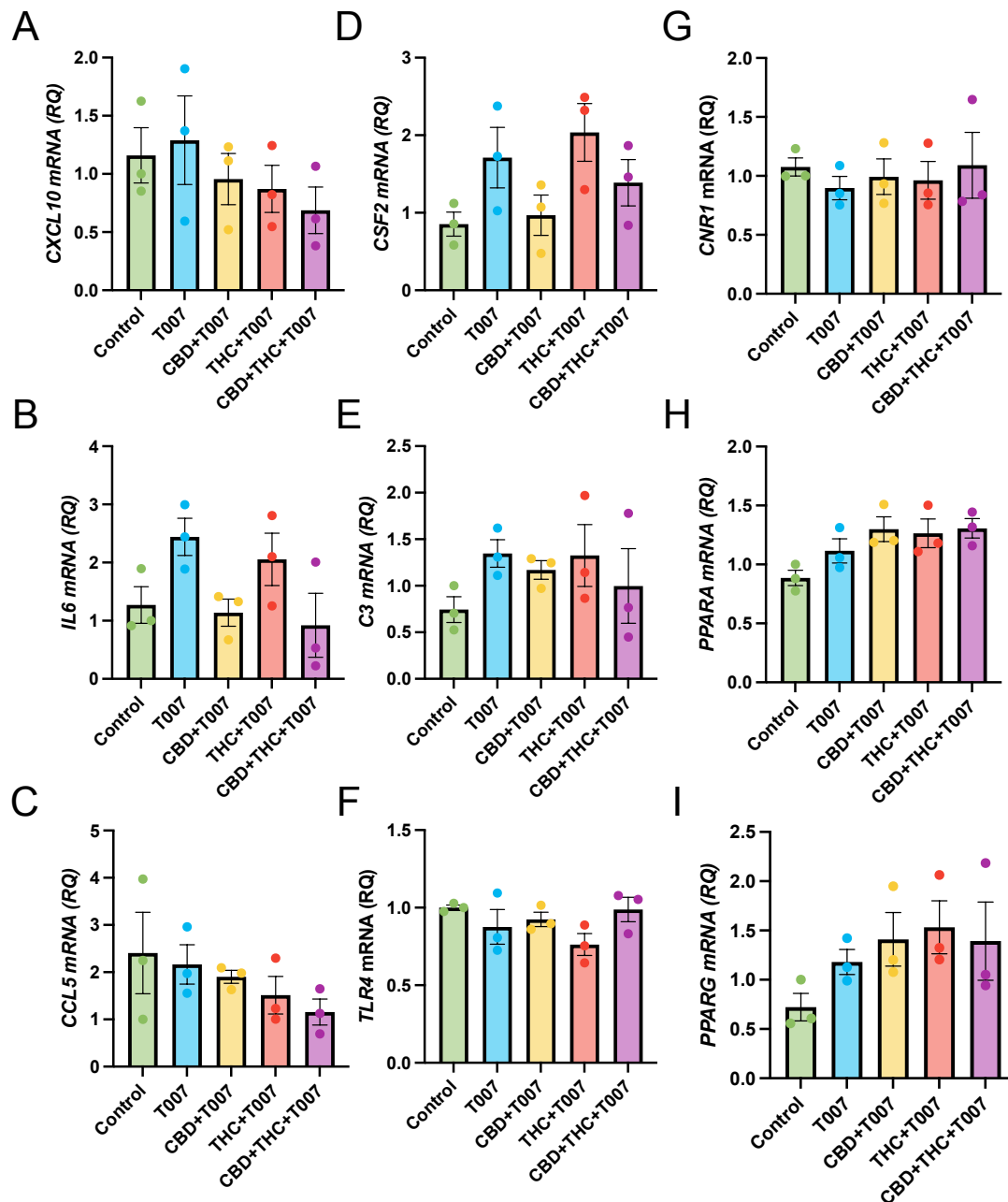
Supplemental Figure 3 Treatment with SR141617 does not have a negative effect on cytokine release or cell viability in CTX-Astros.

CTX-Astros treated with SR141617 (1 μ M) alone, or in combination with cannabinoids, did not release significantly higher levels of CXCL10 (**A**), IL-6 (**B**), RANTES (**C**), or GM-CSF (**D**). Treatment with SR141617, with or without cannabinoids, also did not alter the metabolic activity (**E**) or LDH release (**F**) of CTX-Astros. Data expressed as mean $\pm$ SEM (N=3, r=3). One-way ANOVA.



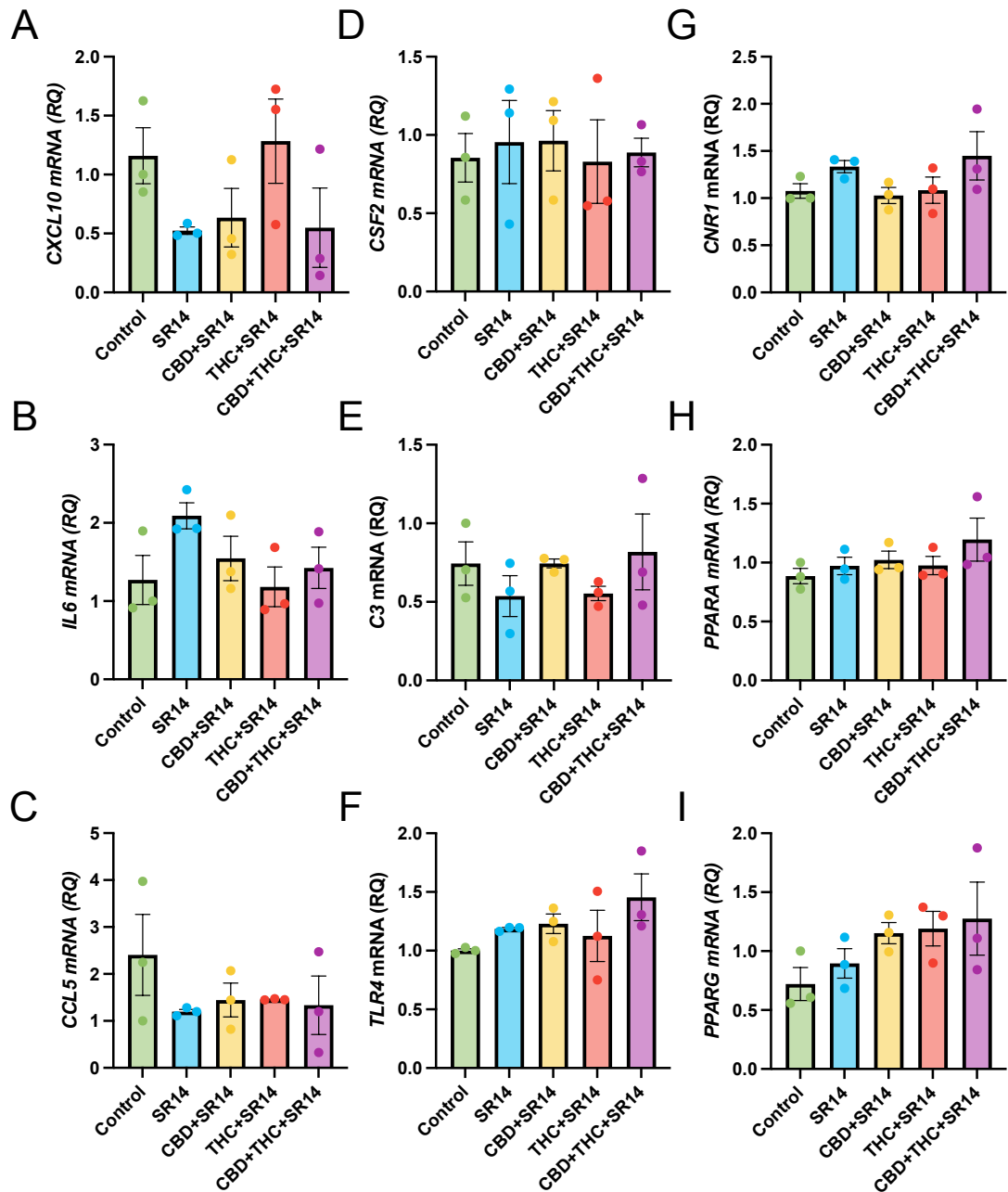
Supplemental Figure 4 Treatment with Pioglitazone hydrochloride does not have a negative effect on inflammatory gene or cannabinoid-related receptor gene expression in CTX-Astros.

CTX-Astros treated with Pioglitazone hydrochloride (1 μ M) alone, or in combination with cannabinoids, did not express significantly higher levels of CXCL10 (A), IL-6 (B), CCL5 (RANTES) (C), CSF2 (GM-CSF) (D), C3 (E) or TLR4 (F) mRNA. Treatment with Pioglitazone, with or without cannabinoids, did not alter the mRNA expression of CNR1(G), PPARA (H) or PPARG (I) in CTX-Astros either. Data expressed as mean $\pm$ SEM (N=3, r=3). One-way ANOVA.



Supplemental Figure 5 Treatment with T0070907 does not have a negative effect on inflammatory gene or cannabinoid-related receptor gene expression in CTX-Astros.

CTX-Astros treated with T0070907 (1 μ M) alone, or in combination with cannabinoids, did not express significantly higher levels of CXCL10 (A), IL-6 (B), CCL5 (RANTES) (C), CSF2 (GM-CSF) (D), C3 (E) or TLR4 (F) mRNA. Treatment with T0070907, with or without cannabinoids, did not alter the mRNA expression of CNR1(G), PPARA (H) or PPARG (I) in CTX-Astros either. Data expressed as mean $\pm$ SEM (N=3, r=3). One-way ANOVA.



Supplemental Figure 6 Treatment with SR141617 does not have a negative effect on inflammatory gene or cannabinoid-related receptor gene expression in CTX-Astros.

CTX-Astros treated with SR141617 (1 μ M) alone, or in combination with cannabinoids, did not express significantly higher levels of CXCL10 (A), IL-6 (B), CCL5 (RANTES) (C), CSF2 (GM-CSF) (D), C3 (E) or TLR4 (F) mRNA. Treatment with SR141617, with or without cannabinoids, did not alter the mRNA expression of CNR1(G), PPARA (H) or PPARG (I) in CTX-Astros either. Data expressed as mean $\pm$ SEM (N=3, r=3). One-way ANOVA.

Appendix 2.

Publications

McComish SF, O’Sullivan J, MacMahon Copas AN, **Imiolek M**, Boyle NT, Crompton LA, Lane JD, Caldwell MA (2024) Reactive astrocytes generated from human iPSC are pro-inflammatory and display altered metabolism. *Experimental Neurology*, 382, 114979. <https://doi.org/10.1016/j.expneurol.2024.114979>

Oral Presentations

2025, Work in Progress (Department of Physiology), “*Modulating astrocyte reactivity as a potential therapeutic for neurodegeneration*”

2025, Neuroscience Research Day, “*The effect of phytocannabinoids and PPAR γ (ant)agonists on reactivity in human-induced pluripotent stem cell-derived astrocytes*”

2024, Physiology Friday for National Schools, “*Making brain cells from skin cells*”

2024, Physiology Department Transition Year Workshops, “*From skin cells to brain cells – modelling Alzheimer’s disease in a dish*”

Poster Presentations

Magdalena D. Imiolek, Melody Cui Sun, Sarah F. McComish, Eric J. Downer, Maeve A. Caldwell – “*The effect of phytocannabinoids and CB1/PPAR γ antagonists on reactivity in human-induced pluripotent stem cell-derived astrocytes*”. All-Ireland Multiple Sclerosis Research Network Symposium, Maynooth University; June 2025.

Magdalena D. Imiolek, Melody Cui Sun, Sarah F. McComish, Eric J. Downer, Maeve A. Caldwell – “*The use of phytocannabinoids and MCC950 as potential treatments of reactivity in human-induced pluripotent stem cell-derived astrocytes*”. Neurodegenerative Diseases: Biology & Therapeutics conference at Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA; December 2024.

Magdalena D. Imiolek, Melody Cui Sun, Sarah F. McComish, Eric J. Downer, Maeve A. Caldwell – “*The effect of cannabinoid treatment on reactive human-induced pluripotent stem cell-derived astrocytes*”. Trinity College Dublin Glia Club; September 2024.

Magdalena D. Imiolek, Melody Cui Sun, Sarah F. McComish, Eric J. Downer, Maeve A. Caldwell – “*The effect of cannabinoid treatment on reactive human-induced pluripotent stem cell-derived astrocytes*”. Trinity College Dublin Glia Club, November 2023

Magdalena D. Imiolek, Melody Cui Sun, Sarah F. McComish, Eric J. Downer, Maeve A. Caldwell – “*The effect of cannabinoid treatment on reactive human-induced pluripotent stem cell-derived astrocytes*”. 71st International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research (GA), Dublin, July 2023

Magdalena D. Imiolek, Melody Cui Sun, Sarah F. McComish, Eric J. Downer, Maeve A. Caldwell – “*The effect of cannabidiol treatment on reactive human-induced pluripotent stem cell-derived astrocytes*”, Young Neuroscience Research Investigators Conference, October 2022.

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