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MicroRNA and Nrf2 pathway manipulation in human astrocytes: towards a therapeutic approach for Amyotrophic Lateral Sclerosis (ALS)

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Abstract

Dysfunction of the Nrf2 antioxidant pathway is a hallmark of ALS, with Nrf2 activation suggested as potential therapeutic strategy. The Nrf2 pathway plays a critical role in glia, with astrocyte-specific activation shown to be neuroprotective in ALS mice. Dysregulation of miRNAs is also reported in ALS, yet their roles in regulating astrocyte reactivity or Nrf2 signalling remain poorly understood. Here, potential links between miRNAs and these pathways were explored in human astrocyte models, to investigate if inhibiting Nrf2-targeting miRNAs could be a viable astrocyte-targeted Nrf2-activating ALS therapy.

Proteomic analysis of cultured human primary astrocytes detected ~4000 proteins, including 55/154 ALS-associated proteins in the ALSoD database. Twelve Nrf2 target gene products were downregulated in 'reactive' (FBS-exposed) astrocytes, compared to 'quiescent' serum-free astrocytes, while no Nrf2 target gene products were upregulated. Reduced expression of *NFE2L2* (the Nrf2 gene) was also detected by RT-qPCR. This reactive astrocyte model therefore offered a disease-relevant platform to explore Nrf2 rescue strategies.

Parallel small non-coding RNA sequencing (sncRNAseq) was conducted, with analysis extended to astrocyte-derived extracellular vesicles (ADEVs) due to their potential as circulating ALS biomarkers. This enabled rational selection of highly expressed Nrf2-targeting miRNAs (miR-340-5p and miR-27b-3p) for inhibition in reactive astrocytes. Transfection of a GFP plasmid proved ineffective, prompting use of cell-permeable antisense miRNA 'power inhibitors' to upregulate Nrf2. However, presumed low inhibitor uptake limited their utility. Lentiviral delivery modestly improved transduction (5-20%), with GFP expression allowing single-cell analysis, though no Nrf2 pathway upregulation was observed. Nucleofection achieved ~50% delivery of a GFP plasmid, however proteomic analysis of reactive astrocytes nucleofected with a miR-340-5p inhibitor revealed no significant protein changes.

Regardless, the reactive astrocyte model proved effective for screening Nrf2 activators, as demonstrated with the commercial activator tBHQ, which upregulated Nrf2 pathway components and increased cell viability, ATP-induced Ca^{2+} signalling, and reduced ROS (non-significantly). These assays were extended to the ALS therapeutics riluzole and edaravone, which modestly upregulated the Nrf2 pathway. Riluzole's effect may reflect oxidative stress, as high doses reduced viability. Edaravone had similar effects to tBHQ, with the enhanced Ca^{2+} response to ATP predicted to promote astrocyte reactivity and glutamate release, potentially contributing to its limited efficacy in ALS.

This thesis evaluated a practical human astrocyte model for screening Nrf2 activators. Multi-omic analysis identified ALS-associated proteins and miRNAs altered in astrocyte reactivity, highlighting possible therapeutic targets to combat neuroinflammation. Although miRNA-based Nrf2 activation was unsuccessful, the astrocytic effects of riluzole and edaravone were evaluated, to work towards identification of astrocyte-targeted treatments for ALS.

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List of Abbreviations

For genes and proteins, only those frequently referred to have been included.

Abbreviation	Full name
18S	18S ribosomal RNA
AD	Alzheimer's disease
ADEV	Astrocyte-derived extracellular vesicle
ALS	Amyotrophic lateral sclerosis
ALSoD	Amyotrophic lateral sclerosis online database
ANOVA	Analysis of variance
APS	Ammonium persulfate
ARE	Antioxidant response element
ASO	Antisense oligonucleotide
AUC	Area under the curve
B2M	Beta-2-microglobulin
BBB	Blood-brain barrier
BCA	Bicinchoninic acid assay
BSA	Bovine serum albumin
C3/C1q	Complement component (3/1q)
C9orf72	Chromosome 9 open reading frame 72
cDNA	Complementary DNA
CISBP	Catalog of Inferred Sequence Binding Preferences
CNS	Central nervous system
CSF	Cerebrospinal fluid
Ct	Cycle threshold
CV	Coefficient of variation
DAPI	4',6-diamidino-2-phenylindole
ddH ₂ O	Double distilled water
DDA	Data dependent acquisition
DIA	Data independent acquisition
DMEM	Dulbecco's modified eagle medium
dNTP	Deoxynucleoside triphosphate
(D)PBS	(Dulbecco's) phosphate buffered saline
DPR	Dipeptide repeat protein
DTT	Dithiothreitol
EAAT2	Excitatory amino acid transporter 2
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine tetraacetic acid
EMA	European Medicines Agency
ER	Endoplasmic reticulum
EV	Extracellular vesicle
fALS	Familial amyotrophic lateral sclerosis
FBS	Foetal bovine serum
FC	Fold change
FDA	US Food and Drug Administration

FTD	Frontotemporal dementia
FUS	Fused in sarcoma
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
hnRNP	Heterogenous nuclear ribonucleoprotein
HRP	Horseradish peroxidase
IL (e.g. IL-1 α)	Interleukin
IPA	Ingenuity pathway analysis
iPSC	Induce pluripotent stem cell
K ⁺ / Na ⁺ / Cl ⁻ /Ca ²⁺	Potassium/ sodium/ chloride/ calcium ions
kDa	Kilodalton
LPS	Lipopolysaccharide
miRISC	MicroRNA induced silencing complex
miRNA	Micro-ribonucleic acid
MISEV	Minimal information for studies of extracellular vesicles
MND	Motor neurone disease
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
mSOD1	SOD1 ^{G93A}
NF- κ B	Nuclear factor kappa B
Nrf2	Nuclear factor erythroid 2-related factor 2
NTA	Nanoparticle tracking analysis
NTC	No template control
padj	Adjusted p value
PD	Parkinson's disease
PFA	Paraformaldehyde
PI	Power inhibitor
Pre-miRNA	Precursor micro-ribonucleic acid
Pri-miRNA	Primary micro-ribonucleic acid
RIPA	Radio-immunoprecipitation assay
RBP	RNA binding protein
RIN	RNA integrity number
ROS	Reactive oxygen species
rpm	Revolutions per minute
RT-qPCR	Real-time quantitative polymerase chain reaction
sALS	Sporadic amyotrophic lateral sclerosis
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
SOD1	Superoxide dismutase 1
<i>SOD1</i> -ALS	ALS caused by SOD1 variants (applicable to other genes)
sncRNAseq	Small non-coding RNA sequencing

SWATH	Sequential window acquisition of theoretical mass
TBS	Tris-buffered saline
TDP-43	Tar DNA binding protein-43
TEAB	Triethylammonium bicarbonate
TEMED	Tetramethylethylenediamine
TNF α	Tumour necrosis factor alpha
UPS	Ubiquitin-proteasome system
UTR	Untranslated region

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Chapter 1. Introduction

Amyotrophic Lateral Sclerosis (ALS) is a terminal neurodegenerative disease characterised by the degeneration of motor neurones. ALS is highly complex, with numerous mechanisms implicated in the disease pathogenesis. Neuroinflammation plays a central role, marked by astrocyte reactivity and their associated neurotoxicity. Oxidative stress further contributes to neurodegeneration, likely in part due to dysregulation of the antioxidant Nrf2 pathway, including in astrocytes and motor neurones. Evidence also implicates microRNA (miRNA) dysregulation in ALS; however, it remains unclear whether these alterations contribute to astrocyte-mediated neurotoxicity and oxidative stress through modulation of the astrocytic Nrf2 pathway or astrocyte reactivity. This thesis will explore the possible interplay between astrocyte reactivity, the Nrf2 pathway and miRNAs, and investigate the potential of therapeutically targeting these pathways. In Chapter 1, these pathways will be discussed, both in the context of normal physiology and in ALS.

1.1 Astrocytes and their interactions with neurones

1.1.1 The nervous system

The nervous system in humans is comprised of the central nervous system (CNS) and the peripheral nervous system (PNS). The CNS consists of the brain and spinal cord, while the PNS represents the nerves branching out from the CNS to the rest of the body. Both nervous systems contain neurones and non-neuronal (glial) cells. The major glial population of the CNS are astrocytes, myelin-producing oligodendrocytes, and microglia, the resident immune cells of the brain (Rasband, 2016). Neurones and astrocytes will be discussed further in sections 1.1.2 and 1.1.3 respectively.

1.1.2 Neurone structure and function

Neurones are polarised cells that transmit information throughout the nervous system via chemical and electrical signals. Neurones receive signals from other neurones via their dendrites, which is processed in the soma and overall either stimulates an action potential (excitation) or prevents it (inhibition). This electrical signal propagates down the axon, with ions diffusing in and out of the cell at gaps in the myelin sheath, known as the nodes of Ranvier. Once the electrical signal reaches the axon terminals, this triggers the release of neurotransmitters into the synapse. Neurotransmitters can then bind to dendrites on other neurones to trigger or inhibit an action potential, therefore facilitating the spread of information throughout the nervous system (Debanne *et al*, 2011).

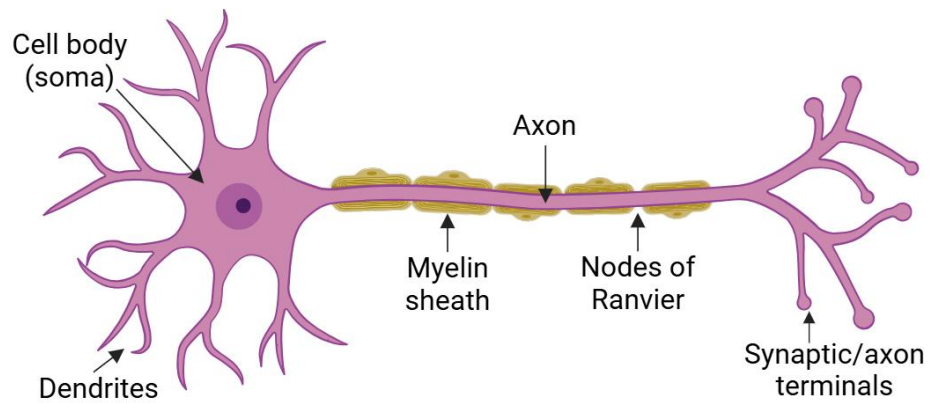


Figure 1.1. General structure of a neurone. Created using BioRender.com.

The three major neurone types are sensory neurones, interneurons and motor neurones. Sensory neurones transmit stimulus information such as pain from the periphery (e.g. the skin) to the CNS, which is relayed via interneurons to motor neurones to carry out a response. Motor neurones transmit information from the CNS to muscle to induce contraction, therefore controlling voluntary and involuntary movements such as walking, breathing, speaking and swallowing. There are two main types: upper motor neurones connect the brain and spinal cord, while lower motor neurones connect the spinal cord to muscle (Ashley & Lui, 2023; Zayia & Tadi, 2020).

1.1.3 Astrocyte structure and function

Astrocytes are the most abundant glial cell in the CNS (Rasband, 2016). They envelope the space between pre-synaptic and post-synaptic neurones to form a tripartite synapse. Here they have many roles in supporting neuronal function, including the release of “gliotransmitters” such as glutamate, GABA and purines, which can regulate neuronal excitability (Sofroniew & Vinters, 2010). This regulation is also achieved by buffering potassium ions (K^+). Diffusion of K^+ out of neurones is a key step of action potential propagation, followed by removal of K^+ from the extracellular space by astrocytes, to ensure the correct membrane potential is maintained (Bellot-Saez *et al*, 2017). Astrocytes are also involved in synaptic pruning, where they remove unnecessary synapses by phagocytosis (Park & Chung, 2023). Astrocytes have an important role in neurotransmitter recycling. After neurotransmitters are released by neurones, it is important to clear the neurotransmitters from the synapse to prevent over-stimulation of receptors on the post-synaptic neurone, as this may cause neuronal death (excitotoxicity). Astrocytes take up excitatory glutamate and inhibitory GABA neurotransmitters from the synapse and metabolise them to form glutamine. Glutamine can then be released back to neurones or converted to glutamate or GABA in astrocytes (Bak *et al*, 2006; Schousboe *et al*, 2013).

Astrocytes are also an integral part of the blood brain barrier (BBB), connecting to blood vessels by their end feet to control blood flow and permeability. This allows astrocytes to regulate blood supply to the brain in response to neuronal

activity, ensuring sufficient glucose is supplied to neurones (MacVicar & Newman, 2015). Since astrocytes can form simultaneous interactions with blood vessels and neurones, they can directly transport glucose, oxygen and other substrates from the blood to neurones (Sofroniew & Vinters, 2010). The lactate shuttle hypothesis proposes that glutamate released by active neurones is taken up and metabolised by astrocytes, which triggers glycolysis and the release of lactate to neurones, therefore providing neurones with an energy source in response to their activity (Pellerin *et al*, 2007). Astrocytes are also vital for redox homeostasis and protection of neurones against oxidative stress via Nrf2 pathway modulation. Nrf2 activation in astrocytes promotes the release of the antioxidant glutathione, which is broken down extracellularly to produce cysteinyl-glycine (CysGly). CysGly is then taken up by neurones to produce their own glutathione in an Nrf2-mediated mechanism (Dringen *et al*, 1999; Oksanen *et al*, 2019). The astrocyte Nrf2 pathway will be described further in section 1.4.2.

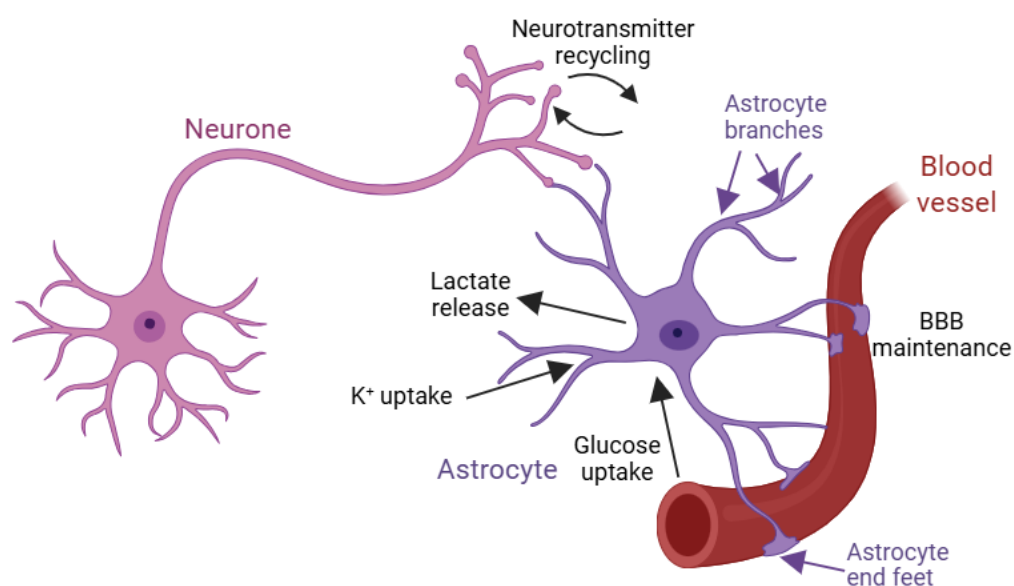


Figure 1.2. Astrocyte functions in the CNS. Astrocytes interact with blood vessels via their end feet to maintain and regulate the BBB and uptake glucose and other nutrients from the blood. They provide metabolic support to neurones by releasing lactate in response to neuronal activity and facilitate neuronal action potentials by taking up K^+ ions. Astrocytes also recycle the neurotransmitters glutamate and GABA from the synaptic cleft. Figure created using BioRender.

1.1.3.1 Reactive astrogliosis

Reactive astrogliosis is a term given for the reaction of astrocytes to disturbed homeostasis in the CNS, which may be caused by injury or neurological disease. This involves changes in astrocyte morphology, function and number, and the overall effect can be beneficial or harmful depending on the context and reactivity trigger (Matusova *et al*, 2023; Sofroniew, 2020). Reactive astrogliosis,

or astrocyte reactivity, is a normal response to protect the CNS from harm, however in certain pathological conditions, including ALS, reactive astrogliosis can be dysfunctional and harmful, persisting long after the initial trigger (Escartin *et al*, 2021).

Reactive astrogliosis is a highly heterogeneous process, with the molecular profile of reactive astrocytes varying according to the stimulus, and even within a cell population. It is therefore recommended that reactive astrocytes should be described by their molecular markers and functionality. Many studies of reactive astrocytes have relied on rodent models, however human astrocytes are known to be more functionally diverse, larger and structurally complex (more branches) (Oberheim *et al*, 2006).

Reactive astrocytes have been described as adopting either an “A1” neurotoxic and pro-inflammatory or “A2” neuroprotective and anti-inflammatory phenotype, similar to the M1-M2 macrophage and microglia polarisation terminology (Escartin *et al.*, 2021; Liddelow *et al*, 2017). It has been proposed inflammation or toxic insults activate A1 astrocytes, while ischemia preferentially activates A2 astrocytes (Escartin *et al.*, 2021; Zamanian *et al*, 2012). Specifically, A1 astrocytes were shown to be induced by lipopolysaccharide (LPS)-activated microglia, due to their release of the pro-inflammatory cytokines IL-1 α , TNF α and complement component C1q (Liddelow *et al.*, 2017). However, the A1 and A2 terminology is often regarded as an oversimplification, with many astrocytes likely falling on a spectrum between these two extreme phenotypes (Anderson *et al*, 2014). Unless A1/A2 terminology is used, ‘reactive astrocyte’ is generally used in this thesis to describe neurotoxic reactive astrocytes, as these are involved in ALS pathogenesis. Other inducers of astrocyte reactivity include reactive oxygen species (ROS), glucose deprivation, infection (often modelled using LPS), ATP and neurodegeneration-associated products such as β -amyloid (Sofroniew & Vinters, 2010).

While single-cell transcriptomics in recent years has provided insight into the molecular profile of different reactive astrocyte subpopulations, many studies still rely on increased glial fibrillary acidic protein (GFAP) expression and cellular hypertrophy as markers of reactivity (Escartin *et al.*, 2021; Matusova *et al.*, 2023). Hypertrophy is an increase in the length, thickness and number of primary astrocyte branches (Wilhelmsson *et al*, 2006). GFAP is an intermediate filament-III cytoskeletal protein, which in the CNS is almost exclusively expressed in astrocytes (Yang & Wang, 2015). However not every reactive astrocyte is characterised by increased GFAP, as expression can vary greatly between location and subpopulation, meaning it should not be considered as a universal marker (Sofroniew & Vinters, 2010). The heterogeneity of astrocyte reactivity means that numerous potential markers of reactive astrocytes have been identified, as reviewed in (Escartin *et al.*, 2021). These include increased expression of other intermediate filaments such as nestin and vimentin, the Ca²⁺ binding protein S100 β and activation of the STAT3 transcription factor (Escartin *et al.*, 2021). Complement factor C3 is often used as a specific marker for A1 astrocytes (Liddelow *et al.*, 2017).

The degree of change undergone by astrocytes upon reactivity is affected by the severity of the injury or pathology. In the most severe cases, such as in spinal cord injury or neurodegeneration, astrocytes proliferate extensively to form a glial scar. These scars act as a physical barrier to protect neurones from insults such as infectious agents and inflammatory cells. However glial scars typically persist long after the initial trigger, becoming harmful by preventing axon regeneration and neuronal repair (Sofroniew & Vinters, 2010; Wang *et al*, 2018).

The functional consequences of neurotoxic astrocyte reactivity in neurodegenerative diseases can be summarised as a loss of homeostatic functions and gain of deleterious functions. Lost homeostatic functions may include the maintenance of the BBB, reduced uptake of glutamate from the synapse and loss of antioxidant support. Gained neurotoxic functions may include the release of soluble factors which promote neuronal death, and increased inflammation (Escartin *et al.*, 2021; Moujalled *et al*, 2017). Such changes have been identified in ALS models, which will be discussed in section 1.2.2.6.1.

1.1.4 Astrocyte-neurone communication by extracellular vesicles

As discussed, astrocytes and neurones communicate to ensure sufficient energy supply to neurones, and to regulate their excitability. This cell-cell communication includes the transfer of neurotransmitters, gliotransmitters, ions and extracellular vesicles (EVs).

EVs are lipid-bilayer enclosed particles which carry cargo such as proteins, lipids, metabolites and nucleic acids between cells and tissues. EVs are secreted from cells in both physiological and pathological states and have been shown to contribute to pathologies including neurodegenerative diseases and cancer. There are three main EV subtypes - microvesicles, exosomes and apoptotic bodies, as summarised in Table 1.1. They can be distinguished by size, biogenesis and release pathways, content and function (Doyle & Wang, 2019). Recent studies have broadened these subtypes to include particles such as exomeres (formed by cleavage of cytoplasmic extensions), ectosomes (vesicles assembled and released at the plasma membrane) and supermeres (nanoparticles formed by an unknown mechanism) (Cocucci & Meldolesi, 2015; Kumar *et al*, 2024). Despite this, the International Society for Extracellular Vesicles (ISEV) recommends using the general term EV, differentiating between small EVs (<200nm diameter) and large EVs (>200nm diameter) where necessary. This is because terms such as exosome and ectosome rely on the knowledge of their biogenesis pathway, which is often not available. EVs can also be described by their cell type of origin, for example astrocyte-derived EVs (ADEVs) (Welsh *et al*, 2024). As well as recommending EV-related terminology, ISEV also determines acceptable EV isolation and measurement methods, and the experimental proof required that EVs have been successfully purified. These guidelines are published and frequently

updated in the Minimal Information for Studies of Extracellular Vesicles (MISEV) (Welsh *et al.*, 2024).

Table 1.1. A summary of the major EV subtypes.

EV subtype	Typical size	Biogenesis
Microvesicles	50-1,000nm (Crescitelli <i>et al.</i> , 2013)	Microvesicles form by the outward blebbing and pinching of the plasma membrane (Yu <i>et al.</i> , 2024).
Exosomes	40-100nm (Crescitelli <i>et al.</i> , 2013)	The endosomal membrane buds inwards, forming multi-vesicular bodies (MVBs), membrane-bound organelles containing multiple smaller vesicles. MVBs then either fuse with the lysosome for degradation, or fuse with the cell membrane, with the vesicles released as exosomes (Yu <i>et al.</i> , 2024).
Apoptotic bodies	800-5,000nm (Crescitelli <i>et al.</i> , 2013)	Apoptotic bodies are produced in the final stage of apoptosis (Yu <i>et al.</i> , 2024).

EVs were initially thought to simply represent cellular waste, however they have since been shown to have functional roles in cell-cell communication, neuronal growth and survival, tumour progression and the spread of pathological proteins in neurodegenerative diseases (Doyle & Wang, 2019; Emmanouilidou *et al.*, 2010; Gomes *et al.*, 2007; Wang *et al.*, 2011b). In particular, apoptotic bodies were presumed to represent cell debris, however they have since been shown to have functions such as triggering anti-inflammatory responses in recipient cells (Mohan *et al.*, 2020).

Regarding cell-cell communication, EVs from a ‘donor’ cell can be taken up by other ‘recipient’ cells and modulate their function. ADEVs from various astrocyte models have been shown to contain neuroprotective proteins, including fibroblast growth factor-2 (FGF2), vascular endothelial growth factor (VEGF), heat shock proteins (HSPs), glutamate transporters and synapsin I (Upadhyay *et al.*, 2020). In contrast, in disease states and upon astrocyte reactivity ADEV cargo may promote neuroinflammation and neurodegeneration. For example, ADEVs from Alzheimer’s disease (AD) patient blood were shown to be enriched with several complement effector proteins and the pro-inflammatory cytokines TNF α and IL-1 β (Goetzl *et al.*, 2018). Overexpression of mutant superoxide dismutase 1 (SOD1) protein, a key player in ALS which will be discussed in section 1.2.1.1, in mouse astrocytes was shown to increase release of ADEVs containing mutant SOD1 (mSOD1) protein. This may reflect an attempted protective mechanism to remove the harmful protein from astrocytes, however the ADEVs transferred mSOD1 protein to motor neurones and induced their death (Basso *et al.*, 2013). Overall, this suggests that ADEVs may directly contribute to ALS pathogenesis by delivering

pathological proteins to motor neurones. Other crucial EV cargo for astrocyte-neurone communication in physiological and pathological states are miRNAs, which will be discussed further in section 1.3.

1.2 Amyotrophic Lateral Sclerosis (ALS)

ALS is a neurodegenerative disease of progressive muscle paralysis, caused by degeneration of upper and/or lower motor neurones in the brainstem, spinal cord, primary motor cortex and corticospinal tracts. It typically causes death within 2-5 years due to respiratory failure (Wijesekera & Leigh, 2009). ALS is the most common form of motor neurone disease (MND), with a disease prevalence of 5.4/100,000 and incidence rate of 2.8/100,000 per year in Europe (Chiò *et al*, 2013), equating to a lifetime risk of around 1/300 (Martin *et al*, 2017). The average age of onset of sporadic (non-inherited) ALS is around 60 years, with a male to female ratio of approximately 1.5:1. Two thirds of patients have spinal onset ALS, where symptoms usually start in the upper and lower limbs, such as muscle weakness. Patients with bulbar onset ALS typically present with difficulty speaking or swallowing in addition to limb symptoms, and have a more rapid disease course (Wijesekera & Leigh, 2009). Riluzole is an anti-excitotoxic therapeutic which can extend survival by 2-3 months, and is currently the only ALS drug approved in the UK (Hinchcliffe & Smith, 2017). Additional treatments which are approved in the US are the antioxidant Edaravone, and the antisense oligonucleotide Tofersen for the ~2% of ALS patients that have SOD1 mutations (*SOD1*-ALS) (Miller *et al*, 2022; Rothstein, 2017).

ALS is a highly heterogenous disease at the cellular and genetic level, and in disease presentation and symptoms. This makes diagnosis challenging and results in many patients experiencing a long delay in diagnosis after symptom onset, resulting in a delay in accessing treatment. There is not currently a single test for ALS/MND, with diagnosis typically relying on physical examination, electromyography to monitor nerve conduction, ruling out similar diseases (for example using MRI) and sometimes genetic testing (Brotman *et al*, 2023).

ALS is often considered on a disease spectrum with Frontotemporal Dementia (FTD), a disease for which the main symptoms are behavioural changes and language difficulties (Bott *et al*, 2014). Around 10-15% of ALS patients meet the diagnostic criteria for FTD (Ahmed *et al*, 2021), but it is thought that up to 50% of ALS patients experience some symptoms of FTD. Conversely, around 30% of FTD patients experience motor symptoms, with 10-15% developing ALS (Abramzon *et al*, 2020; Ahmed *et al*, 2021). There is considerable overlap of genetics and cellular pathways implicated in the two diseases (Abramzon *et al*, 2020).

1.2.1 Genetics of ALS

Most cases of ALS are sporadic (sALS), with no clear family history, while 5-10% cases are familial ALS (fALS), typically described when a first-degree relative has

had ALS. However, the recent view is that there is not a definite distinction between sALS and fALS. sALS can be caused by inheritance of a disease-associated mutation, even if there is no clear family history (Al-Chalabi, 2017).

The Amyotrophic Lateral Sclerosis online Database (ALSoD) collates information on genes that have been connected to ALS, as well as the strength of evidence for their connection (Abel *et al*, 2012). As of December 2024, there were 154 genes in ALSoD, of which 17 are termed ‘definitive ALS genes’, meaning variants in these genes have been shown to significantly increase the risk of ALS. This list of 17 definitive ALS genes will be used as a reference in this thesis, therefore brief summaries of each will now be given, with emphasis on the most common causative ALS genes: *C9Orf72*, *SOD1*, *FUS* and *TARDBP* (Mejzini *et al*, 2019).

1.2.1.1 SOD1

Variants in the *SOD1* gene were the first to be associated with ALS in 1993 (Rosen *et al*, 1993). *SOD1* mutations are reported in 12-20% of fALS patients and 1-2% of sALS patients (Nijs & Van Damme, 2024). The *SOD1* protein functions as a key antioxidant enzyme, catalysing the conversion of superoxide to hydrogen peroxide and oxygen, a process which will be discussed further in section 1.2.2.4. *SOD1* variants reduce enzyme activity by 50-80%, however *SOD1* knockout mice do not develop ALS, suggesting that a gain of toxic function may be the dominant pathogenic mechanism (Mejzini *et al.*, 2019; Reaume *et al*, 1996; Rosen *et al.*, 1993). This is likely due to a conformational change in mSOD1 protein, allowing it to interact with new proteins and exert new functions. For example, mSOD1 can bind to the endoplasmic reticulum (ER) protein Derlin1, which promotes ER stress (Fujisawa *et al*, 2012). mSOD1 also has increased propensity to expose its hydrophobic regions, which increases aggregation of the protein (Münch & Bertolotti, 2010). Inclusions of aggregated *SOD1* protein are observed in *SOD1*-ALS patient motor neurones, as well as in some sALS patients with mutations in other known ALS genes such as *C9Orf72*, suggesting *SOD1* is involved in pathogenesis irrespective of mutation status (Forsberg *et al*, 2019; Forsberg *et al*, 2010). Protein aggregation is a key feature of ALS and other neurodegenerative diseases, which will be discussed further in section 1.2.2.1. The *SOD1*^{G93A} mouse model is one of the most widely studied ALS mouse models, which is referred to as mSOD1 in this thesis.

1.2.1.2 C9Orf72

The most common genetic cause of ALS in European populations is a hexanucleotide (GGGGCC) repeat expansion in intron 1 of the *C9Orf72* gene (Smith *et al*, 2013), with a frequency of 30-60% in fALS and 5-10% in sALS patients (Nijs & Van Damme, 2024). The functions of the *C9Orf72* protein are poorly understood, however they are believed to include roles in autophagy regulation, vesicle trafficking, DNA damage repair and RNA processing, all pathways of relevance to ALS pathogenesis (Sun *et al*, 2020; Zhou & Xu, 2023). There are three main splice variants of *C9Orf72*, of which two retain the expansion-containing intron when missplicing occurs. Healthy individuals

typically have two repeats of the expansion, while *C9Orf72*-ALS patients can have from 24 repeats up to hundreds or thousands (Iacoangeli *et al*, 2019).

Like several ALS-associated gene variants, the *C9Orf72* expansion is likely pathogenic due to both gain and loss of function mechanisms. Similar to *SOD1*, *C9Orf72* knockout mice do not display motor deficits, again suggesting that loss of function is not the major pathogenic mechanism (Koppers *et al*, 2015). One toxic function gained is the clustering of repeat-containing transcripts to form nuclear RNA foci in neurones, and to a lesser extent in astrocytes, oligodendrocytes and microglia (Mizielinska *et al*, 2013). These foci can sequester RNA binding proteins (RBPs), therefore impairing RNA processing and metabolism which has neurotoxic effects (Lee *et al*, 2013). The repeat-containing transcripts can also be translated in a process known as repeat-associated non-ATG (RAN) translation. The mRNA is translated in both the sense and antisense directions, resulting in the production of aggregation-prone dipeptide repeat proteins (DPRs), which are found in neuronal inclusions and the cerebrospinal fluid (CSF) of *C9Orf72*-ALS patients (Ash *et al*, 2013; Krishnan *et al*, 2022). The DPR poly-glycine-alanine (poly-GA) has been shown to impair the proteasome, resulting in ER stress and cell death (Zhang *et al*, 2014).

1.2.1.3 TARDBP

The *TARDBP* gene encodes TDP-43, an RBP and DNA binding protein which is identified in cytoplasmic inclusions of 97% of ALS patients. This TDP-43 pathology is therefore a hallmark of the disease, regardless of any mutation (Prasad *et al*, 2019). In normal physiology, TDP-43 shuttles between the nucleus and cytoplasm but is primarily found in the nucleus, as its functions include the regulation of transcription, mRNA stability, splicing and non-coding RNAs (Mejzini *et al*, 2019). However, in ALS motor neurones, TDP-43 mislocalises to the cytoplasm where it aggregates. This results in both a loss of nuclear TDP-43 function and gain of toxic cytoplasmic functions, key examples of which will be discussed further in sections 1.2.2.2 and 1.3.3.4. Unlike with *C9Orf72* and *SOD1*, it is believed that the loss of function is just as important as gain of function for disease pathogenesis, although this is still cause for debate (Mejzini *et al*, 2019).

Variants in *TARDBP* are also causative of ALS (3-5% frequency in fALS, 1% frequency in sALS patients (Nijs & Van Damme, 2024)) further supporting that TDP-43 dysregulation is a key pathogenic mechanism (Gitcho *et al*, 2008). Most ALS-associated *TARDBP* variants are in the C-terminal domain (TDP-43 domain structure illustrated in Figure 1.3) which is important for many of its functions, such as splicing regulation (Mejzini *et al*, 2019). Astrocytes isolated from TDP-43^{Q331K} mice showed defects in functions such as stress-induced glutathione production, while TDP-43^{A315T} astrocytes displayed reduced glutamate uptake and ATP accumulation. This implicates TDP-43 dysregulation as a non-cell autonomous mechanism of neurodegeneration, whereby dysfunction in other cell types contributes to motor neurone loss (Barton *et al*, 2020; Moujalled *et al*, 2017).

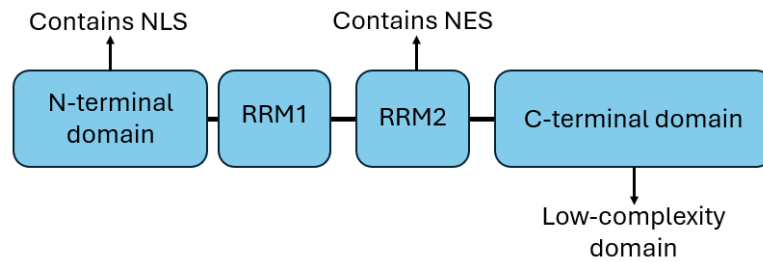


Figure 1.3. Schematic of the domain structure of TDP-43 protein. RRM = RNA recognition motif. NLS = nuclear localisation signal. NES = nuclear export signal. Figure adapted from (Chien *et al*, 2021).

TDP-43 aggregates may even be present in non-CNS organs such as the skin decades before symptom onset, according to a pre-print study (Waldron *et al*, 2025). Detection of TDP-43 inclusions in astrocytes of ALS patients appears to vary between studies (Nolan *et al*, 2020; Zhang *et al*, 2008). Notable examples of ALS subtypes without TDP-43 pathology are in *SOD1*-ALS and *FUS*-ALS patients, where SOD1 and FUS proteins aggregate, respectively (Mackenzie *et al*, 2007; Rakhit *et al*, 2002; Vance *et al*, 2009).

1.2.1.4 FUS

Variants in the *FUS* gene are associated with early onset, aggressive ALS and have frequencies of 2-4% in fALS and 1% in sALS patients (Moens *et al*, 2025; Nijs & Van Damme, 2024). *FUS* encodes the fused in sarcoma (FUS) RBP, which shares many functions with TDP-43 such as regulating splicing and DNA repair. *FUS* is also implicated in miRNA biogenesis and selecting miRNAs for loading into EVs, functions which will be discussed further in section 1.3.3.4 (Garcia-Martin *et al*, 2022; Morlando *et al*, 2012; Zhang *et al*, 2018). Like TDP-43, *FUS* is mostly localised to the nucleus, but shuttles between the nucleus and cytoplasm. Many ALS-associated variants are in the nuclear localisation signal, resulting in cytoplasmic mislocalisation and aggregation (Vance *et al*, 2009; Vance *et al*, 2013).

In addition to *FUS* aggregates, there is evidence that cytoplasmic soluble *FUS* may also be toxic, giving rise to the hypothesis that *FUS* aggregation may be an attempted protective mechanism against soluble *FUS* (Mejzini *et al*, 2019). While loss of nuclear *FUS* function may contribute to ALS pathogenesis, it is unlikely to be a driving mechanism, as *Fus* knockout mice develop abnormalities distinct from ALS (Kino *et al*, 2015).

1.2.1.5 Other definitive ALS genes

According to ALSod, the 13 other definitive ALS genes are: *ANXA11*, *CHCHD10*, *EPHA4*, *HNRNPA1*, *KIF5A*, *NEK1*, *OPTN*, *PFN1*, *TBK1*, *UBQLN2*, *UNC13A*, *VAPB* and *VCP* (Abel *et al*, 2012). Variants in these genes are generally rare (<5% of cases) except for *UNC13A*. *UNC13A* variants are reported in ~15% of sALS patients, although they appear to have relatively small effect size (Nijs & Van Damme, 2024). A brief description of the function of these genes will be given, with possible contributions of their mutation to pathogenesis.

Annexin A11 (encoded by *ANXA11*) is a Ca^{2+} -dependent phospholipid-binding protein which has roles in calcium signalling and vesicle trafficking. ALS-associated *ANXA11* variants have been shown to cause dysregulation of stress granule dynamics (membraneless organelles which form in response to stress), calcium homeostasis and intracellular protein trafficking. Cytoplasmic aggregates of ANXA11 have been detected in *ANXA11*-ALS patients neurones, as well as in *FUS*-ALS patients, suggesting the gene may be involved in pathogenesis even if not mutated (Nahm *et al*, 2020; Smith *et al*, 2017).

CHCHD10 encodes a mitochondrial protein that may promote mitochondrial and synaptic integrity and the nuclear retention of TDP-43. In a *C. elegans* model, ALS-associated *CHCHD10* variants resulted in mitochondrial and synaptic damage, and cytoplasmic TDP-43 accumulation (Woo *et al*, 2017). While *CHCHD10* variants are not a common cause of ALS, the protein may still be implicated in sALS pathogenesis (Consortium *et al*, 2018; McCann *et al*, 2020). A reduction in CHCHD10 protein has been reported in ALS patient spinal cord and FTD frontal cortex, which could be hypothesised to contribute to TDP-43 mislocalisation (McCann *et al*, 2020).

EPH receptor A4 (*EPHA4*) is a receptor tyrosine-kinase with several roles in the nervous system, such as regulating axon guidance and reactive astrogliosis (Goldshmit & Bourne, 2010). *EPHA4* has been described as a disease modifier, as *EPHA4* expression in ALS patients inversely correlates with disease onset and survival, while loss of function mutations are associated with longer survival (Van Hoecke *et al*, 2012).

HNRNPA1 encodes heterogeneous nuclear riboprotein A1, another RBP which is involved in RNA processing, metabolism and transport. *HNRNPA1* variants have been shown to give rise to a range of clinical phenotypes all linked by muscle weakening, including atypical slow-progressing ALS, the MND distal hereditary motor neuropathy (dHMN) and inclusion body myositis (Beijer *et al*, 2021). TDP-43 regulates splicing of *HNRNPA1* mRNA, with nuclear depletion of TDP-43 resulting in dysregulated *HNRNPA1* splicing and increased production of an isoform that contains an elongated prion-like domain, known as hnRNPA1B. As a result, hnRNPA1B cytoplasmic aggregates have been identified in ALS patients, which are distinct from TDP-43 inclusions (Deshaies *et al*, 2018). hnRNPA1 also interacts with the ALS-associated protein UBQLN2, an interaction which is inhibited by hnRNPA1 mutations (Gilpin *et al*, 2015).

KIF5A encodes the kinesin family member 5A, a motor protein important for axonal transport. ALS-associated variants result in the inclusion of a novel C-terminal sequence, facilitating altered RNA and protein interactions. This results in gene expression and splicing alterations, disrupted intracellular trafficking and ultimately neuronal death (Baron *et al*, 2022).

NEK1 encodes NIMA related kinase 1, a protein with roles in the DNA damage response, meiosis and microtubule regulation. ALS-associated loss of function variants disrupt microtubule homeostasis and nucleocytoplasmic transport (Mann *et al*, 2023). Missense *NEK1* variants have been reported in patients

with other ALS gene variants, including *SOD1*, *TARDBP* and *C9orf72*, perhaps suggesting a “second hit” model where NEK1 mutations may enhance the effects of driving mutations (Nguyen *et al*, 2018).

Optineurin (*OPTN*) plays important roles in protein degradation pathways, acting as an autophagy receptor to recruit cellular cargo (including damaged mitochondria) for lysosomal degradation, and a scaffold protein for ubiquitination to facilitate proteasomal degradation. These pathways will be discussed in section 1.2.2.1.2. *OPTN* variants therefore likely cause accumulation of malfunctioning mitochondria which contributes to oxidative stress (Zhao *et al*, 2023). Cytoplasmic *OPTN* protein aggregates are reported in *OPTN*-ALS and some sALS patients, sometimes co-localised with TDP-43 or *SOD1* (Maruyama *et al*, 2010). Additional pathogenic effects of *OPTN* pathology may include neuroinflammation, altered vesicular transport and necroptosis (inflammatory cell death). *OPTN*-ALS is typically characterised by slow progression (Zhao *et al*, 2023).

Profilin-1 (*PFN1*) is a nucleotide exchange factor involved in cytoskeleton dynamics (including in axons), membrane trafficking and synapse regulation. ALS-mutant profilin-1 binds less efficiently to actin, therefore inhibiting axon growth (Wu *et al*, 2012). Mutant profilin-1 may also gain toxic functions, for example by altering stress granule dynamics and forming aggregates (Figley *et al*, 2014). Profilin-1 is also involved in autophagy regulation, therefore *PFN1* variants may exert similar pathogenic effects as *OPTN* variants (Teyssou *et al*, 2022).

ALS variants in *TBK1* (encoding TANK binding kinase protein 1) are generally considered to be loss of function, either by reducing its expression or kinase activity (de Majo *et al*, 2018; Freischmidt *et al*, 2015). *TBK1* variants may contribute to neuroinflammation, as *TBK1* is an inducer of type 1 interferons in innate immune cells such as macrophages (Hui *et al*, 2025; Oakes *et al*, 2017). *TBK1* also regulates autophagy by phosphorylating autophagy receptors including the ALS-associated proteins p62 (SQSTM1) and *OPTN*, facilitating the recruitment of cargo for degradation (Heo *et al*, 2015). *TBK1* has also been implicated in autophagosome maturation, a later stage in autophagy, overall suggesting that *TBK1* variants may contribute to autophagy failures in ALS (Pilli *et al*, 2012). Microtubule dynamics and the motor protein dynein have also been shown to be regulated by *TBK1*, perhaps implicating *TBK1* in the cytoskeletal defects seen in ALS (Pillai *et al*, 2015). *TBK1* variants have been shown to occur more frequently in those with ALS-FTD than ALS alone (Le Ber *et al*, 2015).

Ubiquilin-2 (*UBQLN2*) is also involved in protein degradation, as it facilitates the ubiquitylation of target proteins to induce their degradation by the proteasome or autophagy. *UBQLN2* variants have also been shown to cause defects in the import of proteins into the mitochondria, resulting in mitochondrial dysfunction (Lin *et al*, 2021). Ubiquilin-2 inclusions have been detected in ALS patients with and without *UBQLN2* variants, suggesting universal involvement in pathogenesis (Deng *et al*, 2011).

The UNC13A protein is involved in the priming and docking of synaptic vesicles for releasing neurotransmitters into the synapse, by reducing the energy required for vesicles to fuse to the presynaptic membrane. ALS-associated *UNC13A* variants are loss of function, with mouse studies demonstrating that a lack of UNC13A results in a reduced rate of synaptic vesicle release and impaired neurotransmission (Quade *et al*, 2019; Varoqueaux *et al*, 2002; Willemse *et al*, 2023). ALS-associated *UNC13A* variants are typically intronic, but these can be included in the final transcript as an effect of TDP-43 pathology, a phenomenon which will be discussed further in section 1.2.2.2. TDP-43 pathology can also result in dysregulation of wild-type UNC13A (Willemse *et al*, 2023).

The *VAPB* gene encodes Vamp associated protein B, an integral membrane protein in the ER. VAPB functions include regulating lipid transport and homeostasis, vesicle and membrane trafficking and the unfolded protein response, a pathway to reduce the buildup of unfolded proteins in the ER (Lev *et al*, 2008). Mutant VAPB is prone to aggregation and is non-functional, therefore *VAPB* mutations may cause ER stress, although more work is required to confirm this (Borgese *et al*, 2021).

VCP encodes valosin-containing protein, a protein involved in regulating the cell cycle, membrane fusion and the ubiquitin-proteasome system (UPS), further implicating failed protein degradation in ALS pathogenesis (Chu *et al*, 2023). In addition to pure ALS, *VCP* mutations can cause multisystem proteinopathy, a syndrome which can involve different clinical combinations of ALS, Paget's disease of bone, FTD and inclusion body myopathy. Multisystem proteinopathy has also been associated with the ALS genes *SQSTM1*, *HNRNPA1* and *HNRNPA2B1* (Chompoopong *et al*, 2023).

While it is not possible to describe the functions of every gene that has been linked to ALS, many of them, including those described above, share cellular pathways that have been implicated in ALS pathogenesis. Several of these gene products are also involved in regulating Nrf2 signalling. Briefly, this includes OPTN, TBK1 and the non-definitive ALS gene *SQSTM1* (p62), due to their roles in regulating KEAP1-mediated repression of Nrf2, a process which will be discussed in section 1.4.1 (Deng *et al*, 2020; Foster *et al*, 2020; Hu *et al*, 2023). Nrf2 signalling has also been linked to TDP-43 and C9orf72 (Bono *et al*, 2021; Moujalled *et al*, 2017), which will be discussed in section 1.4.3. The major cellular pathways and pathogenic mechanisms believed to be important for the development of ALS will now be presented.

1.2.2 Pathogenic mechanisms

The comprehensive pathogenesis of ALS is not fully understood. Many pathogenic mechanisms have been identified, however it is likely that some have a greater role in driving disease progression than others. Like most neurodegenerative diseases, aberrant cytoplasmic protein aggregation is a hallmark of the disease. Numerous factors may contribute to this aggregation, including dysregulated protein degradation mechanisms and protein

misfolding (impaired proteostasis), and the mislocalisation of nuclear proteins to the cytoplasm due to nucleocytoplasmic transport defects. Many of the proteins involved in ALS pathogenesis are RBPs, therefore metabolism of RNAs, including miRNAs, is also impacted. Oxidative stress is likely induced in ALS patients due to mitochondrial dysfunction and an ineffective antioxidant defence system, resulting in damage to a range of macromolecules. Cytoskeletal defects in motor neurones disrupts axonal transport of molecules and organelles. Another pathogenic mechanism is excitotoxicity, where excessive excitatory glutamatergic neurotransmission results in Ca^{2+} overload and motor neurone death. This is in part due to failures of astrocytes to clear excess glutamate from the synapse. Astrocytes also become reactive and secrete neurotoxic factors to promote motor neurone degeneration, which along with microglia contributes to neuroinflammation in ALS (Hu *et al*, 2024; Zhou & Xu, 2023). These major pathogenic mechanisms are depicted in Figure 1.4 and will be discussed in the following sections.

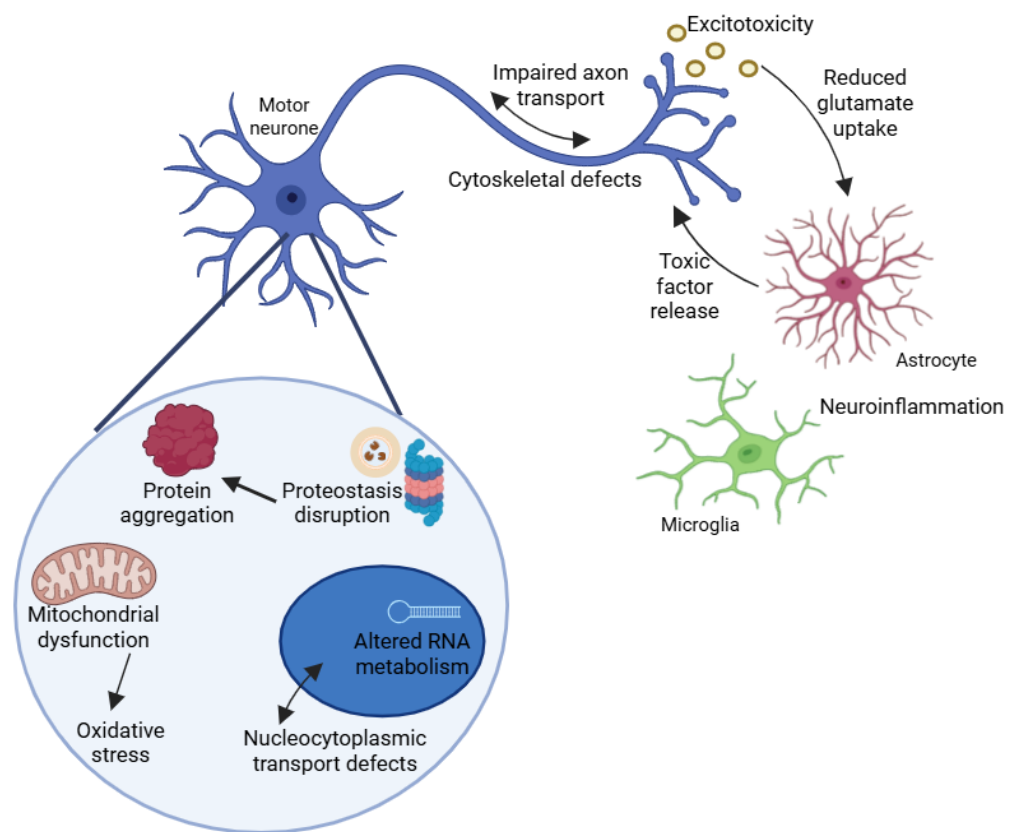


Figure 1.4. Graphical summary of the main pathogenic mechanisms underlying ALS. Created using BioRender.

1.2.2.1 Proteotoxicity and proteostasis disruption

Protein misfolding and aggregation underpins many neurodegenerative diseases, including ALS. The resulting impairment to cellular function and neurotoxicity is referred to as proteotoxicity. Protein aggregates generally form when soluble proteins form insoluble filamentary and oligomeric polymers which accumulate (Ruz *et al*, 2020). Protein quality control systems are in place to protect cells from proteotoxicity, notably chaperone proteins which ensure

correct folding, and autophagy and the UPS to degrade misfolded or aggregated proteins. The correct balance of protein synthesis, folding, disaggregation and degradation is referred to as proteostasis (protein homeostasis) (Balch *et al*, 2008). In ALS, disrupted proteostasis causes protein aggregates to persist and induce neurotoxicity (Lambert-Smith *et al*, 2022).

1.2.2.1.1 Protein misfolding

Prion-like propagation of protein misfolding has been suggested to potentiate misfolding in ALS, meaning that existing protein aggregates can sequester wild-type proteins, promoting their misfolding or aggregation. These aggregates then may spread between cells, promoting the spread of disease. For example, SOD1 aggregates have been shown to be released from neuronal cells (Neuro2a/NSC34 cells) and transferred to neighbouring neuronal cells, where they induce the misfolding and aggregation of additional SOD1 (Grad *et al*, 2014; Münch *et al*, 2011).

Due to their role in assisting correct protein folding, certain chaperone proteins have been suggested to protect against ALS (Parakh & Atkin, 2016). Chaperones typically promote correct folding by interacting with a hydrophobic region of the target protein, which in the folded protein resides in the hydrophobic core, allowing non-covalent interactions to form (Banach *et al*, 2020; Hartl *et al*, 2011). Some chaperones have found to be upregulated in ALS, likely as an attempt to ameliorate protein aggregation. For example, protein disulfide isomerase (PDI) and heat shock protein B1 (HSPB1) are upregulated in the spinal cord of mSOD1 mice and ALS patients (Ilieva *et al*, 2007; Vleminckx *et al*, 2002). PDI and HSPB1 also associate with inclusions in cultured mSOD1 mouse neurones, and PDI co-localises with TDP-43 and FUS inclusions in ALS patient tissue, perhaps further indicating an attempt to reduce aggregation (Atkin *et al*, 2006; Farg *et al*, 2012; Honjo *et al*, 2011; Kato *et al*, 1997). Rare variants in genes encoding chaperone proteins such as PDI, HSPB1 and DnaJC7 have been found in some ALS patients, therefore directly implicating protein folding dysregulation in ALS pathogenesis (Capponi *et al*, 2016; Farhan *et al*, 2019; Kwok *et al*, 2013).

Protein synthesis itself may also be affected in ALS. Certain C9Orf72-derived DPRs affected expression and processing of ribosomal RNAs in human astrocytes, and decreased the rate of translation in mouse astrocytes (Kaneura *et al*, 2016; Kwon *et al*, 2014).

1.2.2.1.2 Protein degradation failure

There is evidence for disruption of the two major protein degradation pathways in ALS – autophagy and the UPS. Protein aggregates, including those positive for TDP-43, are also positive for ubiquitin in ALS patient motor neurones (Medinas *et al*, 2017b; Neumann *et al*, 2006). Similarly, astrocytes from SOD1^{G85R} mice were shown to be positive for ubiquitinated SOD1, and many ALS patient spinal cord astrocytes are positive for ubiquitin (Bruijn *et al*, 1997; Mendonça *et al*, 2006). Since tagging a protein with ubiquitin typically marks it for degradation via the autophagy or UPS pathways, this suggests

failure of protein degradation mechanisms as the aggregates have not been cleared (Layfield *et al*, 2005; Medinas *et al*, 2017a).

Autophagy is a degradation pathway in which substrates such as misfolded proteins and their aggregates, damaged organelles or intracellular pathogens are degraded by the lysosome (Glick *et al*, 2010). The most common form of autophagy in mammals is macro-autophagy (referred to as autophagy), where substrates are delivered to the lysosome in a membrane-bound vesicle named the autophagosome (Glick *et al*, 2010). Autophagy receptors can bind to substrates in a selective manner to mediate their recruitment to the autophagosome (Vicencio *et al*, 2020).

Many genes with ALS-linked variants have roles in autophagy, such as autophagy receptors *SQSTM1* (p62) and *OPTN* and the kinase *TBK1* which activates these autophagy receptors. Proteins encoded by genes such as *C9orf72*, *FUS* and *TARDBP* (TDP-43) have also been shown to have roles in regulating autophagy (Nguyen *et al*, 2019). There is accumulation of autophagosomes (structures of early autophagy), but not autolysosomes (formed by fusion of the autophagosome with the lysosome at a later stage of autophagy) in patient spinal motor neurones (Sasaki, 2011). This illustrates that rates of autophagy are likely elevated in ALS patients, however the pathway is impaired at some stage, preventing formation of later structures of autophagy (Amin *et al*, 2020). ALS patient-derived induced pluripotent stem cell (iPSC)-astrocytes and reactive astrocytes (induced by cortical injury) from wild-type and mSOD1 mice have also been shown to impair autophagy in motor neurones via a non-cell autonomous mechanism (Madill *et al*, 2017; Tripathi *et al*, 2017). In the mouse model this was due to increased secretion of TGF- β 1, which increased mTOR signalling and inhibited autophagy, resulting in neurotoxicity (Tripathi *et al*, 2017).

For proteins to be degraded by the UPS, first they are covalently tagged with multiple ubiquitin molecules, typically via selected lysine residues. The 26S proteasome complex (consisting of a 20S catalytic core and 19S regulatory complex) then recognises the polyubiquitin chain and degrades the tagged protein, releasing ubiquitin for further use (Glickman & Ciechanover, 2002). Genetic evidence for UPS impairment in ALS includes the discovery of ALS-linked variants in the *UBQLN2* and *VCP* genes (Deng *et al*, 2011; Koppers *et al*, 2012). Both proteins bind to poly-ubiquitin chains to transfer substrates to the proteasome (Dai & Li, 2001; Ko *et al*, 2004).

Additionally, the UPS was shown to be impaired in motor neurones of mSOD1 mice after symptom onset, and mSOD1 itself has been shown to directly impair proteasome activity in NSC34 motor neurone-like cells (Cheroni *et al*, 2009; Sau *et al*, 2007). UPS impairment has also been implicated in *C9Orf72*-ALS, as poly-GA repeat proteins encoded by the hexanucleotide repeat expansion have been shown to recruit proteasomes, resulting in their accumulation and functional impairment in rat neurones (Guo *et al*, 2018). mSOD1 mouse astrocytes were found to express higher levels of two proteasome subunits (PSMB4 and PSMD8) than wild-type astrocytes, and ALS postmortem tissue

analysis suggests upregulation of the UPS in both motor neurones and astrocytes (Mendonça *et al.*, 2006; Stella *et al.*, 2021). Like autophagy, this data may therefore suggest both upregulation and simultaneous impairment of the UPS in the disease state.

1.2.2.2 Aberrant RNA metabolism

Dysregulation of multiple aspects of RNA metabolism have been implicated in ALS pathogenesis, including transcription, alternative splicing and RNA transport (Butti & Patten, 2018). MiRNA biogenesis is also implicated, which will be discussed in section 1.3.3.4. ALS-associated genes that have roles in RNA metabolism include but are not limited to *TARDBP*, *FUS*, *C9orf72*, *SOD1*, *ATXN2*, *TAF15*, *hnRNPA1*, *hnRNPA2B1*, *MATR3*, *EWSR1*, *TIA1*, *SETX* and *ANG* (Butti & Patten, 2018).

RNA-regulating roles of TDP-43 include the stabilisation of mRNA including for neurofilament light chain (*NEFL*), a key axonal structural protein, including in motor neurones. Dysregulation of this process has therefore been suggested as a driving disease mechanism in *TARDBP*-ALS (Butti & Patten, 2018; Strong *et al.*, 2007). TDP-43 also has a role in mRNA transport along axons, which is impaired by *TARDBP* variants (Alami *et al.*, 2014). TDP-43 also binds to mRNA to regulate gene expression, including for several ALS-related genes such as *FUS* and *ATXN2* (Sephton *et al.*, 2011). In rat astrocytes, TDP-43 knockdown impaired RNA homeostasis, which promoted a proinflammatory phenotype and astrocyte reactivity (LaRocca *et al.*, 2019).

However, TDP-43's function that has gained the most interest in recent years is its role in splicing regulation. In healthy neurones, TDP-43 regulates splicing by repressing the inclusion of cryptic exons – exons which are usually excluded from mature mRNA (Donde *et al.*, 2019). However, in ALS, loss of nuclear TDP-43 function can result in cryptic exons being included in selected mature mRNA transcripts. This often results in a premature stop codon or reduced RNA stability (Brown *et al.*, 2022). Two examples of genes which have reduced expression due to cryptic exon inclusion upon TDP-43 nuclear depletion are *UNC13A* and *STMN2* (encoding stathmin-2), which are both important for neuronal function (Klim *et al.*, 2019; Willemse *et al.*, 2023). Cryptic exon regulation has received considerable attention in recent years as a possible therapeutic strategy in ALS, to replace the physiological function of TDP-43 and restore *UNC13A* and stathmin-2 homeostasis (Hayes & Kalab, 2022; Wilkins *et al.*, 2024).

FUS is also important for splicing regulation, and it has been suggested to regulate transcription via RNA polymerases II and III (Butti & Patten, 2018; Schwartz *et al.*, 2012; Tan & Manley, 2010). Consequently, *FUS* mutation or manipulation in several models results in large transcriptomic changes, including expression of other RBPs (De Santis *et al.*, 2017; Nakaya *et al.*, 2013). *C9orf72* is also implicated in RNA dysregulation due to the toxic nuclear RNA foci formed by transcription of the repeat expansion (Gendron *et al.*, 2013). These foci can sequester RBPs, affecting RNA metabolism further (Lee *et al.*, 2013). RNA foci have been detected in postmortem cultured *C9orf72*-ALS

astrocytes, although to a lesser extent than in neurones (Conlon *et al*, 2016; Rossi & Cozzolino, 2021). Astrocytic RNA foci were shown to colocalise with the RBP heterogeneous nuclear ribonucleoprotein H (hnRNP H), which caused (along with other cell types) aberrant alternative splicing in ALS patient brains (Conlon *et al*, 2016). Aberrant splicing of the key glutamate transporter EAAT2 has been detected in both ALS patients and human astrocytes exposed to synthetic C9orf72-derived DPRs, suggesting that splicing dysregulation may contribute to excitotoxicity (Kwon *et al*, 2014; Lin *et al*, 1998b).

While SOD1 does not contain RNA-binding motifs, SOD1 mutations have been shown to alter RNA metabolism. For example, mSOD1 protein can bind to the 3'UTR of mRNAs such as *VEGF* and *NEFL*, reducing their expression (Chen *et al*, 2014; Lu *et al*, 2007). As noted earlier, this gain of SOD1 function may be because protein misfolding exposes regions that can bind non-specifically to RNA (Butti & Patten, 2018). mSOD1 protein has also been shown to be recruited to RNA-rich structures such as stress granules, and SOD1 inclusions contain mRNA in mSOD1 mice (Da Ros *et al*, 2021). Aberrant intron retention has also been observed in *SOD1*-variant iPSC-motor neurones, suggesting a possible role of SOD1 in splicing regulation (Luisier *et al*, 2018). However, Da Ros *et al* did not find evidence for SOD1 directly binding RNA, instead suggesting these associations are indirect, contrary to the studies by Chen and Lu *et al*.

Aberrant alternative splicing may also promote or underlie astrocyte reactivity in ALS. iPSC-astrocytes derived from *VCP*, *SOD1* and *C9orf72*-ALS patients displayed decreased intron retention, leading to increased expression of many genes involved in immune activation, which may be involved in regulating astrocyte reactivity. This was also observed when astrocytes were stimulated with TNF α , IL-1 β and C1q, suggesting reduced intron retention may be a general feature of reactive astrocytes, whether induced by pro-inflammatory stimulus or an ALS genetic variant (Rossi & Cozzolino, 2021; Ziff *et al*, 2021).

1.2.2.3 Aberrant axonal transport

Transport of intracellular cargo is important for all cellular functions, but it is particularly important in neurones due to their polarisation and long axons. Cargo moves along microtubules or neurofilaments along the axon, driven by the molecular motors such as dynein and kinesin family proteins (Hirokawa *et al*, 2010). Transport of cargo such as vesicles along the axon allows communication between the cell body and the synaptic terminals, so that neurones can react to their surroundings. Furthermore, synthesis of organelles, proteins, mRNA and lipids mostly occurs in the cell body, therefore requiring transport along the axon in a spatiotemporal-specific manner (De Vos & Hafezparast, 2017).

Axonal transport is impaired in ALS motor neurones, with evidence including abnormal axonal accumulation of structures such as phosphorylated neurofilaments, mitochondria and lysosomes in postmortem tissue (Hirano *et al*, 1984; Rouleau *et al*, 1996). Rare variants in cytoskeletal genes (encoding cytoskeletal filaments or accessory/motor proteins) also implicate impaired

intracellular trafficking in ALS. ALS-associated variants in the following genes have all been linked to disrupted axonal transport: *ALS2*, *C9Orf72*, *CHMP2B*, *DCTN1*, *FUS*, *SPG11*, *SOD1*, *TARDBP*, *TUBA4A*, *VAPB*, *KIF1A*, *KIF5A*, *SPAST*, *AR* (De Vos & Hafezparast, 2017). Notable examples include TDP-43, which is involved in the transport of mRNA along the axon, with *TARDBP* variants impairing this in multiple models (Al-Chalabi *et al*, 1999). This list also includes two kinesin family members, *KIF1A* and *KIF5A*, an α -tubulin gene *TUB4A*, and a subunit of the dynein-activating dynactin complex (*DCTN1*). In ALS patients with variants in a key cytoskeleton gene, disrupted axonal transport may be a primary cause of disease (De Vos & Hafezparast, 2017; Letournel *et al*, 2003). In some cases, axonal transport defects may be induced by astrocytes. *C9Orf72*-ALS patient-derived iPSC-astrocytes reduced the motility of mitochondria along the axons of co-cultured motor neurones, which was found to be due to direct cell-cell contact, not secreted factors (Maria *et al*, 2024).

1.2.2.4 Oxidative stress

Oxidative stress refers to the toxic cellular state caused by the imbalance between oxidant and antioxidant mechanisms. Increased production of oxidants and/or a dysfunctional antioxidant system result in oxidative stress. Many cellular components can be damaged by oxidative stress. It can lead to DNA mutations, changes to protein structure and function and lipid peroxidation, leading to cell membrane and lipoprotein damage (Halliwell, 2007). Oxidative stress can be triggered by exogenous stimuli such as pathogens, cigarette smoke, drugs or environmental pollutants. It can also be induced by endogenous processes including inflammation, aging and within diseases including cancer and ALS (Bono *et al.*, 2021). Here, general mechanisms of the antioxidant response and oxidative stress will be described before focussing on their role in CNS cells and ALS.

1.2.2.4.1 Reactive oxygen species

The stress-inducing agents induced by these exogenous and endogenous processes include ROS and reactive nitrogen species (RNS). ROS and RNS are collective terms for free radicals and non-radical species called oxidants, which can easily become radicals themselves (Pham-Huy *et al*, 2008). Free radicals are molecules with unpaired electrons in their outer shell formed by the breakage of a chemical bond, rendering them highly unstable and able to modify cellular structures. ROS species include the oxidants hydrogen peroxide (H_2O_2), ozone (O_3), singlet oxygen ($^1\text{O}_2$), and the free radicals hydroxyl ($\text{OH}\bullet$) and superoxide ($\text{O}_2\bullet$) (Genestra, 2007). While ROS can be beneficial for cellular function at low levels, for example they have roles in signalling cascades and the immune system, at high concentrations they can induce damaging cellular oxidative stress (Pham-Huy *et al.*, 2008).

ROS are formed by both enzymatic and non-enzymatic reactions *in vivo*, commonly during mitochondrial respiration. One example of ROS generation is that the superoxide anion radical ($\text{O}_2^{\bullet-}$) is generated via several oxidase enzymes, before itself participating in the generation of other ROS. This includes the production of the most reactive free radical $\text{OH}^{\bullet}$, which is formed

by a Fe^{2+} or Cu^+ catalysed reaction between $\text{O}_2^{\cdot-}$ with H_2O_2 , known as the Fenton reaction (Pham-Huy *et al.*, 2008).

1.2.2.4.2 Antioxidant defence mechanisms

Antioxidant defence mechanisms are vital to scavenge free radicals, prevent excessive ROS accumulation and ultimately avoid oxidative stress. These mechanisms can also be enzymatic and non-enzymatic, of which the enzymatic defence system will be discussed first. A key enzyme is superoxide dismutase (SOD) which converts the highly reactive $\text{O}_2^{\cdot-}$ radical to H_2O_2 . In humans there are three SOD forms, including the ALS-associated protein SOD1. The resulting H_2O_2 can be decomposed to water and O_2 by catalase (CAT) or glutathione peroxidase (GPX) enzymes (Lee *et al.*, 2020). Another enzymatic system is the thioredoxin system (Silva-Adaya *et al.*, 2014). Thioredoxin (TRX) is an oxidoreductase enzyme which reduces peroxiredoxin (PRDX) enzymes, facilitating the reduction of peroxides. Thioredoxin reductase (TRXR) reduces a disulfide at the thioredoxin active site using NADPH. It also reduces other substrates such as peroxides and PDI chaperone proteins (Lundström & Holmgren, 1990; Silva-Adaya *et al.*, 2014).

Non-enzymatic antioxidants are an equally important part of the antioxidant defence system, including enzyme cofactors such as selenium, zinc and coenzyme Q (CoQ). The main role of selenium is in selenoproteins, which have selenocysteine in their active site. More than half of the 25 selenoproteins have antioxidant activity, including glutathione peroxidases and thioredoxin reductases (Solovyev, 2015). Zinc is a cofactor of SOD1 and SOD3, is an indirect glutathione peroxidase cofactor, and has also been shown to increase catalase activity (Kloubert & Rink, 2015). Furthermore, it inhibits pro-oxidant enzymes such as inducible nitric oxide synthetase (iNOS), which produces nitric oxide, and NADPH oxidase, which produces superoxide radicals from NADPH and O_2 (Oteiza, 2012). The antioxidant transcription factor Nrf2, which will be discussed in detail in section 1.4, is also activated by zinc (Jarosz *et al.*, 2017). The lipid CoQ is best known as an electron transporter for oxidative phosphorylation in mitochondria, but its antioxidant roles extend beyond this. It is a free radical scavenger, and its reduced form (ubiquinol) can recycle other antioxidants such as ascorbate (vitamin C) and tocopherol (vitamin E) (Bhagavan & Chopra, 2006). Vitamin C, E and A are ROS and RNS scavengers with numerous roles in antioxidant defence. For example, vitamin E is especially important for the protection of cell membrane integrity, and vitamin C can repair other scavengers (Lee *et al.*, 2020).

Several transcription factors are activated by specific cellular redox states and activate expression of specific genes in response. Redox-regulated transcription factors include AP-1, NF- κ B, Nrf1, and crucially for this thesis, Nrf2 (Biswas & Chan, 2010; Meyer *et al.*, 1994). The mechanism of Nrf2-mediated activation of antioxidant gene expression will be discussed in section 1.4.1.

1.2.2.4.3 Oxidative stress in ALS

The brain consumes 20% of the body's O₂, meaning it has a high rate of oxidative metabolism and production of ROS, with similar O₂ levels in the spinal cord (Hernandez-Gerez *et al*, 2019; Lee *et al.*, 2020). The brain also has a high lipid content, which is a key target for ROS damage via lipid peroxidation (Lee *et al.*, 2020). However, the brain has relatively low antioxidant capacity, with reduced activity of enzymes such as SODs, glutathione peroxidase and catalase, making the brain more susceptible to oxidative stress (Dringen *et al*, 2000). Neurones have high metabolic demands due to their large size, again resulting in elevated ROS production (Hemerková & Vališ, 2021).

There is significant evidence for oxidative stress in ALS patients. For example, indicators of oxidative damage to DNA and protein were elevated in sALS motor cortex, fALS and sALS spinal cord (Ferrante *et al*, 1997). A product of lipid peroxidation (4-hydroxy-2,3-nonenal) was also shown to be increased in serum and CSF of sALS patients, correlating with disease severity (Simpson *et al*, 2004). Astrocytes may also have a role in oxidative stress, for example C9Orf72-ALS patient-derived iPSC-astrocytes exhibited downregulation of antioxidant proteins such as SOD1, SOD2 and glutathione synthetase (GSS). This increased oxidative stress (ROS) in astrocytes, which promoted oxidative stress in wild-type motor neurones via release of soluble factors (Birger *et al*, 2019).

This oxidative stress in ALS patients may in part be due to failures of the antioxidant defence system, such as Nrf2, which will be discussed in section 1.4.3, in addition to overproduction of ROS due to mitochondrial dysfunction.

1.2.2.4.4 Mitochondrial dysfunction

A major contributor to oxidative stress in ALS patients is mitochondrial dysfunction, as ROS are a byproduct of mitochondrial oxidative phosphorylation. Mitochondria are also especially vulnerable to oxidative damage by ROS due to proximity, suggesting a possible feedback loop where mitochondrial damage results in oxidative stress and *vice versa* (Smith *et al*, 2019). Evidence for damaged mitochondria in ALS patient tissue includes the detection of swollen and aggregated mitochondria with abnormal morphologies in dorsal root ganglia (Sasaki & Iwata, 2007). Abnormal axonal transport of mitochondria has also been reported in ALS, for example in the sciatic nerve of TDP-43^{A315T} and mSOD1 mice, which may contribute to mitochondrial accumulation (Magrané *et al*, 2013).

Genetic evidence for mitochondrial dysfunction in ALS includes the discovery of causative variants in the CHCHD10 gene, as described in section 1.2.1.5, which result in a loss of cristae (folds in the inner mitochondrial membrane), which are vital for mitochondrial function (Genin *et al*, 2016). Loss or deformation of cristae has been reported in several ALS models, including in C9Orf72-ALS patient fibroblasts, mSOD1 mouse motor neurones and TDP-43^{A513T} mice (Kirkinezos *et al*, 2005; Onesto *et al*, 2016; Stribl *et al*, 2014). CHCHD10 mutations also prevent repair of the mitochondrial genome following oxidative stress in fibroblasts (Genin *et al.*, 2016). Evidence for

mitochondrial genome dysregulation has also been observed in postmortem sALS patient spinal cord (Wiedemann *et al*, 2002).

Mitochondrial dysfunction impacts respiration in ALS patients, for example in postmortem spinal cord of sALS patients there was reduced activity of all four electron transport complexes (Wiedemann *et al*, 2002). A possible contributing factor to reduced cellular respiration in ALS is the reduction of energy sources available to neurones, such as lactate. Expression of the astrocyte lactate efflux transporter Slc16a4 was shown to be reduced in mSOD1 mice, causing a decrease in spinal cord lactate levels and contributing to motor neurone toxicity in culture. This decrease in Slc16a4 expression was also detected in spinal astrocytes isolated from three SOD1-ALS patients (Ferraiuolo *et al*, 2011).

Mitochondria are also important for maintaining calcium homeostasis. The uptake of calcium by mitochondria is important for energy production, buffering increases in cytosolic calcium and regulating apoptosis (Contreras *et al*, 2010). Mitochondrial calcium handling may be affected in ALS, as loss of calcium homeostasis is reported in several ALS models and motor nerve terminals of ALS patients (Siklós *et al*, 1996; Smith *et al*, 2019). Mitochondria are often associated with the ER, allowing calcium exchange, but these interactions have been reported as disrupted in several ALS models, perhaps contributing to the loss of calcium homeostasis (Smith *et al*, 2019; Stoica *et al*, 2014; Stoica *et al*, 2016b).

1.2.2.5 Excitotoxicity

Glutamate is the major excitatory neurotransmitter in the brain. It binds to glutamate receptors such as α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), N-methyl-D-aspartate (NMDA) and kainite receptors on post-synaptic neurones to stimulate calcium influx and neurone firing. Excess glutamate is removed from the synapse by the astrocytic EAAT2 glutamate transporter, preventing overstimulation of the post-synaptic neurone (Niciu *et al*, 2012). Excitotoxicity occurs when overstimulation of glutamate receptors results in excessive neurone firing which leads to cell death (Dong *et al*, 2009). Excitotoxicity and oxidative stress may be in a vicious feed-forward cycle in ALS patients, as increased calcium influx into motor neurones can lead to mitochondrial dysfunction and ROS production. ROS may then be released from the cell and impair glutamate uptake by astrocytes, resulting in increased synaptic glutamate levels and further motor neurone stimulation (Rao & Weiss, 2004).

Mechanisms contributing to excitotoxicity in ALS patients may include the increased release of glutamate from the presynaptic neurone, although most studies have been limited to mSOD1 mice (Arnold *et al*, 2024; Raiteri *et al*, 2004). AMPA receptors also become more permeable to calcium, resulting in increased calcium uptake by neurones and therefore increased firing. This is because the calcium-impermeable GluR2 subunit of AMPA becomes permeable to calcium, due to impaired editing of its mRNA transcript by the ADAR2 enzyme (Arnold *et al*, 2024; Hosaka *et al*, 2021; Takuma *et al*, 1999).

This is likely due to the nucleocytoplasmic mislocalisation of ADAR2, which has been reported in postmortem ALS patient spinal cord tissue and several model systems (Arnold *et al.*, 2024; Hosaka *et al.*, 2021).

Astrocytes are important drivers of excitotoxicity in ALS patients, as they take up less glutamate from the synapse due to decreased expression of the glutamate transporter EAAT2, with a loss of 30-95% of EAAT2 reported in 60-70% of sALS patients. Aberrant processing of *EAAT2* mRNA has also been described, with abnormal transcripts detected only in disease-affected brain regions. Rapid degradation of these abnormal mRNAs may contribute to the loss of functional EAAT2 (Lin *et al.*, 1998a). Furthermore, sALS and *SOD1*^{A4V}-ALS, but not *C9orf72*-ALS, patient-derived iPSC-astrocytes release excessive glutamate (Mohamed *et al.*, 2019). The same was shown in mSOD1 mice, where increased GABA-induced glutamate release largely preceded symptom onset (Milanese *et al.*, 2010). Inhibiting astrocytic exocytosis prevented mSOD1 astrocyte-mediated motor neurone toxicity in mouse *in vitro* co-cultures and delayed onset in mSOD1 mice (Kawamata *et al.*, 2014). ATP stimulation of astrocytes via the P2X7 purinergic receptor also promotes astrocytic glutamate release, with activation of this receptor in mSOD1 astrocytes shown to be toxic to co-cultured motor neurones (Gandelman *et al.*, 2010).

Motor neurones have a low Ca²⁺ buffering capacity and high numbers of Ca²⁺-permeable AMPA receptors. While important for normal function, these properties may render them vulnerable to excitotoxicity and underly the selective nature of their degeneration in ALS (Van Den Bosch *et al.*, 2006). Motor cortex hyperexcitability has been detected in ALS patients using transcranial magnetic stimulation (TMS) technology, even before symptom onset in fALS patient (Odierna *et al.*, 2024), suggesting hyperexcitability and the associated excitotoxicity may be a driving disease mechanism. Further evidence for the importance of excitotoxicity in ALS pathogenesis includes the fact that riluzole, which modestly slows disease progression and prolongs survival, is an anti-excitotoxic drug. Riluzole blocks glutamatergic neurotransmission by inhibiting glutamate release and non-competitively blocking NMDA receptors (Doble, 1996).

1.2.2.6 Neuroinflammation

Neuroinflammation is a complex process which aims to limit CNS damage induced by pathogens, injury or neurodegeneration-associated products such as misfolded proteins. In ALS, chronic inflammation becomes detrimental and promotes non-cell autonomous neurodegeneration, whereby other cell types contribute to motor neurone demise in ALS. Neuroinflammation is mainly orchestrated by CNS-resident microglia and astrocytes (Stacchiotti *et al.*, 2025). However, damage to the BBB has been identified in ALS patients (Pan & Nicolazzo, 2022), suggesting that peripheral immune cells may enter the CNS to contribute to neuroinflammation, recruited by glia-released pro-inflammatory cytokines (Stacchiotti *et al.*, 2025).

Elevation of inflammatory mediators has been detected in several studies of ALS patient biofluids, indicating their potential as ALS biomarkers, such as this

panel in CSF: IL-9, IL-4, IL-7, IL-17, IL-13, IL-6, IL-1 β , TNF, IL-2 and GCSF (granulocyte colony stimulating factor) (Chen *et al*, 2018; Ehrhart *et al*, 2015; Femiano *et al*, 2024). There also may be an association between pro-inflammatory molecules such as IL-2, IL-6 and IFN γ and parameters of disease progression, suggesting increased neuroinflammation as ALS progresses (Femiano *et al*, 2024; Lu *et al*, 2016).

Studies in mSOD1 mice typically indicate an anti-inflammatory immune response in presymptomatic and early-stage ALS, involving anti-inflammatory microglia and regulatory T cells. In late-stage ALS, pro-inflammatory immune responses dominate, involving pro-inflammatory microglia and astrocytes, inflammatory monocytes, natural killer cells, neutrophils and cytotoxic CD8+ T cells (as reviewed in (Béland *et al*, 2020)). T cells have been demonstrated to infiltrate in the spinal cord of ALS patients, with CD4+ T cell infiltration shown to play a neuroprotective role in mSOD1 mice, perhaps by controlling microglial activation (Chiu *et al*, 2008; Engelhardt *et al*, 1993; Komine & Yamanaka, 2015).

As mentioned, evidence suggests there is microglial activation in some ALS patients, however there may be heterogeneity between genetic subtypes (Bond *et al*, 2025). Microglial activation was demonstrated to contribute to neurodegeneration in mSOD1 mice (Clarke & Patani, 2020; Xiao *et al*, 2007). Like astrocytes, microglial activation can have both neuroprotective and neurotoxic effects. In normal physiology, microglia promote neuronal health by providing immune surveillance, releasing anti-inflammatory and neurotrophic factors and clearing protein aggregates. In ALS, they may still promote neuroprotection by removing protein aggregates, however they can also release ROS and pro-inflammatory cytokines to promote neurotoxic astrocyte reactivity, potentiating neuroinflammation (Bond *et al*, 2025; Clarke & Patani, 2020).

1.2.2.6.1 Astrocyte-mediated neurotoxicity

The element of neuroinflammation most relevant to this thesis is the reactivity and associated neurotoxicity of human astrocytes. Astrocytes become reactive in response to neuronal degeneration, with levels of astrogliosis typically reported to be at their highest at the end stage of ALS (Vargas & Johnson, 2010). However, astrogliosis may not always be secondary to motor neurone degeneration, as astrocyte-specific SOD1^{G86R} expression in mice caused astrogliosis without motor neurone pathology (Gong *et al*, 2000). One hypothesis is that in pre-symptomatic ALS, astrocytes slowly transform to the A2 neuroprotective reactivity state, which promotes motor neurone survival and delays disease onset. Later, perhaps after additional insults, astrocytes may transform to the A1 neurotoxic state, worsening neurodegeneration (Izrael *et al*, 2020).

TDP-43 pathology may contribute to astrocyte reactivity in ALS, as TDP-43 overexpression in mouse primary astrocytes caused an augmented response to LPS, illustrated by over-production of proinflammatory cytokines and neurotoxic mediators (Swarup *et al*, 2011). TDP-43 knockdown also promoted astrocyte reactivity in rat astrocytes (LaRocca *et al*, 2019), perhaps suggesting

that fine control of TDP-43 is required for 'normal' astrocyte function. Since TDP-43 is depleted in the nucleus and accumulated in the cytoplasm in ALS, a possible hypothesis is that both factors may promote astrocyte reactivity.

In many studies, ALS mutant astrocytes have been demonstrated to contribute to, and sometimes be critical for, motor neurone degeneration. These studies include those using the mSOD1 mouse model, fALS and sALS patient postmortem astrocytes, skin fibroblast-derived and iPSC-derived astrocytes (Birger *et al.*, 2019; Di Giorgio *et al.*, 2007; Haidet-Phillips *et al.*, 2011; Marchetto *et al.*, 2008; Meyer *et al.*, 2014; Nagai *et al.*, 2007). For example, mouse embryonic stem cell (ESC)-derived motor neurones survive for less time in coculture with mSOD1 astrocytes than wild-type astrocytes. mSOD1 astrocytes release soluble neurotoxic factors that are specifically toxic to motor neurones, not spinal GABAergic, dorsal root ganglion neurones or interneurones. This suggests that astrocytes may be one reason for motor neurone-specific degeneration in ALS. mSOD1 fibroblasts, microglia, cortical neurones and myocytes do not induce the same neurotoxicity, further highlighting the important role of astrocytes (Nagai *et al.*, 2007).

The astrocytic contribution to non-cell autonomous neurodegeneration is likely due to both a loss of supportive functions and gain of neurotoxic functions. A neuroprotective function which may be impaired in ALS astrocytes is the release of neurotrophic factors (Komine & Yamanaka, 2015). Rat astrocytes exposed to ALS patient CSF released lower levels of VEGF and glial-derived neurotrophic factor (GDNF) (Mishra *et al.*, 2016). Furthermore, astrocytes from mSOD1 mice demonstrated an impaired capacity to increase brain-derived neurotrophic factor (BDNF) and GDNF in response to stimulation with high-mobility group box 1 (HMGB1) protein. HMGB1 is shown to have neuroprotective functions in wild-type astrocytes, including the upregulation of BDNF and GDNF, a process which therefore appears to be disrupted in mSOD1 astrocytes (Brambilla *et al.*, 2018).

Another astrocyte function which may be disrupted in ALS is the release of lactate to neurones, as discussed in section 1.2.2.4.4 (Ferraiuolo *et al.*, 2011). Astrocytes derived from both sALS and C9Orf72-ALS patients have been shown to have defects in energy production, such as fructose, glycogen and adenosine metabolism, which may reduce nutritional support for motor neurones (Allen *et al.*, 2019; Rossi & Cozzolino, 2021).

Secretion of soluble toxic factors by ALS astrocytes has been shown to play an important role in motor neurone toxicity (Di Giorgio *et al.*, 2007; Haidet-Phillips *et al.*, 2011; Nagai *et al.*, 2007). For example, mSOD1 astrocytic release of nerve growth factor (NGF) has been shown to contribute to motor neurone death, illustrating how different neurotrophic factors can have contrasting effects (Ferraiuolo *et al.*, 2011; Pehar *et al.*, 2004). Pehar *et al.* found that the mechanism involved the formation of the RNS nitric oxide and peroxynitrite (Pehar *et al.*, 2004). There may also be a decrease in astrocytic secretion of antioxidant proteins, as demonstrated by decreased SOD1, SOD2 and glutathione synthetase secretion from C9Orf72-ALS patient-derived iPSC-

astrocytes (Birger *et al.*, 2019). Although extracellular roles of these proteins have not been fully described, mSOD1 protein has previously been shown to be released via ADEVs (Marton *et al.*, 2023). Overall, these studies illustrate that both oxidative stress and a disrupted antioxidant response in astrocytes may contribute to motor neurone death in ALS.

1.3 microRNAs

MiRNAs are small non-coding (snc)RNAs ranging from 21-24 nucleotides in length, which are major regulators of post-transcriptional gene expression. Approximately 2,600 mature miRNAs are encoded by the human genome and miRNA expression patterns can be highly specific to tissues, cell types, subcellular compartments and pathologies (Guo *et al.*, 2014; Kozomara *et al.*, 2018; Xu *et al.*, 2022). This includes ALS, where miRNA dysregulation has been widely reported in ALS models and patient samples (Liu *et al.*, 2022). MiRNAs can be intragenic, meaning they are transcribed from within protein coding genes (usually introns), or intergenic, meaning they are separate from protein coding genes so are controlled by their own promoters (O'Brien *et al.*, 2018). A miRNA family refers to miRNAs with high sequence similarity resulting in similar functions (Gregorova *et al.*, 2021).

1.3.1 miRNA biogenesis and function

The canonical pathway of miRNA biogenesis is shown in Figure 1.5. It begins with the RNA polymerase II-catalysed production of intramolecularly double stranded primary (pri)-miRNA. The microprocessor complex, comprised of Drosha and DGCR8, cleaves pri-miRNA to produce precursor (pre)-miRNA. An exportin 5/RanGTP complex exports pre-miRNA into the cytoplasm. Here it is further processed by Dicer, an RNase III endonuclease, in association with proteins such as transactivation response RNA binding protein (TRBP) to form the mature miRNA duplex. The miRNA is loaded in an ATP-dependent manner onto a member of the Argonaute (AGO) family, which is AGO1-4 in humans. One of the strands, termed the passenger strand, is removed and degraded. The strand remaining associated with AGO is the guide strand. This guide miRNA is termed the 5p strand if it originates from the 5' end of pre-miRNA, while the 3p strand is from the 3' end (O'Brien *et al.*, 2018). In general, the predominant guide strand of a specific miRNA either has a uracil residue at position 1, or has the least thermodynamically stable 5' ends in the duplex (Khvorova *et al.*, 2003).

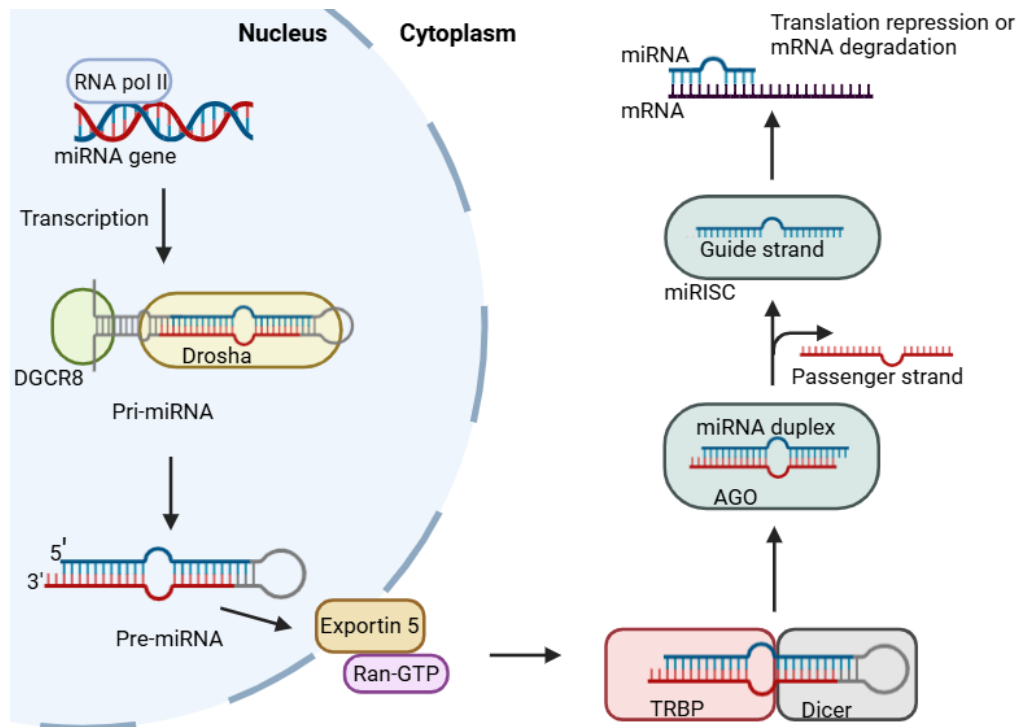


Figure 1.5. The canonical pathway of miRNA biogenesis. RNA polymerase II transcribes miRNA genes to produce pri-miRNA, which is then processed in the nucleus by Drosha/DCR8. This produces a 70nt pre-miRNA with a 2nt 3' overhang, which is transported to the cytoplasm by exportin 5. Dicer (in association with other proteins such as TRBP) then processes it further to produce a mature miRNA duplex (22nt with a 2nt 3' overhang). One of the miRNA strands (guide strand) binds to AGO proteins to form the miRISC complex. The seed sequence of the miRNA can then bind to MREs in mRNA sequence, resulting in mRNA degradation or translation repression. Created using BioRender.

The complex containing the guide strand and AGO, along with several other associated proteins, is called the minimal miRNA-induced silencing complex (miRISC or RISC). MiRISC interacts with sequences complementary to the miRNA within target mRNAs, known as miRNA response elements (MREs) which are usually in the 3' untranslated region (UTR) of the mRNA. Generally, the functional interaction occurs via the seed region (nucleotides 2-8 of the 5' end) of the miRNA. Further sequence complementarity at the 3' end helps with the stability and specificity of the interaction. If miRNA:MRE sequences are completely complementary, the mRNA is cleaved by AGO2 (O'Brien *et al.*, 2018). However, in animals most miRNA:MRE interactions are not completely complementary, so instead the mRNA is poly-deadenylated and de-capped, preventing its translation (Behm-Ansmant *et al.*, 2006). Overall, the mechanism of how miRNAs induce repression of their target mRNAs is incompletely understood (O'Brien *et al.*, 2018). There is also evidence for miRNAs in some instances promoting rather than repressing gene expression, adding further complexity to their mechanisms of action (Valinezhad Orang *et al.*, 2014).

MiRNAs are regulated both transcriptionally and post-transcriptionally. Since miRNAs have similar promoter regions to protein-coding genes, their transcription is regulated in a similar way by transcription factors, enhancers and chromatin modifications (Ozsolak *et al*, 2008). There are checkpoints at every step of the miRNA biogenesis pathway depicted in Figure 1.5. Signalling pathways crucial for miRNA biogenesis include the MAPK and GSK3 β pathways. For example GSK3 β -mediated phosphorylation of Drosha stimulates its nuclear localisation, and MAPK signalling results in nuclear localisation of the p68 Drosha component (Hong *et al*, 2013; Tang *et al*, 2011).

MiRNAs are very stable in biological fluids such as blood and CSF, allowing them to circulate throughout the body (Weber *et al*, 2010). While around 90% of circulating miRNAs are in complexes with proteins such as AGO2, the other 10% are encapsulated by EVs (Condrat *et al*, 2020). Local miRNA transfer via EVs is a method of cell-cell communication, including bi-directionally between neurones and astrocytes. For example, EVs from mouse neurone-conditioned medium contain the neuronal miRNA miR-124a. Astrocytes can internalise these EVs, causing an increase in astrocytic miR-124a, as well as its indirect target GLT1 (EAAT2 in humans). MiR-124a is downregulated in mSOD1 mouse spinal cord, likely due to neuronal loss. A miR-124a mimic may be a viable therapeutic strategy to combat excitotoxicity by increasing astrocytic EAAT2 expression (Morel *et al*, 2013). Muscle-derived EV miRNAs may also be important for neuroprotection, as inhibiting muscle release of the *TARDBP*-targeting EV-miR-126a-5p promoted presynaptic accumulation of TDP-43, which induced neuromuscular junction degeneration in iPSC-derived motor neurone/myotube co-cultures (Ionescu *et al*, 2025).

1.3.2 miRNA target prediction

There are many bioinformatic tools available which predict miRNA-mRNA interactions. Web-based tools include TargetScan, miRDB and DIANA Tools (MicroT-CDS) (Chen & Wang, 2019; McGeary *et al*, 2019; Tastsoglou *et al*, 2023). MiRNA sequences are typically obtained from the miRbase database, which as of 2019 contained 1917 hairpin precursor sequences and 2654 mature sequences (Kozomara *et al*, 2018). Target prediction can be complemented by tools such as TarBase, which collates experimentally validated miRNA-mRNA interactions (Skoufos *et al*, 2023).

While the algorithms for prediction vary between target prediction software, generally miRNAs are predicted to target a mRNA if its seed region is perfectly complementary to a sequence in the mRNA 3'UTR (Gu *et al*, 2009). Target prediction tools typically also provide the level of conservation of the seed sequence, as if this is highly conserved between species, the prediction is considered to be a more reliable and biologically advantageous (Friedman *et al*, 2009). TargetScan ranks predicted interactions, with the strongest interactions referred to as 8mer (mRNA sequence complementarity to miRNA positions 2-8 with an adenosine opposite position 1), followed by 7mer-m8 (complementary to positions 2-8), then 7mer-A1 (complementary to positions

2-7 with an adenosine opposite position 1) (Agarwal *et al*, 2015). The preference of an adenosine at position 1 does not depend on the miRNA nucleotides, as it is due to the recognition of adenosine by a binding pocket of Argonaute (Agarwal *et al.*, 2015; Schirle *et al*, 2014).

1.3.3 miRNAs in ALS

MiRNA expression changes or dysregulation in ALS have been widely documented in studies of patient tissue and biofluids, as well as cell and animal models (Foggin *et al*, 2019; Wang *et al*, 2024b). MiRNA dysregulation may be a consequence of disease, for example due to alterations in miRNA-regulating proteins TDP-43 and FUS, a driving disease mechanism, or a combination of both. Regardless, miRNAs isolated from patient biofluids are promising ALS biomarkers to aid diagnosis and monitor disease progression (Banack *et al*, 2024; Wang *et al.*, 2024b). MiRNAs may also be therapeutic targets for treating ALS, as they provide an opportunity to alter expression of many genes with one treatment.

1.3.3.1 ALS miRNA biomarkers

In recent years, the search for miRNA biomarkers in ALS has received increasing attention. Biomarkers may be used for disease diagnosis, prognosis or patient stratification for precision medicine. Several characteristics of miRNAs make them promising biomarkers. MiRNAs are stable in many biological fluids, so they can be attained from patients in a minimally invasive manner (Weber *et al.*, 2010). MiRNAs are often highly specific, for example to tissue of origin or pathology, and the technology for detecting panels of miRNA biomarkers is already established (Condrat *et al.*, 2020).

Many reviews have collated information on miRNAs which are dysregulated in ALS patients. This includes a previous literature review by our research group, which highlighted that the direction of dysregulation of many miRNAs in ALS patients often varied between studies (Foggin *et al.*, 2019). Tissues profiled included whole blood, serum, plasma, CSF, spinal cord and muscle. They also found that dysregulated miRNAs were not predicted to target ALS-associated genes more than a random selection of miRNAs, perhaps suggesting that miRNAs are not major disease drivers, or that target prediction is not accurate enough without support from experimental data. Despite this, Foggin *et al* highlighted miRNAs which were most commonly dysregulated in ALS patient studies at that time, with miR-133a-3p, let-7a-5p, miR-127-3p, miR-155-5p, miR-206-3p, miR-26-5p, miR-455-3p, miR-9-5p and miR-124-3p, each dysregulated in at least 6/27 studies (Foggin *et al.*, 2019).

EVs also represent a potential source of miRNA biomarkers. EVs that are released by degenerating motor neurones and other pathologically relevant cell types circulate in body fluids, which can then be isolated. Recently, Banack *et al* identified an 8-miRNA ALS signature from neural-enriched EVs isolated from plasma (Banack *et al.*, 2024). In ALS patients, they report upregulation of miR-199a-3p, miR-151a-3p, miR-151a-5p, miR-199a-5p, miR-146a-5p and

downregulation of miR-29b-3p, miR-10b-5p and miR-4454. Importantly, the direction and magnitude of dysregulation of these miRNAs have been consistent across four independent patient cohorts (Banack *et al*, 2020; Banack *et al*, 2022). A range of sample collection procedures were used, and samples were analysed by two laboratories, indicating the reproducibility of the test. Furthermore, the test distinguishes ALS from primary lateral sclerosis (PLS) and Parkinson's disease (PD) as well as healthy controls. Since PLS and PD are 'mimic' diseases that can share symptoms with ALS, it highlights the potential of the test to help clinicians to diagnose ALS, in conjunction with the current clinical metrics (Banack *et al*, 2024).

1.3.3.2 miRNAs in ALS astrocyte models

The use of ALS mouse models for studying miRNAs is limited by the fact that many miRNAs are not conserved between human and mouse, with poorly conserved miRNAs often considered less biologically important (Friedman *et al*, 2009). However, many studies still use these animal models, and some findings have been reflected in ALS patients. For example, dysregulation of miR-124-3p, miR-19b-3p, miR-218 and miR-335 has been reported in both the mSOD1 mouse model and in SOD1-ALS patient-derived iPSC-motor neurones (Dash *et al*, 2024; Wang *et al*, 2024b).

Some studies have investigated miRNA dysregulation in cultured ALS astrocytes and their secretome. The EV miRNA cargo of C9orf72-ALS patient fibroblast-derived astrocytes (iAstrocytes) was found to be altered, including those important for regulating axonal maintenance. This included downregulation of miR-494-3p, a negative regulator of semaphorin 3A, an important neuronal protein. Treating motor neurones with miR-494-3p mimic partially rescued the motor neurone death induced by C9orf72-ALS iAstrocyte conditioned medium (Varcianna *et al*, 2019). Depleted expression of miR-146a in mSOD1 mouse astrocytes and their derived EVs may have deleterious effects on motor neurones and microglia (Barbosa *et al*, 2021; Gomes *et al*, 2019). This contrasted findings by Jovičić *et al*, who found that mSOD1 expression did not affect the miRNA profile of mouse astrocyte-derived EVs (Jovičić & Gitler, 2017). MiR-146a expression was also variable in mSOD1 and sALS patient-derived iAstrocytes, exemplifying the limited translatability of the mSOD1 mouse model for miRNA studies (Gomes *et al*, 2022).

mSOD1 rat ADEVs were also shown to be significantly enriched for miR-155-5p, with this elevation contributing to mSOD1 ADEV-induced motor neurone death (Marton *et al*, 2023). MiR-124 was shown to be associated with astrocytic differentiation of neural stem cells in mSOD1 mice due to Sox2 and Sox9 targeting (Zhou *et al*, 2018), which could be predicted to increase the number of neurotoxic astrocytes in ALS patients. Hoye *et al* demonstrated that dying iPSC-motor neurones release miR-218, which can be taken up by mouse astrocytes and is sufficient to downregulate the glutamate transporter GLT1 (EAAT2). Anti-miR-218 treatment in mSOD1 mice reduces this loss of EAAT2, therefore may increase glutamate reuptake from the synapse and reduce excitotoxicity (Hoye *et al*, 2017; Hoye *et al*, 2018). As discussed in section 1.3.1,

EVs from mouse neurone-conditioned medium contain miR-124a, which when internalised by astrocytes instead cause an increase in its indirect target GLT1. MiR-124a replacement may therefore be another viable EAAT2-upregulating therapeutic strategy (Morel *et al.*, 2013).

A miR-29b-3p mimic may be another viable astrocyte-targeted ALS therapeutic, as it was shown to increase NQO1 antioxidant activity in patient-derived iAstrocytes, leading to reduced motor neurone toxicity (Provenzano *et al.*, 2022). Overall, these studies illustrate the possible important role of astrocyte miRNAs in ALS pathogenesis, and the therapeutic potential of an astrocyte-targeted miRNA modulating therapy.

1.3.3.3 miRNAs as therapeutic targets in ALS

As illustrated in ALS astrocyte studies, miRNAs are attractive therapeutic targets. Since each miRNA typically targets a range of mRNAs, often in the same biological pathway, the inhibition or overexpression of a single miRNA may have significant widespread effects, potentially affecting whole biological processes. While this creates a concern about off-target effects, this can also be the case with single-gene targeting therapies (Hanna *et al.*, 2019). While several miRNA-based therapies are in clinical trials for various conditions, none are yet FDA-approved, with technical challenges including ensuring sensitive and selective targeting, improving delivery methods and preventing an immunogenic response (Seyhan, 2024). Recently, an adeno-associated virus (AAV)-mediated delivery of an artificial miRNA based on pri-miR-451, named AMT-130 appeared to be the first effective treatment for Huntington's disease in a Phase I/II trial, although the full study data has not yet been published (Bokinni, 2025; Kotowska-Zimmer *et al.*, 2022).

Artificial miRNAs (mimics) have shown potential for ALS treatment, with two similar studies reporting their potential for treating *SOD1*-ALS. An artificial *SOD1*-targeting miRNA was transmitted to mSOD1 mice using the rAAVrh10 AAV, which prolonged survival in both studies (Borel *et al.*, 2016; Wang *et al.*, 2014). The treatment reduced mSOD1 gene expression in non-human primate lower motor neurones in one study (Borel *et al.*, 2016), and in transduced cells (neurones, astrocytes and oligodendrocytes) of the mSOD1 mouse spinal cord in the other study (Wang *et al.*, 2014). Following these successes, the AAV-delivered artificial *SOD1*-targeting miRNA APB-102 was developed (also called AMT-162). This therapy was tested in two patients with familial *SOD1*-ALS, who were treated with a single intrathecal injection. Although it is difficult to draw conclusions from two patients, SOD1 protein levels were unchanged by the treatment. As of 2023, the company UniQure was acquiring the rights for phase I/II clinical trials (Tzeplaeff *et al.*, 2023).

Regarding miRNA inhibitors for ALS treatment, further to the potential astrocyte-specific treatments discussed in the previous section, additional examples of miRNA inhibitors for ALS treatment include intraventricular delivery of anti-miR-155 in mSOD1 mice which prolonged survival, and anti-miR-29a treatment at day 70 mSOD1 mice which was associated with a trend towards increased lifespan in males (Koval *et al.*, 2013; Nolan *et al.*, 2014).

While no miRNA-based therapies are currently approved for ALS treatment, there has recently been significant progress in the RNA therapeutics field. Antisense oligonucleotide (ASO) technology is being actively investigated for ALS treatment, following success in treating other the neurological diseases spinal muscular atrophy and Duchenne muscular dystrophy (Crawford *et al*, 2023; Syed, 2016). ASOs are short, modified nucleic acid molecules which bind to target RNA sequences to prevent or alter transcription. A recent breakthrough in the ALS field was the development of the ASO tofersen (trade name Qalsody), which was FDA-approved for treatment of *SOD1*-ALS in April 2023. Tofersen reduces synthesis of SOD1 protein by inducing RNase H-mediated degradation of its mRNA (Everett & Bucelli, 2024; Miller *et al.*, 2022). An ASO for *FUS*-ALS known as jacifusen (ION363) is currently undergoing clinical trials, with initial findings in 12 patients demonstrating safety and possible efficacy, including unprecedented functional recovery in one patient (Boros *et al*, 2022; Shneider *et al*, 2025). These successes with ASO treatments highlight the potential of other nucleic acid-based therapeutics for ALS treatment.

1.3.3.4 Aberrant miRNA processing in ALS

Several ALS-associated genes, notably *TARDBP* and *FUS*, have a role in RNA metabolism, including the processing of miRNAs. TDP-43 promotes efficient binding of Drosha to certain primary miRNAs including pri-miR-558-3p, pri-miR-574-3p, pri-miR-132-3p/5p, and pri-miR-143-3p/5p. In the cytoplasm, TDP-43 binds to the terminal loops of pre-miR-143 and pre-miR-574 to promote their Dicer-mediated cleavage. TDP-43 was found to regulate neuronal outgrowth of Neuro2A cells, at least in part due to its role in miRNA production, supporting the hypothesis that TDP-43-mediated miRNA regulation may be important for neurone health and function (Kawahara & Mieda-Sato, 2012). Several TDP-43 regulated miRNAs were found to be dysregulated in sALS patient CSF and/or serum, supporting that TDP-43 pathology may impact miRNA processing in ALS (Freischmidt *et al*, 2013).

Like TDP-43, *FUS* binds to specific pri-miRNAs and assists with recruitment of Drosha. High-throughput RT-qPCR revealed that 166/377 (44%) of measured miRNAs were deregulated by >15% upon *FUS* knockdown in the neuroblastoma SK-N-BE cell line. Of these deregulated miRNAs, 90% were downregulated, illustrating the importance of *FUS* for miRNA production. Several miRNAs with important roles in neuronal function, differentiation and synaptogenesis were downregulated, including the TDP-43 regulated miRNAs miR-9 and miR-132. The ALS mutants *FUS*^{R521C} and *FUS*^{P525L} reduced levels of the *FUS*-regulated miRNAs miR-9, miR-132 and miR-192 in these neuroblastoma cells, with *FUS*^{P525L} having the greatest effect. *FUS*^{P525L} exhibits a much stronger cytoplasmic mislocalisation phenotype than *FUS*^{R521C}, therefore consistent with reduced *FUS* in the nucleus in *FUS*-ALS patients preventing the efficient processing of these miRNAs (Morlando *et al.*, 2012).

FUS was also shown to promote the mRNA-targeting activity of certain miRNAs, by associating with AGO2 and miRNAs in the miRISC complex, as well as their

target mRNA transcripts. Specifically, Zhang *et al* showed that FUS promotes the miR-200c-mediated silencing of *ZEB1*, which is reduced by the ALS-associated FUS^{R495X} mutation (Zhang *et al.*, 2018). FUS also promotes expression of miR-141 and miR-200a, miRNAs which target the 3'UTR of FUS, therefore creating a regulatory loop. This loop may be deregulated in ALS patients with the severe FUS^{G48A} mutation, as the mutation is found in the 3'UTR sequence complementary to the two miRNAs (Dini Modigliani *et al*, 2014).

As noted in section 1.2.1.4, FUS has also been demonstrated to have a role in miRNA selection for loading into EVs from several non-CNS mouse cell lines, with preferential loading of miRNAs containing the mouse-specific CGGGAG sequence, termed an 'EXOmorf' (Garcia-Martin *et al.*, 2022). Following this study, FUS was suggested to have a role in selecting miRNAs for ADEV loading, since ~15% of human iPSC-derived ADEV miRNAs were reported to contain other sequences recognised by FUS (Gordillo-Sampedro *et al*, 2024).

1.3.4 miRNAs and astrocyte reactivity

Little is known about how miRNAs regulate astrocyte reactivity. MiR-181 was shown to be anti-inflammatory in mouse primary astrocytes, for example miR-181 knockdown increased the LPS-induced production of pro-inflammatory cytokines TNF α , IL-6, IL-1 β and IL-8. Meanwhile, miR-181 overexpression in mouse astrocytes increased expression of IL-10, an anti-inflammatory cytokine (Hutchison *et al*, 2013). However miR-181a-5p inhibition was shown to protect the rat hippocampus from astrocyte and microglia activation, contrasting the previous findings (Kong *et al*, 2020).

MiR-125b is another miRNA which may be involved in astrocyte reactivity, although similarly its specific role appears to vary between models. LPS and IFN- γ -induced astrogliosis was associated with decreased miR-125b expression in marmoset astrocytes, whereas IL-6-induced astrogliosis induced miR-125b upregulation (Mor *et al*, 2011; Pogue *et al*, 2010). MiR-125b inhibition attenuated IL-6-induced astrocyte proliferation, therefore this may be represent a potential astrocyte reactivity-targeted therapeutic strategy (Pogue *et al.*, 2010). MiR-125b has also been shown to promote activation of ALS microglia, with miR-125b inhibition promoting motor neurone survival in mouse cell cultures (Parisi *et al*, 2016). MiR-21 and miR-145 are negative regulators of astrogliosis induced by spinal cord injury, for example reducing glial scarring (Bhalala *et al*, 2012; Wang *et al*, 2015).

TNF α and IL-1 β have been shown to alter miRNA expression in a range of astrocyte models, as reviewed in (Chu & Williams, 2021), with upregulation of miR-155-5p and miR-146a-5p reported in multiple studies (Iyer *et al*, 2012; Junker *et al*, 2009; van Scheppingen *et al*, 2016). The pro-inflammatory effects of miR-155 are in part due to its targeting of suppressor of cytokine signalling 1 (SOCS1), an anti-inflammatory regulator (Cardoso *et al*, 2012). MiR-146a is an anti-inflammatory miRNA, for example due to its targeting of *IRAK-1* and *TRAF6* genes, which are components of the NF- κ B pathway (Iyer *et al.*, 2012).

1.4 The Nrf2 pathway

1.4.1 Nrf2 – structure, regulation and function

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor encoded by the *NFE2L2* gene. Nrf2 is best known as the master regulator of the cellular response to oxidative stress, or the antioxidant response (Vomund *et al*, 2017). This includes in the CNS, where it is particularly abundant in glial cells (Navarro & Esteras, 2024). The Nrf2 protein is a member of the Cap'n'Collar subfamily of basic leucine zipper (bZIP) transcription factors, of which other members include NRF1 and NRF3 (Tonelli *et al*, 2018).

Under basal conditions, Nrf2 remains in the cytoplasm associated with the protein Kelch-like ECH-associated protein 1 (KEAP1) via the actin cytoskeleton (Itoh *et al*, 1999). KEAP1 is an adaptor protein for the Cullin-3-based E3 ligase, therefore in unstressed conditions it continuously targets Nrf2 for ubiquitin-mediated proteasomal degradation (Cullinan *et al*, 2004). Under oxidative stress, the Nrf2-KEAP1 interaction is inhibited and KEAP1 is degraded by autophagy, releasing Nrf2 so it can translocate to the nucleus to activate gene expression. This suppression of the Nrf2-KEAP1 interaction can occur by various mechanisms. For example, endogenous electrophiles modify cysteine residues of KEAP1, or kinases phosphorylate Nrf2 at serine and threonine residues. This induces a conformational change to facilitate the release of Nrf2 from KEAP1 (Dinkova-Kostova *et al*, 2002; Kobayashi & Yamamoto, 2006). Once in the nucleus, Nrf2 forms heterodimers with small Maf proteins before binding to antioxidant response elements (ARE) within the promoters of specific antioxidant genes, activating gene expression (Hirotsu *et al*, 2012).

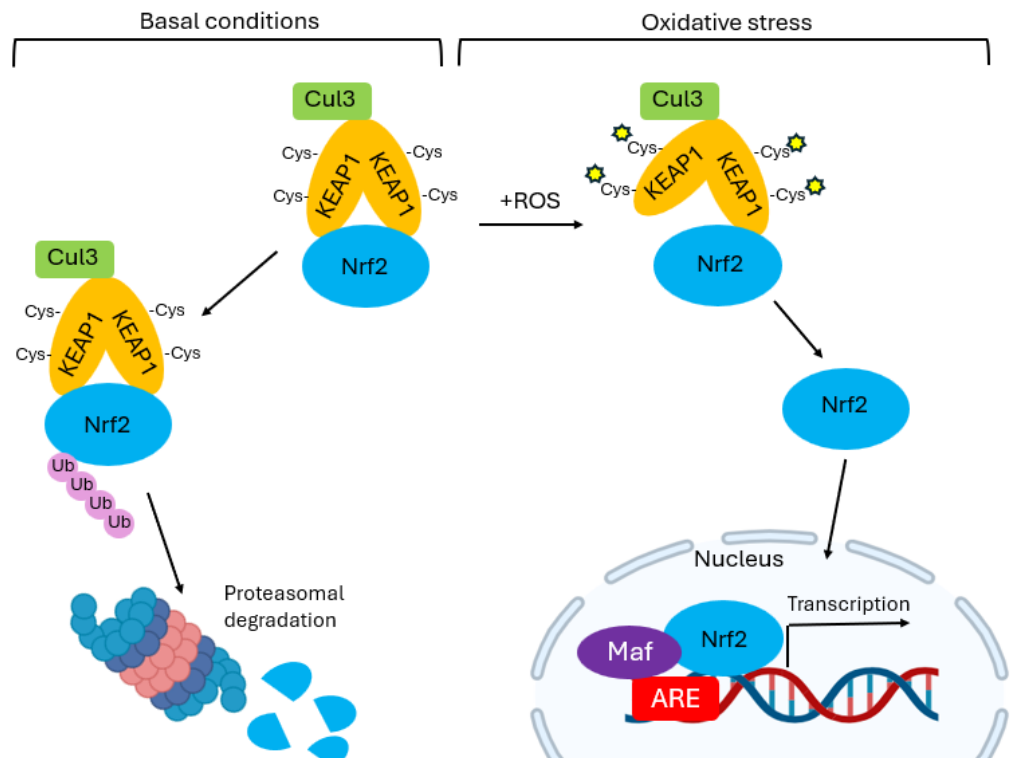


Figure 1.6. The KEAP1-Nrf2 pathway. Under basal conditions, Nrf2 is bound to a KEAP1 homodimer and ubiquitinated by the Cul3 ubiquitin ligase (for which KEAP1 is an adapter), targeting Nrf2 for proteasomal degradation. ROS modify key cysteine residues of KEAP1, inducing a conformational change which releases Nrf2. Nrf2 is then free to translocate to the nucleus where it binds with Maf proteins to ARE regions in promoters to activate target gene expression. Created using BioRender.

As noted earlier, antioxidant genes induced by Nrf2 include those in the thioredoxin and glutathione antioxidant defence pathways (Liddell, 2017). However, Nrf2 target gene functions extend beyond the antioxidant response, including various metabolic pathways and proteostasis. While not an exhaustive list, a summary of Nrf2 target genes with associated biological functions presented in Table 1.2, many of which have been validated in human cells.

Table 1.2. Nrf2 target genes and their associated functions.

General Biochemical Function	Genes
Biotransformation and detoxification (phase I, II and III)	<i>ABCB6, ABCC1, ADH7, ALDH, CBR1, CYP1B1, EPHX1, NQO1, UGT1A1</i>
Antioxidant response	<i>GCLC, GCLM, GPX1, GSR1, PRDX1, SRXN1, TXN1</i>
Carbohydrate metabolism and NADPH generation	<i>G6PD, HDK1, IDH1, ME1, PGD, TALDO1, TKT</i>
Lipid metabolism	<i>ACOT7, ACOX1, SCD2</i>
Haem and iron metabolism	<i>BLVRA, BLVRB, FTH1, FTL1, HMOX1</i>
Purine biosynthesis	<i>PPAT</i>
Protein degradation	<i>ATF4, PSMA1, PSMB5, SQSTM1, ATG5, ATG7, LC3B</i>
Apoptosis	<i>BCL2</i>
Growth factors	<i>FGF, NRG1</i>
Mitochondrial function	<i>NRF1</i>
Protein folding	<i>DNAJB1</i>

Table is adapted from (Lewczuk et al, 2023; Song et al, 2021). Note that several genes have multiple of the listed biochemical functions.

The Nrf2 protein consists of seven conserved NRF2-ECH homology domains (Neh), as depicted in Figure 1.7 (Tonelli et al., 2018). The Neh1 domain can bind to DNA and associate with small Maf proteins (Fuse & Kobayashi, 2017). The Neh2 domain mediates the interaction between KEAP1 and the Nrf2 lysine residues that are ubiquitylated to induce proteasomal degradation (Itoh et al., 1999). The Neh3 domain has transactivation activity, working with Neh4 and Neh5 to activate transcription of target genes (Nioi et al, 2005). The Neh6 domain is important for the KEAP1-independent regulation of Nrf2 stability. Specifically, GSK3 β phosphorylates a region of the Neh6 domain, which facilitates the binding of β -transducing repeat-containing protein (β -TrCP). This induces the recruitment of the ubiquitin ligase complex Skp1-Cul1-F-box protein (SCF) to Nrf2, therefore promoting the proteasomal degradation of Nrf2 (Rada et al, 2011). The Neh7 domain is also involved in Nrf2 repression, as retinoic X receptor alpha (RXR α) binding to Neh7 represses Nrf2 activity (Wang et al, 2013).

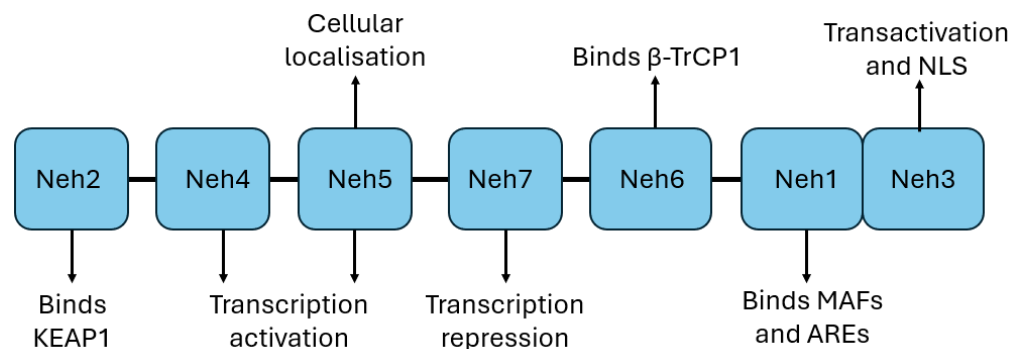


Figure 1.7. Schematic of the domain structure of Nrf2 protein.

Nrf2 can be regulated at the transcriptional or post-transcriptional level. Expression of the *NFE2L2* gene is induced by a range of stimuli, including the inflammatory stimulus LPS and the transcription factor NFκB, the cancer-related BRCA1, Kras, B-Raf and Myc proteins, and PI3K-Akt and Notch signalling pathways (Tonelli *et al.*, 2018). Transcriptional regulation can also occur via promoter regulation, for example hypermethylation may reduce Nrf2 expression (Schmidlin *et al.*, 2019; Yu *et al.*, 2010). The *NFE2L2* promoter also contains two ARE-like sequences, creating an autoregulatory loop (Kwak *et al.*, 2002). Nrf2 post-transcriptional regulation includes alternative splicing, for example transcript variants missing exons 2, or exons 2 and 3, have been observed in certain cancers. These variants are missing the KEAP1 interaction domain, therefore Nrf2 is stabilised and over-active (Goldstein *et al.*, 2016). MiRNAs are another source of Nrf2 post-transcriptional regulation, which will be discussed further in section 1.4.5.

As discussed, Nrf2 protein stability is regulated by its interaction with KEAP1, therefore several factors that influence KEAP1 stability can regulate Nrf2. These include the ALS-associated gene products SQSTM1 (p62) and TBK1, as TBK1 phosphorylates p62 to activate p62-mediated autophagic degradation of KEAP1, promoting Nrf2 activation (Deng *et al.*, 2020). ALS variants in *TBK1* reduce p62 phosphorylation and autophagic activity, while *SQSTM1* variants reduce Nrf2 activation in reporter assays (Deng *et al.*, 2020; Goode *et al.*, 2016).

1.4.2 Nrf2 in astrocytes

Astrocytes are an important source of Nrf2 signalling in the CNS, as neurones rely on antioxidant support from astrocytes (Vargas & Johnson, 2009). In neurones, Nrf2 is epigenetically repressed during early development, which has been shown to be necessary for correct neuronal development (Bell *et al.*, 2015). Many Nrf2-regulated genes are preferentially activated in astrocytes over neurones, resulting in a more efficient antioxidant defence system in astrocytes (Vargas & Johnson, 2009). For example, there is greater expression of enzymes in the glutathione synthesis pathway in astrocytes, resulting in more efficient glutathione synthesis. This allows the secretion of glutathione from astrocytes to neurones, which is a critical neuroprotective mechanism (Shih *et al.*, 2003). Greater expression of Nrf2 pathway components in glial cells than motor neurones has also been confirmed in mSOD1 mice (Mimoto *et al.*, 2012). In mixed cortical cultures, pharmacological Nrf2 activation via the commercial activator tBHQ has been found to selectively activate Nrf2 in astrocytes, but not neurones, with this astrocytic effect sufficient for neuroprotection (Kraft *et al.*, 2004). A similar effect was observed in mSOD1 mice, where astrocytic Nrf2 activity can also increase in response to elevated neuronal activity, to provide sufficient antioxidant support. Neuronal secretion of glutamate and other unknown soluble factors results in astrocyte glutamate receptor activation and a Ca²⁺ signalling cascade, resulting in Nrf2 activation (Habas *et al.*, 2013).

1.4.2.1 Nrf2 in astrocyte reactivity

Several studies have suggested that Nrf2 protects against astrocyte reactivity. In the brains of an AD mouse model, Nrf2 deletion promoted astrocyte reactivity, while increasing Nrf2 expression blocked reactivity by reducing NF- κ B signalling (Nakano-Kobayashi *et al*, 2023). Nrf2 activation has been shown to convert neurotoxic A1 astrocytes to neuroprotective A2 astrocytes, in both wild-type mouse primary astrocytes and AD mice, using dimethyl fumarate (DMF) and AAV-mediated Nrf2 overexpression, respectively. In the mouse model, again this was found to be due to NF- κ B pathway inhibition (Guo *et al*, 2025). Furthermore, astrocyte-specific Nrf2 overexpression via the GFAP promoter in mSOD1 mice reduced glial reactivity (Vargas *et al*, 2008). DMF or ginseng-mediated Nrf2 activation also reduced reactive gliosis in astrocytes and microglia following cerebral ischemia in mice (Liu *et al*, 2020). Nrf2 activation via KEAP1 knockout reduced astrocyte reactivity in a mouse model of spinal cord injury (Zhao *et al*, 2022). Finally, Nrf2 has been shown to suppress pro-inflammatory responses of astrocytes in mice with CNS autoimmune disease (modelling multiple sclerosis) (Wheeler *et al*, 2020).

In contrast, there is limited evidence that Nrf2 may promote, or not affect, astrocyte reactivity. Nrf2 overexpression in mouse astrocytes induced a strong upregulation of genes which had previously been found to be induced in astrocytes by the reactivity-inducing stimuli LPS and/or ischemia in mice (Jiwaji *et al*, 2022; Zamanian *et al.*, 2012). The demyelinator cuprizone causes reactive gliosis in wild-type mice, however Nrf2 hyperactivation in astrocytes (via KEAP1 knockout) did not counteract this reactivity (Draheim *et al*, 2016). Astrocyte-specific Nrf2 overexpression via the GFAP promoter protected mice from lesions induced by the neurotoxic mitochondrial inhibitor malonate, however it did not protect against astrocyte reactivity (Calkins *et al*, 2010). There may be cell-type specific effects of Nrf2 on neuroinflammation, as after LPS-induced systemic inflammation in mice, genetic knockout of Nrf2 in microglia and astrocytes, but not endothelial cells, reduced inflammatory responses (indicated by RNAseq). Without inflammation, Nrf2 promoted expression of pro-inflammatory genes only in microglia (He *et al*, 2025).

In summary, Nrf2 appears to protect against astrocyte reactivity, however there are subtleties between models. Since astrocyte reactivity is highly heterogeneous depending on stimulus, the studies mentioned likely represent different reactivity states and thus different Nrf2 phenotypes.

1.4.3 Nrf2 in ALS

Impairment of the Nrf2 pathway in ALS has been demonstrated in patients and cell culture models (Arslanbaeva & Bisaglia, 2022). Nrf2 protein was downregulated in postmortem spinal cord and motor cortex of ALS patients, whereas KEAP1 was upregulated (Sarlette *et al*, 2008). The Nrf2 target gene and mitochondrial protein peroxiredoxin 3 (PRDX3) was downregulated in postmortem spinal motor neurones from sporadic MND and mSOD1-MND patients, evidencing impaired mitochondrial antioxidant defence (Wood-Allum

et al, 2006). Nrf2 expression was also reduced in mSOD1 rat primary motor neurones and motor neurone-like NSC34 cells transfected with mSOD1, in which Nrf2 target genes were also downregulated (Kirby *et al*, 2005; Pehar *et al*, 2007). As discussed in section 1.2.2.4.3, C9Orf72-ALS patient-derived iPSC-astrocytes had reduced expression of the Nrf2 target antioxidant proteins glutathione synthetase and peroxiredoxins (Birger *et al.*, 2019). Astrocytes from TDP-43^{Q331K} mice were shown to have evidence of oxidative stress and increased expression of Nrf2 target gene transcripts, but reduced levels of glutathione. This suggests disrupted translation of Nrf2 target genes, which may be due to aberrant binding of hnRNP RBPs to antioxidant mRNA (Moujalled *et al.*, 2017). This disconnection between Nrf2 pathway activation and antioxidant gene translation has also been demonstrated in mutant TDP-43 NSC34 cells (Duan *et al*, 2010). Since TDP-43 pathology is a hallmark of ALS, this may contribute to Nrf2 pathway impairment in most ALS patients.

Impairment of the Nrf2 pathway in older ALS patients may also in part be due to age. Expression of Nrf2 positive regulators (PI3K, p62, CBP and BRCA1) generally decreases with age, while negative regulators (KEAP1, BACH1 and c-Myc) increase with age, in a range of cellular and animal models, as reviewed in (Zhang *et al*, 2015). This includes in mouse astrocytes, where expression of Nrf2 and its target genes NQO1 and HO-1 severely decline with age, which may contribute to the reduced neuroprotective ability and increased vulnerability to oxidative stress of older astrocytes (Duan *et al*, 2009). DNA methylation also increases with age, which may be predicted to reduce Nrf2 expression by hypermethylation of its promoter (Schmidlin *et al.*, 2019).

In contrast, Nrf2 activity has been reported as upregulated in the muscle and spinal cord of mSOD1 rodents (Kraft *et al*, 2007; Mimoto *et al.*, 2012), including in spinal cord reactive astrocytes (Vargas *et al*, 2005). Measures of Nrf2 activity included enzyme activity of the Nrf2 target gene product hPAP (human placental alkaline phosphatase), and expression of Nrf2, KEAP1 and HO-1 (Kraft *et al.*, 2007; Mimoto *et al.*, 2012; Vargas *et al.*, 2005). Nrf2 activation may increase with disease progression, likely as an attempted neuroprotective mechanism (Kraft *et al.*, 2007; Mimoto *et al.*, 2012). Despite this Nrf2 pathway upregulation, knockout of Nrf2 in mSOD1 mice does not have great impact on disease onset or lifespan, perhaps due to activation of antioxidant genes by alternate mechanisms (Guo *et al*, 2013; Vargas *et al*, 2013). The fact that Nrf2 is upregulated in mSOD1 mice, but that its deletion has little effect, may further support that Nrf2 signalling is impaired (Liddell, 2017).

It must be noted that most of these studies rely on the mSOD1 mouse model, which is an imperfect model of many ALS cases, with many clinical trials not being able to replicate findings from mSOD1 mice (Vinsant *et al*, 2013). Importantly, Nrf2 is itself a regulator of SOD1, therefore Nrf2 pathway phenotypes may be specific to this model, and Nrf2 therapeutic activation should be investigated in non-SOD1 models, which is addressed in this thesis (Kraft *et al.*, 2007).

Finally, there is also genetic evidence for Nrf2 pathway involvement in ALS pathogenesis. In a study of Swedish sALS patients, rare variants in the *NFE2L2* and *KEAP1* genes were identified which delayed disease onset or reduced sALS risk, suggesting that the Nrf2 pathway may modulate disease pathogenesis (Bergström *et al*, 2014). Further genetic evidence comes from studies of p62 (SQSTM1), where ALS-associated mutations in the KEAP1-interacting domain reduced Nrf2 signalling in HEK293T cells (Goode *et al.*, 2016).

The effects of Nrf2 pathway impairment in ALS are likely to be far-reaching, due to the broad functions of Nrf2 and its target genes. Analysis of ALS patient transcriptomic data revealed multiple pathways that may be impacted by Nrf2 dysregulation, including redox balance, protein quality control, RNA processing, inflammation resolution, intracellular trafficking, vascular homeostasis, and excitotoxicity (Jiménez-Villegas *et al*, 2021). Nrf2 activation could therefore be a single-hit approach to target many pathogenic mechanisms and induce neuroprotection.

1.4.4 Therapeutic targeting of Nrf2

There are many commercial Nrf2 activators, for which the mechanisms of action often include electrophilic modification of KEAP1 to induce dissociation of Nrf2, or inhibition of Nrf2 transcriptional competitors such as BACH1. These include the natural products curcumin, resveratrol and sulforaphane, and the synthetic activators DMF and tBHQ (tert-butylhydroquinone) (Liu *et al*, 2023b; Pouremamali *et al*, 2022). Several Nrf2 activators have entered clinical trials for other indication, although as of October 2025 the only FDA approved activators are DMF for treatment of multiple sclerosis, and omaveloxolone for Friedrich's ataxia (Wang *et al*, 2024a). Nanocurcumin, a more soluble form of curcumin, was shown in a pilot trial to be safe and potentially improve survival of sALS patients in conjunction with riluzole (Ahmadi *et al*, 2018). Another ALS trial investigated combined curcumin (200mg) and resveratrol (75mg) treatment for at least 6 months, however the results are not yet published (NCT04654689). Other Nrf2 activators investigated in pre-clinical models include the compounds CDDO ethylamide, CDDO trifluoroethylamines (CDDO-TFEA), WN1316, γ -oryzanol and CPN-9 (Kanno *et al*, 2012; Neymotin *et al*, 2011; Tanaka *et al*, 2014; Zhang *et al*, 2019a).

The FDA-approved ALS therapeutic edaravone is also an antioxidant and appeared to increase Nrf2 protein levels in several models, for example in neurone-like PC12 cells, human corneal epithelial cells and a mouse model of asthma (Li *et al*, 2017; Pan *et al*, 2020; Shou *et al*, 2019), which will be discussed more in Chapter 6. An Nrf2 activator M102 (S[+]-Apomorphine), a non-toxic enantiomer of a clinically approved dopamine receptor agonist, has been shown to be brain penetrant and promising for ALS treatment. M102 improved glutathione secretion from mouse primary astrocytes in co-culture with motor neurones and protected mSOD1 mice from neuromuscular junction denervation or declining motor function (Mead *et al*, 2013). M102 has also been shown to rescue TDP-43 proteinopathy, oxidative stress, and astrocyte-

induced motor neurone toxicity in co-cultures of patient-fibroblast-derived astrocytes and mouse motor neurones. Neuromuscular function was also improved in mSOD1 and TDP-43^{Q331K} ALS mouse models (Keerie *et al.*, 2025). In addition to Nrf2, M102 activates the chaperone protein heat shock factor 1 (HSF-1), therefore also targeting the proteostasis disturbances observed in ALS (Kampinga & Bergink, 2016; Keerie *et al.*, 2025).

Nrf2 activation specifically in astrocytes also demonstrates therapeutic potential. Multiple studies show that mSOD1 rodent astrocytes are toxic to co-cultured wild-type motor neurones *in vitro*, however this neurotoxicity was completely reversed by astrocytic overexpression of Nrf2 via the astrocyte-specific GFAP promoter (Nagai *et al.*, 2007; Vargas *et al.*, 2008; Vargas *et al.*, 2006). This was due to increased secretion of glutathione, which likely improves the antioxidant defence of motor neurones. Astrocytic Nrf2 overexpression increased lifespan and delayed disease onset in mSOD1 mice, illustrating its potential as a therapeutic strategy (Vargas *et al.*, 2008). In contrast, when Nrf2 was upregulated in neurones or muscle of mSOD1 mice, the disease onset was delayed but survival was not increased (Vargas *et al.*, 2013). These studies indicate that Nrf2 upregulation confers strong neuroprotection in ALS models primarily when it occurs in astrocytes (Liddell, 2017).

Importantly for this thesis, the Nrf2 pathway has previously been activated in ALS astrocytes by a miRNA-regulating therapeutic, to facilitate neuroprotection. Transfection of SOD1-ALS and C9orf72-ALS patient-derived iAstrocytes with a miR-29b-3p mimic activated the Nrf2 pathway via inhibition of the Nrf2 negative regulator BACH1, which improved the survival of co-cultured mouse motor neurones. A similar effect was also produced by mesenchymal stem cell-derived EVs by a miRNA-mediated mechanism (Provenzano *et al.*, 2022).

1.4.5 miRNA-mediated regulation of Nrf2

Nrf2 expression and activity must be tightly controlled to facilitate timely response to oxidative stress. In addition to Nrf2 protein regulation by KEAP1, post-transcriptional regulation by miRNAs has been shown to have an important role. Many miRNAs have been demonstrated to bind to the 3'UTR of Nrf2 (*NFE2L2*) to inhibit expression, of which many are described in Table 1.3. Target prediction software (accessed 2024) reveal other potential targeting miRNAs, with 163 Nrf2-targeting miRNAs according to TargetScan (no additional criteria) and 109 according to DIANA-Micro T-CDS (>0.7) (McGeary *et al.*, 2019; Tastsoglou *et al.*, 2023). Nrf2 signalling can also be impacted by miRNAs that target Nrf2-regulating proteins. For example, *KEAP1*-targeting miR-200a and miR-7 were shown to regulate Nrf2 signalling in breast cancer cells and SH-SY5Y cells, respectively (Eades *et al.*, 2011; Kabaria *et al.*, 2015). Similarly, Cullin 3-targeting miR-101 activated Nrf2 signalling in human umbilical vein endothelial cells (HUVECs) (Kim *et al.*, 2014). MiRNA targeting of Nrf2 is likely to be a fine-tuned process with autoregulatory loops. Papp *et al.*

predicted that 35 Nrf2-regulated transcription factors activate expression of 63 Nrf2-targeting miRNAs (Papp *et al*, 2012).

Table 1.3. Studies of experimentally validated Nrf2-targeting miRNAs.

MiRNAs	Cell types	Sources
miR-153, miR-27a, miR-142-5p, miR-144	SH-SY5Y	(Narasimhan <i>et al</i> , 2012)
miR-129-5p, miR-450a, miR-507, miR-634	HeLa	(Yamamoto <i>et al</i> , 2014)
miR-28	HEK293T, mammary epithelial cells and MCF-7 breast cancer cells	(Yang <i>et al</i> , 2011)
miR-340-5p	Osteosarcoma cells (patient derived) and HepG2 hepatocellular carcinoma cells	(Fan <i>et al</i> , 2021; Shi <i>et al</i> , 2015)
miR-144	K562 erythroleukemic cells	(Sangokoya <i>et al</i> , 2010)
miR-27b-3p	EC9706 esophageal carcinoma cells	(Han <i>et al</i> , 2020)
miR-140-5p	HEK293T	(Liao <i>et al</i> , 2017)
miR-155-5p	HEK293T	(Chen <i>et al</i> , 2019)

All studies were in human cells, and utilised reporter assays (e.g. luciferase), sometimes with additional validation assays.

1.4.6 Nrf2-mediated regulation of miRNAs

Whilst Nrf2 has long been described as a transcription factor that regulates expression of protein coding genes, more recently there has also been evidence that suggests Nrf2 can regulate expression of miRNAs. Nrf2 was shown to indirectly regulate miR-1 and miR-206, two miRNAs which share the same seed sequence (Singh *et al*, 2013). Investigation in multiple cancer cell lines revealed that sustained activation of Nrf2 signalling reduces miR-1 and miR-206 expression, resulting in increased expression of their target metabolic (pentose phosphate pathway) genes. Conversely, loss of Nrf2 was found to reduce the levels of HDAC4, a redox-sensitive histone deacetylase, which resulted in increased miR-1 and miR-206 expression by epigenetic regulation. This therefore reduced the activity of the pentose phosphate pathway and formed a feedback loop wherein miR-1 and miR-206 repressed further HDAC4 expression (Singh *et al.*, 2013). Overall, this demonstrates a redox-sensitive pathway whereby Nrf2 indirectly regulates miRNA expression.

Nrf2 can also directly activate expression of miRNAs. This includes miR-129-3p in Huh7 hepatoma cells and miR-125b-1 and miR-29b-1 in acute myeloid leukaemia cells (Shah *et al*, 2015; Sun *et al*, 2019). Chromatin-immunoprecipitation sequencing (ChIP-seq) of sulforaphane (Nrf2 activator)-

treated human lymphoblastoid cells identified likely Nrf2 binding close to the following miRNAs: miR-365-1/miR-193b, miR-617, miR-592, miR-29b-1, miR-1207, miR-32, miR-181c, miR-200c and miR-550-1 (Chorley *et al*, 2012). Of the four miRNAs expressed in the lymphoblast cell line, only miR-29b-1 significantly changed (decreased) after sulforaphane treatment, consistent with previous studies where oxidative stress suppressed miR-29b in human trabecular meshwork cells and epithelial ovarian cancer cells (Hou *et al*, 2017; Luna *et al*, 2009). Furthermore, miR-29b inhibition suppressed oxidative stress and reduced apoptosis in mouse and cellular models of ischemic stroke, perhaps suggesting Nrf2-mediated miR-29b suppression could be a protective mechanism against oxidative stress (Ma *et al*, 2022).

1.5 Aims and hypotheses of the thesis

ALS is a complex disease involving many pathogenic mechanisms and genes. Nrf2 activation has received considerable attention as a way to potentially target several disease pathomechanisms simultaneously, including oxidative stress, neuroinflammation and proteostasis dysregulation. Despite this, no specific Nrf2 activating therapeutics have been clinically approved for ALS treatment. I, and others, hypothesise that Nrf2 activation specifically in astrocytes may be an improved therapeutic strategy, which is explored in this thesis by miRNA targeting. In addition, I aimed to improve understanding of how astrocyte miRNA and protein expression may be altered in ALS, using simple and accessible models of quiescent and FBS-induced reactive astrocytes. After demonstrating the utility of this reactive astrocyte model for Nrf2 activation studies, I also investigated the effects of the ALS therapeutics riluzole and edaravone, to improve understanding of their astrocytic mechanism of action. While specific aims are provided at the start of chapters 3-6, these can be summarised to the following:

- Characterise simple models of human primary reactive and quiescent astrocytes by proteomic analysis, with a view to studying Nrf2 pathway activation.
- Identify miRNA changes associated with FBS-induced human astrocyte reactivity in this same model and integrate with literature analyses to highlight miRNAs which may be involved in neuroinflammation in ALS.
- Optimise delivery of miRNA therapeutics to human primary astrocytes, to investigate inhibition of an Nrf2-targeting miRNA as a future astrocyte-targeted therapeutic strategy.
- Broadly profile the effects of Nrf2 activation on the human reactive astrocyte antioxidant response, viability and excitability.
- Compare astrocytic responses to Nrf2 activation with those elicited by edaravone and riluzole, towards developing astrocyte-targeted therapeutic strategies for ALS.

Chapter 2. Materials and Methods

2.1 Cell culture

2.1.1 Culture of quiescent and reactive human primary astrocytes

All cells were incubated under humidity at 37°C with 5% CO₂ and were handled under aseptic conditions in a cell culture hood. Commercial foetal human primary cortical astrocytes [SC1800] were purchased from ScienCell [US] via Caltag Medsystems [UK] and stored in liquid nitrogen upon arrival. Ethical approval was not required for the studies on humans in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used

In the Chapters 2-6 of this thesis, ‘reactive astrocytes’ refers to these human primary astrocytes cultured continuously in medium containing 2% FBS, unless otherwise specified. FBS has previously been shown to induce reactive-like changes in astrocytes as it can contain factors such as cytokines, infectious agents, significant levels of lipopolysaccharides and EVs, whereas astrocytes do not typically contact serum *in vivo* under normal physiological conditions (Perriot *et al*, 2018; Prah *et al*, 2019; Santos *et al*, 2023). Astrocytes are cultured in a serum-free medium to achieve a ‘quiescent’ phenotype. The media compositions which required for the culture of reactive and quiescent astrocytes are shown in Table 2.1. These models of reactive and quiescent astrocytes were previously developed and published by our research group, for which I was a contributing author (White *et al.*, 2024). This work will be discussed in more detail in Chapter 3.

Table 2.1. A summary of the media compositions used for different aspects of human primary astrocyte culture, and their abbreviations.

Abbreviation	Media composition	Use
Astrocyte medium	Astrocyte medium [ScienCell, 1801] supplemented with 2% FBS, 1% Astrocyte Growth Supplement (AGS) [ScienCell, 1852] and 1% Penicillin/Streptomycin (P/S).	Standard culture of reactive astrocytes
Serum-free astrocyte medium	Astrocyte medium supplemented with 1% AGS and 1% P/S. No FBS.	Culture of reactive astrocytes for 3 days prior to EV purification.
Advanced DMEM	Advanced DMEM/F-12 [Gibco, 12634010] supplemented with 1% P/S [Sigma, P0781] and 1% L-Glutamine [Sigma, G7513].	Used briefly during the plating of quiescent astrocytes, and for washes to remove lentivirus (Chapter 5).
G5 media	Advanced DMEM/F-12, supplemented with 1% P/S, 1% L-Glutamine and 1% G5 supplement [Gibco, 17503012] (composition in Appendix Table 9.1.)	Standard culture of quiescent astrocytes, including for EV purification (FBS free).

2.1.1.1 Expansion and cryostorage of astrocytes

A purchased vial of human astrocytes [SC1800] was expanded and frozen to create astrocyte stocks that could then be cultured in either reactive or quiescent-inducing conditions. To induce growth, the purchased vial had to initially be cultured in reactive-inducing conditions (in astrocyte medium) and passaged before cryostorage. However, upon thawing, astrocytes could be cultured in either reactive or quiescent-inducing conditions. Approximately 5 vials were purchased for this thesis.

To passage cells, medium was removed followed by a wash with ~4ml DPBS [Gibco, 14190144]. Trypsin-EDTA [Sigma-Aldrich, T3924] was then added (2ml in a T75 flask, <1ml in a T25 flask) and the cells were incubated at 37°C for ~5 minutes. Detachment of cells was confirmed using a light microscope, before trypsin was neutralised with astrocyte medium in at least a 1:1 trypsin:media ratio. Cells were then split across flasks as required and topped up with astrocyte medium (10ml in a T75 flask, 4ml in a T25 flask). Cells were typically passaged in a ratio between 1:4 and 1:6.

For expansion, the vial was first split evenly across two T75 flasks, which when confluent cells were each passaged once at a 1:6 ratio, therefore resulting in 12 total flasks. When confluent, the cells from each flask split into two vials for cryostorage, resulting in 24 vials frozen at p2.

To cryopreserve cells, they were passaged from the confluent T75 flasks as described, then the neutralised cell suspension was centrifuged at 1500rpm for 5 minutes to pellet cells. After removing the supernatant, each pellet was resuspended in 2ml of 90% (v/v) FBS, 10% (v/v) DMSO, and split evenly between two cryovials. The cryovials were frozen using the Mr Frosty™ Freezing Container [Nalgene] at -80°C for ~24 hours before transfer to liquid nitrogen. For cell culture, vials were thawed in warm water (~37°C) before immediate addition to the required media.

2.1.1.2 Reactive human astrocytes

To culture reactive astrocytes, primary astrocytes were cultured in astrocyte medium, as shown in Table 2.1. Cells cultured in tissue-culture treated flasks were passaged twice a week and were used up to passage 7 (p7), representing ~3 weeks in culture and modelling chronic astrocyte reactivity.

To seed experiments, cells were counted after passaging. 10µl of the cell suspension (after trypsin neutralisation) was resuspended with 10µl of trypan blue, and cells were counted using the TC20™ automated cell counter [Bio-Rad, 1450102]. Seeding densities of reactive astrocytes varied between experiments, often depending on incubation time, dish size, passage number and batch-to-batch variation. Densities are therefore provided for each experiment in Chapters 3-6.

2.1.1.3 Quiescent human astrocytes

To culture quiescent astrocytes, thawed expanded astrocytes (in 1ml FBS/DMSO) were added to 5ml Advanced DMEM (details in Table 2.1) and centrifuged at 300g for 5 minutes to remove FBS. The cell pellet was resuspended in G5 medium then seeded at a density of 125,000-150,000 cells/well of a 6-well plate, or 5,000-8,000 cells/well in µ-Slide 8 Well^{high} microscopy dishes [ibidi] (referred to as ibidi dishes). Quiescent astrocytes do not proliferate therefore they did not require passaging, so they remained at passage 2 with bi-weekly media changes. Cells were cultured for a maximum of 3 weeks but were typically used for assays within 1-2 weeks.

2.1.2 Culture of HEK293T and HEK293S cells

HEK293T and HEK293S cells, used for certain optimisation experiments, were a gift from Professor Ian Kerr [University of Nottingham] and were cultured under humidity at 37°C and 5% CO₂. Cells were cultured in DMEM (1X) + GlutaMAX [Gibco] supplemented with 10% FBS and 1% P/S, and passaged approximately twice a week, as described for reactive astrocytes in section 2.1.1.1. Cells were seeded at higher densities than astrocytes due to their smaller size, as indicated in Figure legends.

2.1.3 Purification of human astrocyte-derived extracellular vesicles (ADEVs)

ADEVs were isolated from reactive and quiescent astrocyte conditioned medium for small non-coding RNA sequencing (sncRNAseq) and to use as a vehicle to enhance cellular uptake of synthetic miRNA power inhibitors. For EV isolation and purification, conditioned medium was collected from astrocytes, followed by ultrafiltration and size exclusion chromatography (SEC). Nanoparticle tracking analysis (NTA) was then performed to confirm EV presence in the expected size range, and measure particle concentration.

2.1.3.1 Collection of human astrocyte conditioned media

Reactive astrocytes were cultured in astrocyte medium containing 2% FBS as in section 2.1.1.1 and split into four T75 flasks. The following day, cells were changed to serum-free astrocyte medium, as FBS has previously been shown to contain EVs (Prah *et al.*, 2019). After 3 days, all medium (~40ml) was collected and RNA was isolated from the corresponding cells if samples were used for sncRNAseq. Quiescent astrocytes were cultured in G5 medium in 6-well plates as in section 2.1.1.3, with medium collected every 3-4 days. Four collections were required to collect ~40ml medium. All conditioned medium was centrifuged at 300g to remove cells then stored at -80°C until required for EV isolation. Freezing of conditioned medium has been shown to maintain EV integrity and cargo better than freezing purified EVs, and avoiding multiple freeze-thaw cycles maintains the EV yield (Ahmadian *et al.*, 2024). For EV RNA extraction, two ~40ml samples had to be combined for due to low EV RNA yield. The two samples were concentrated together before RNA extraction, as described in section 2.3.2.

2.1.3.2 Ultrafiltration and size-exclusion chromatography

For ADEV isolation and purification, ~40ml of astrocyte conditioned medium was thawed overnight at 4°C, then centrifuged at ~527g (1500rpm) for 5 minutes to remove any remaining cells. The medium was then concentrated to 0.5ml using 10K MWCO Pierce Protein Concentrators [ThermoFisher Scientific, 88539] by centrifuging at 3739g at 20°C (ultrafiltration). The Izon qEV Automatic Fraction Collector (AFC) was used to isolate EVs by SEC, with the qEVoriginal/70nm Gen 2 Columns [Izon Science LTD, ICO-70]. EV fractions were to be collected based on mass, therefore the AFC was first calibrated using a provided metal weight of known mass (10g). The column was then flushed with DPBS, the 0.5ml sample was added as prompted by the AFC, followed by further DPBS. The first 2.5ml to elute was the contents of the column (void fraction), followed by 3x 0.4ml EV fractions. Columns were washed with ~8ml NaOH (0.5M) then ~20ml DPBS between isolations, and the columns were used for a maximum of 5 isolations. When collecting EVs for sncRNAseq, columns were only re-used for samples of the same type (e.g. quiescent ADEVs only). If not frozen, EVs were stored at 4°C for a maximum of 1 week before being used for downstream applications (Ahmadian *et al.*, 2024).

2.1.3.3 Nanoparticle tracking analysis

The Zetaview® [Analytik] was used for NTA of purified ADEVs. Before use, the Zetaview was flushed as prompted with particle-free water and DPBS, then calibrated using polystyrene beads of known concentration and size. EV samples were then diluted in DPBS, typically 1:100. If EVs had been concentrated (as described in section 2.3.2 for RNA extraction), EVs were typically diluted 1:500. 1ml of the diluted EVs was injected into the Zetaview, which imaged at 11 positions with the 488nm laser set to scatter. Two technical replicates were completed per sample and the average number of EVs between the two replicates was calculated.

2.2 Protein expression measurements

For western blot and proteomic analysis, protein was extracted from cells by cell lysis and quantified by BCA assay. Alternatively, cells were fixed for immunocytochemistry to quantify protein expression by fluorescence intensity.

2.2.1 Cell lysis

All cell lysis was completed on ice, with centrifugations at 4°C. After removal of medium and a PBS wash, cells were manually scraped into fresh PBS. Cells were pelleted by centrifugation at 1200g for 5 minutes. PBS was removed and the pelleted cells were lysed for 15 minutes in ~60-100µl RIPA lysis buffer (150mM NaCl, 1% (v/v) IGEPAL CA-630, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 50mM Tris, pH 8) with Protease and Phosphatase Inhibitors (1:1000) [Sigma-Aldrich, P8340 and P5726] followed by sonication for 10 seconds. Cell debris was pelleted by centrifugation at 16,200g for 20 minutes then cell lysate (supernatant) was transferred to a fresh Eppendorf and stored at -20°C or used immediately.

2.2.2 BCA assay for protein quantification

To prepare protein standards for protein quantification, a 2mg/ml solution of BSA in diluted RIPA buffer (1:10 in double-distilled water (ddH₂O)) was used for a 1:2 serial dilution, resulting in 8 standards ranging from 2mg/ml to 15.625µg/ml. The 1:10 RIPA buffer solution was used as a blank. Cell lysates were diluted 1:10 in ddH₂O. 25µL of standards and diluted samples were loaded in triplicate in a clear 96-well plate. Pierce™ BCA Protein Assay Reagents A and B [Thermo Fisher] in a ratio of 50:1 were added to each well (200µL per well). The plate was incubated for 30 minutes at 37°C with rocking, then absorbance was read at 562nm using the SPECTROstar Nano microplate spectrophotometer [BMG Labtech].

Average absorbances were calculated for each sample across the three technical replicates. The concentration of each protein standard was plotted against absorbance on a graph. The simple linear regression equation of the

graph was then used to calculate the concentration of cell lysate samples. Since samples had been diluted 1/10, the calculated concentration was multiplied by 10.

2.2.3 Western blotting

After protein concentrations of whole cell lysates were measured by a BCA assay, samples containing 1-20µg protein were heated at 95-100°C for 5 minutes with 4x gel application buffer (0.15M Tris, 8M urea, 2.5% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, 3% (w/v) DTT). The amount of protein required was optimised for each experiment and is therefore reported in the appropriate Figure legend.

A 5-20% gradient polyacrylamide SDS-PAGE gel was then prepared, with the gel composition shown in Table 2.2. First the resolving gel was made by mixing the 5% and 20% solutions in a gradient mixer and pouring it into a glass cassette (1mm thickness). After the gel had polymerised, the stacking gel (composition shown in Table 2.2) was poured on top and a comb inserted to create wells. Either a wide comb (12 wells, ~60µl capacity) or narrow comb (20 wells, ~30µl capacity) was used depending on the sample volume. Once the stacking gel had polymerised, the cassette was loaded into a gel tank and topped up with electrode buffer (25mM Tris, 0.19M Glycine, 0.1% (w/v) SDS). The first well was loaded with 3µl of BLUEye Prestained Protein Ladder [MERCK, Sigma Aldrich, UK], with samples loaded to the other wells. Gels were electrophoresed at 45 milliamps (mA) per gel (i.e. 90mA if two gels in the tank), until the dye front reached the bottom of the gel (~1.5 hours).

Table 2.2. Composition of a gradient 5-20% gel for SDS-PAGE.

Gel component	5%	20%	Stacking gel
Buffer A (1.1M Tris, 0.1% (w/v) SDS, 30% (v/v) glycerol, pH 8.8)	-	3.33ml	-
Buffer B (1.1M Tris, 0.1% (w/v) SDS, pH 8.8)	3.33ml	-	-
Stacking Buffer (0.14M Tris, 0.1% (w/v) SDS, pH 6.8)	-	-	5ml
ddH ₂ O	5ml	-	-
30% (w/w) Acrylamide-bisacrylamide	1.67ml	6.67ml	1ml
10% (w/v) SDS	100µl	100µl	-
10% (w/v) APS	100µl	100µl	100µl
TEMED	10µl	10µl	10µl

First the 5-20% resolving gel was made, with the polyacrylamide gradient allowing better separation of proteins. Once polymerised, the stacking gel was added to the top with a comb to create wells for sample loading, ensuring the proteins entered the resolving gel at the same time.

After SDS-PAGE, proteins were transferred overnight onto a nitrocellulose membrane [Cytiva, 10600008] in a transfer tank containing western transfer buffer (25mM Tris, 0.192M glycine, 20% (v/v) methanol) at 40mA. Ponceau stain (0.1% (w/v) Ponceau S, 10% (v/v) glacial acetic acid) was added to the membrane to reversibly stain and confirm protein transfer efficiency, then removed with ddH₂O and three 5-minute TBS Tween washes (10% (v/v) Tris-Buffered Saline (10mM Tris, 150mM NaCl, pH 7.5) and 0.1% (v/v) Tween® 20 [Fisher, BP337-500]). All washes and incubations were at room temperature with rocking, unless specified otherwise. The membrane was incubated for 1 hour in blocking buffer (5% (w/v) Marvel milk powder in TBS Tween), then with primary antibody in blocking buffer at 4°C overnight. The primary antibody was removed with three TBS Tween washes, then secondary antibody in blocking buffer was incubated with the membrane for 1 hour. The membrane was washed with three TBS Tween washes, then most of the liquid was removed. A 1:1 mix of western Lightning Plus ECL reagents [PerkinElmer, ORT2655] was added for ~1 minute, then dried. Protected from light, the membrane was exposed to Amersham Hyperfilm® ECL film [Cytiva, 28906837]. Typically, a range of exposure times were tested (minimum 2 seconds to maximum 20 minutes) to achieve visible but not overexposed protein bands. The film was then developed using Ilford PQ universal [Ilford photos], washed with tap water then fixed using Ilford Hypam fixer [Ilford photos], washed again and dried. Western blot images were scanned using the AltaLink C8155 [Xerox].

Table 2.3. Antibodies used for western blotting, with their dilutions and supplier information.

Protein	Dilution	Source
β-actin	1:10000	Sigma, A1978-200UL
GAPDH	1:5000	Proteintech, 60004-1-Ig
GFAP	1:1000	Abcam, ab4648
HO-1	1:1000	Proteintech, 10701-1-AP
NQO1	1:1000	Proteintech, 11451-1-AP
NRF2	1:1000	Santa Cruz Biotechnology, sc-13032
NRF2	1:1000	Proteintech, 16396-1-AP
NURR1	1:500	Perseus Proteomics, PP-N1404-OC
Rabbit anti-mouse HRP secondary antibody	1:3000	Dako, P0260
Swine anti-rabbit HRP secondary antibody	1:3000	Dako, P0217

2.2.4 Proteomic analysis

Proteomic sample preparation and analysis was performed at the John van Geest Cancer Research Centre, Nottingham Trent University. I prepared astrocyte whole cell lysates by S-trap digestion to produce tryptic peptides. Sequential Window Acquisition of all Theoretical Mass Spectra (SWATH-MS) and data normalisation was then performed by Dr Clare Coveney and Dr David Boocock [Nottingham Trent University]. Further analysis was completed (by me) using StatsPro.

2.2.4.1 S-Trap digestion for SWATH-MS

For the proteomic analyses in Chapter 3, 5µg of astrocyte whole cell protein was used for SWATH-MS, while 20µg was used in Chapter 5, with the volumes required of each cell lysate calculated by BCA assay. Each sample was first dried down at 60°C in a vacuum concentrator, then 25µl of 5% (w/v) SDS/50mM triethylammonium bicarbonate (TEAB) was added. Samples were sonicated in a water bath for 10 minutes then 1µl 0.5M DTT was added, followed by incubation for 20 minutes at 56°C with shaking. After cooling to room temperature, 2µl of 0.5M iodoacetamide was added and incubated at room temperature for 15 minutes in the dark. 2.8µl of 12% (v/v) aqueous phosphoric acid was added, followed by 185µl of S-Trap buffer (90% (v/v) aqueous methanol/100mM TEAB pH 7.1). The samples were then added to S-trap™ micro spin columns and centrifuged at 4000g for 30 seconds. Three washes with 150µl S-trap buffer were then performed. Samples on the columns were incubated with 25µl digestion buffer (0.02ng/µl trypsin/50mM TEAB pH 7.5-8) for 1.5 hours at 47°C. Peptides were eluted in three steps, each time adding 40µl of the appropriate solution and centrifuging for 30 seconds at 4000g. First, 50mM TEAB was added, then 0.2% (v/v) formic acid, then 50% (v/v) acetonitrile/0.2% (v/v) formic acid. Samples were dried down again at 60°C, then diluted to 100ng/µl in 5% (v/v) acetonitrile/0.2% (v/v) formic acid.

2.2.4.2 Sequential Window Acquisition of all Theoretical Mass Spectra (SWATH-MS)

Samples were run through the Sciex ZenoTOF 7600 mass spectrometer using zSWATH for data independent acquisition (DIA). While the same amount of protein was loaded for each sample (as quantified by the initial BCA assay of whole cell lysates), another normalisation step was completed by normalising overall traces between samples using the data-independent acquisition by neural networks (DIA-NN) software version 1.8.1. Peptides were mapped to known proteins in the SwissProt database. Overall, this produced a list of protein groups detected in each sample, with MaxLFQ values providing the relative quantification of proteins between samples. Protein groups consisted of unique proteins, where peptide sequences can be assigned to one protein with certainty (e.g. UBA6), and ambiguous protein groups, where peptide sequences may belong to multiple proteins which are often isoforms (e.g. POTEJ;POTEI;POTEJ).

2.2.4.3 Proteomic data analysis using StatsPro

Statistical analysis of proteomic data was completed using the web-based version of the R package StatsPro, accessed via <https://www.omicsolution.com/wukong/StatsPro/> (Yang *et al*, 2022). StatsPro was used to compare two conditions, each with three biological replicates. The first parameter to control the stringency of the analysis was the NA value, which refers to the number of missing values allowed across replicates for the protein to be included for further analysis. The NA ratio was set at 0.334 (rounded to 0.4) and NA values were not counted separately for each group. This means that for a protein to be kept for analysis, it must be detected in 4/6 samples across the two groups, allowing for the variable nature of protein detection in proteomic analyses. The lower the NA value, the more stringent the analysis. Missing values were imputed by StatsPro for proteins with detection in 4/6 or 5/6 samples using the k-nearest neighbour method, which estimates values based on genes with similar expression patterns (Troyanskaya *et al*, 2001; Yang *et al.*, 2022). The second parameter to control stringency was the coefficient of variance (CV) value. This allowed proteins with highly variable values to be removed from the analysis. The CV value was set at 0.3, meaning that only proteins with <30% variance across replicates were included for further analysis. Again, the lower the CV value, the more stringent the analysis. Data was not median normalised (as normalisation had already been performed in DIA-NN) and was not Log transformed at this stage.

The linear model for microarray data (limma) test (an R package exposed by StatsPro) was used to identify proteins which were statistically significantly differentially expressed between groups (Ritchie *et al*, 2015). The limma test has previously been used for SWATH-MS data (Vadakekolathu *et al*, 2022). P values were adjusted using the Benjamini-Hochberg procedure. Differentially expressed proteins were considered as those with an adjusted p value (p_{adj}) <0.05 and Log2 fold change (FC) >±0.5. This equates to a fold change of >±1.41, meaning protein expression has increased or decreased by at least 141%. In Chapter 5, a lower significance threshold of p value <0.05, Log2FC >±0.3 was also considered.

2.2.5 Immunocytochemistry

Human quiescent or reactive astrocytes were cultured in ibidi dishes or standard tissue culture-treated 96-well plates, as detailed in Chapters 3 and 5. Medium was removed from cells before washing once with PBS. Cells were fixed for 30 minutes in 4% (w/v) paraformaldehyde (PFA) [Thermo Fisher Scientific] (in PBS). PFA was removed, followed by three ~5-minute PBS washes (all subsequent washes are for ~5 minutes). Fixed cells were then incubated in PBS/10mM glycine/0.2% (v/v) Triton X-100 for 30 minutes, followed by a wash in PBS/0.1% (v/v) Triton X-100. Cells were then blocked by incubation with 3% (w/v) BSA (in PBS) for 30 minutes, then incubated with primary antibodies in 3% (w/v) BSA overnight at 4°C.

Primary antibodies were removed by three washes in PBS/0.1% (v/v) Triton X-100. Cells were then incubated for 1-2 hours at room temperature with secondary antibodies and DAPI (100nM) in 3% (w/v) BSA. Phalloidin (1:500) was sometimes added here to stain F-actin (red). Secondary antibodies were removed by three PBS/0.1% (v/v) Triton X-100 washes, then one PBS wash. Ibidi dishes were set using ibidi mounting medium and stored at 4°C if imaging was delayed. 96-well plates were stored in PBS and imaged immediately.

Table 2.4. Antibodies used for immunocytochemistry, with their dilutions and supplier information.

Antibodies	Dilution	Source
Nrf2	1:300	Proteintech, 16396-1-AP
Nrf2	1:75/1:100 as indicated	Santa Cruz Biotechnology, sc-13032
HO-1	1:150/1:200 as indicated	Proteintech, 10701-1-AP
NQO1	1:150/1:200 as indicated	Proteintech, 11451-1-AP
GFP	1:500	Roche, 11063100
Alexa Fluor™ 568 goat anti-rabbit	1:300	Thermo Fisher Scientific, A11011
Alexa Fluor™ 488 goat anti-mouse	1:300	Thermo Fisher Scientific, A11029
Alexa Fluor™ 488 donkey anti-rabbit	1:400	Thermo Fisher Scientific, A21206

2.2.5.1 Cell imaging and image analysis

Images were taken using an inverted fluorescent microscope with an LED light source (Axio Vert.A1 FL-LED, Zeiss™). This included imaging of stained cells after immunocytochemistry and imaging of live cells by brightfield and in the green fluorescent protein (GFP) fluorescent channel (e.g. after transfection with a pmaxGFP plasmid). Detection ranges were set at 385-425nm for DAPI (blue channel), 469-514nm for GFP/Alexa Fluor 488 (green channel) and 555-632nm for Alexa Fluor 568 (red channel). Laser intensities (in %) and exposures (in milliseconds) were kept constant within an experiment and are provided in Figure legends for immunocytochemistry experiments. If not provided, the default was 20% laser and 150ms exposure, while the light source for brightfield images was manually adjusted to achieve optimal cell visibility. Magnification is provided in Figure legends and was generally 10x for brightfield images and 20x for immunocytochemistry images, except for lentivirus immunocytochemistry experiments (Chapter 5) where 40x magnification was used. Zen 2.6 (blue edition) [Zeiss] imaging software was used to capture images with the attached microscope, which produced CZI files.

Fiji software (Image J) was used for all image analysis, including the production of colour composite images and addition of scale bars. To measure protein expression, whole cells, nuclei (identified using DAPI) or cytoplasm, as required, were traced around using the freehand selection tool to measure mean grey value. Three background areas per image were also traced, near to measured cells. The average background mean grey value was then subtracted from each corresponding cell or compartment value. The minimum number of cells and images analysed are provided in figure legends.

2.3 Gene expression measurements and sncRNAseq analysis

Real-time quantitative polymerase chain reaction (RT-qPCR) was used to measure mRNA expression. MiRNA expression was measured by both RT-qPCR and sncRNAseq, the latter of which was performed by Novogene. In addition to analyses in whole-cells, EV miRNA expression was also determined by sncRNAseq. All methods began with RNA extraction. RNA was used for sncRNAseq, while RNA was converted to complementary cDNA (cDNA) for RT-qPCR. RT-qPCR data was analysed using the ΔC_t method, while sncRNAseq data was analysed by Novogene using DESeq2, which used readcounts as a quantitative measure. Further sncRNAseq data analysis included identification of RBP motifs in miRNA sequences, and integration with proteomic data.

2.3.1 RNA extraction from human astrocytes

First, medium was removed from cells, then 1ml TRIzol® [ThermoFisher Scientific, 15596026] was added per sample (e.g. one confluent T75 flask) and incubated for 5 minutes. When fewer cells were used, all subsequent reagent volumes were reduced accordingly. For example, 500µl TRIzol was used to extract RNA from 2 wells of a 6-well plate, and 250µl from one well of a 24-well plate. All following volumes correspond to 1ml TRIzol. Cells were scraped and collected, before adding 200µl chloroform and vortexing for 10 seconds. Samples were centrifuged at 12,000rpm for 15 minutes at 4°C (all centrifugations at 4°C), separating the samples into 3 layers. The top aqueous layer was collected, to which 500µl isopropanol was added. After a 10-second vortex, the samples were incubated for 10 minutes. RNA was pelleted by centrifugation at 12,000rpm for 15 minutes. The supernatant was removed and 800µl 70% (v/v) ethanol (diluted using Hyclone™ water [Cytiva, SH30538.03]) was added, inverting to wash the pellet. Samples were centrifuged at 12,000rpm for 5 minutes, then all ethanol was removed. The pellet was left to dry then resuspended in 10-20µl Hyclone water, depending on pellet size. RNA concentration and quality (260/230 and 260/280 ratios) was measured using the Nanodrop™ 2000 spectrophotometer [Thermofisher Scientific]. RNA was stored at -80°C. For samples used for sncRNAseq (Chapter 4), in addition to Nanodrop an RNA ScreenTape® assay was performed by DeepSeq [University of Nottingham].

2.3.2 RNA extraction from extracellular vesicles

Immediately after EV isolation (described in section 2.1.3.2), the purified EV fractions (400µl each) were pooled and further concentrated (ultrafiltrated) to ~250µl by centrifuging at 4000g at 20°C in Amicon Ultra-0.5 Centrifugal Filter Units [Sigma Aldrich, UFC501024], which have a 10kDa molecular weight cutoff. As mentioned, EVs from two ~40ml conditioned medium batches were pooled for RNA extraction of each sample, therefore six EV fractions were concentrated here, not three. 750µl TRIzol® LS Reagent [ThermoFisher Scientific, 10296028] was added to the concentrated EV samples (~250µl) and mixed. 200µl chloroform was added and vortexed for 30 seconds then incubated at room temperature for 3 minutes. Samples were centrifuged at 12,000g for 15 minutes (all centrifugations at 4°C), then the top aqueous fraction was collected. 500µl isopropanol was added, mixed then incubated at -20°C overnight. Samples were then centrifuged at 12,000g for 30 minutes and the supernatant was removed. 1ml 75% (v/v) ethanol (diluted in Hyclone water) was added then vortexed, before centrifuging at 7,500g for 5 minutes. The supernatant was removed and the pellet left to dry, before adding 10µl Hyclone water and incubating in a heat block at 58°C for 15 minutes then resuspending. RNA was stored at -80°C. RNA quality and concentration was measured using a High Sensitivity RNA Screentape® assay [DeepSeq, University of Nottingham].

2.3.3 cDNA synthesis (for mRNA)

First, 20ng/µl stocks of RNA were made by diluting in Hyclone water, using the concentrations determined by Nanodrop. For each cDNA reaction, the reagents listed in Table 2.5 were combined. To anneal the primer to the RNA, the 13µl reaction was heated to 65°C for 5 minutes in the Mastercycler® thermocycler [Eppendorf], then cooled on ice. The 7µl reverse transcription reaction mix in Table 2.6 was then added. The resulting 20µl reaction mix was heated in the thermocycler to 50°C for 10 minutes, then 80°C for 10 minutes to create cDNA. cDNA was stored at -20°C for up to 6 weeks or used immediately.

Table 2.5. Components of each cDNA reaction for annealing the oligo d(T)₂₀ primer to template RNA.

Component	Volume per reaction (µl)	Source
20ng/µl RNA template	5	-
50µM Oligo d(T) ₂₀ primer	1	Thermo Fisher Scientific, 18418-020
10mM dNTPs	1	New England Biolabs
Hyclone water	6	Cytiva, SH30538.03

Table 2.6. Components of each cDNA reaction for reverse transcription.

Component	Volume per reaction (μl)	Source
5x SSIV buffer	4	Thermo Fisher Scientific, 18090010
100mM DTT	1	Thermo Fisher Scientific, 18090010
RNaseOUT™ Recombinant RNase Inhibitor	1	Thermo Fisher Scientific, 10777-019
200U/μl SuperScript® IV Reverse Transcriptase	1	Thermo Fisher Scientific, 18090010

2.3.4 RT-qPCR (for mRNA)

Firstly, a mastermix of the first three reagents listed in Table 2.7 (PowerUp™ SYBR™ green, primer mix and Hyclone water) was prepared in excess for each required primer set. The primer sequences used in this thesis are shown in Table 2.8. 8.5μl of the mastermix was then loaded to each well of a 96-well PCR plate [Starlab, E1403-8200]. cDNA was diluted 50:50 in Hyclone water then 1.5μl was added to the appropriate well. Two technical replicates (wells) were completed per condition, including a no template control (NTC) for each primer, where 1.5μl of Hyclone water was added instead of 1.5μl cDNA.

Table 2.7. RT-qPCR reaction components.

Component	Volume per reaction (μl)
PowerUp™ SYBR™ Green Master Mix (2x) [Thermo Fisher Scientific]	5
10μM forward and reverse primer mix	0.4
Hyclone water	3.1
cDNA (diluted 50:50 in Hyclone water)	1.5

Table 2.8. Primer sequences used for RT-qPCR.

Gene	Forward primer (5'->3')	Reverse primer (5'->3')
18s	CGCGGTTCTATTTTGTGGT	AGTCGGCATCGTTTATGGTC
B2M	AAGTGGGATCGAGACATGTAAG	GGAATTCATCCAATCCAAATGCG
GAPDH	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC
HMOX1	GGCCTCCCTGTACCACATC	AGACTGGGCTCTCCTTGTG
IDH1	TTGGCTGCTTGCAATAAAGGTT	GTTTGGCCTGAGCTAGTTTGA
NQO1	CCTGCCATTCTGAAAGGCTGGT	GTGGTGATGGAAAGCACTGCCT
NRF2	CACATCCAGTCAGAAACCAGTGG	GGAATGTCTGCGCCAAAAGCTG
NURR1	AAACTGCCAGTGGACAAGCGT	GCTCTTCGGTTTCGAGGGGCAAA

Primer sequences (all for human genes) were obtained from OriGene (available via www.origene.com) or published literature. Primers were synthesised by Sigma Aldrich.

The PCR plate was sealed using ThermoSeal® RT2RR™ sealing films [Excel scientific, TS-RT2RR] and centrifuged at 500g for 5 minutes to eliminate bubbles. The QuantStudio™ Real-Time PCR System was used to perform the RT-qPCR reaction in Fast Cycling mode using the Comparative Ct method, with the cycling conditions shown in Table 2.9.

Table 2.9. Thermocycler conditions for RT-qPCR.

Step	Temperature	Duration	Cycles
UDG activation	50 °C	2 minutes	1
Dual-Lock™ DNA polymerase	95 °C	2 minutes	1
Denature	95 °C	1 second	40
Anneal/extend	60 °C	30 seconds	
Melt curve analysis	95 °C	1 second	1
	60 °C	20 seconds	
	95 °C	1 second	

Fast cycling mode using the QuantStudio™ Real-Time PCR System.

Results were analysed using QuantStudio design and analysis software [ThermoFisher, UK]. In each amplification curve, NTC samples were checked to ensure there was no signal. The threshold for analysis for each gene was set in the exponential phase of the curve. Melt curves were checked to confirm the presence of a single peak (mRNA) and no primer dimers.

The $2^{-(\Delta\Delta Ct)}$ method was used for data analysis, where $\Delta\Delta Ct = \Delta Ct(\text{target sample}) - \Delta Ct(\text{reference sample})$ (Livak & Schmittgen, 2001). First, an average of the two technical replicate Ct values was calculated for each sample. The geomean of housekeeper Ct values for the respective sample was then subtracted to give a ΔCt value. GAPDH, B2M and 18S primers were included in each plate as housekeepers, however only the housekeepers with a maximum difference in Ct value between samples of <2 were included in subsequent analysis, sometimes resulting in only two housekeepers used for normalisation. After housekeeper normalisation, the average control ΔCt value for the respective

gene was subtracted from all ΔCt values (both control and treatment groups) to produce $\Delta\Delta\text{Ct}$ values. Fold changes compared to the average control were then calculated for each sample using the formula $2^{-(\Delta\Delta\text{Ct})}$.

2.3.5 cDNA synthesis (for miRNA)

To synthesise cDNA for RT-qPCR measurement of miRNA expression, first 5ng/ μl stocks of whole-cell RNA were made by diluting in Hyclone water. For each cDNA reaction, the reagents listed in Table 2.10 were combined, which are components of the miRCURY LNATM RT kit [Qiagen, 339340]. In the thermocycler, the reactions were then heated at 42°C for 60 minutes, followed by 95°C for 5 minutes to heat-inactivate the reverse transcriptase. The reactions were then cooled to 4°C and used for RT-qPCR immediately or stored at -20°C.

Table 2.10. Components of miRNA cDNA synthesis reactions.

Component	Volume per reaction (μl)
5x miRCURY RT SYBR® Green Master Mix	2
10x miRCURY RT Enzyme Mix	1
Nuclease-Free Water	5
5ng/ μl RNA	2

2.3.6 RT-qPCR (for miRNA)

First, a mastermix was made with the first three reagents listed in Table 2.11, which are components of the miRCURY LNATM SYBR green PCR kit [Qiagen, 339346]. 7 μl of this mastermix was loaded to each well in a 96-well plate. cDNA and ROX reference dye (a dye unaffected by amplification, allowing for detection of errors/artifacts) were both diluted 1/60 in nuclease-free water (58 μl water, 1 μl cDNA, 1 μl ROX). For the NTC for each primer set, only ROX was diluted 1/60 (59 μl water, 1 μl ROX). 3 μl of the diluted cDNA or NTC was loaded to each well, resulting in a 10 μl reaction volume.

Table 2.11. Components of miRNA RT-qPCR reactions.

Component	Volume per reaction (μl)
2x miRCURY LNA SYBR® Green Master Mix	5
Nuclease-Free Water	1
miRCURY LNA miRCURY PCR Assay (primers)	1
Diluted cDNA (1/60 cDNA and ROX Reference Dye in nuclease-free water)	3

The PCR plate was sealed and centrifuged at 500g for 5 minutes. The QuantStudioTM Real-Time PCR System was used to perform the RT-qPCR reaction in Standard Cycling mode and the Comparative Ct method, with the cycling conditions shown in Table 2.12.

Table 2.12. Thermocycler conditions for miRNA RT-qPCR.

Step	Temperature	Duration	Cycles
Polymerase activation/denaturation	50°C	2 minutes	1
Anneal/extend	95°C	10 seconds	40
	56°C	1 minute	
Melt curve analysis	95°C	15 seconds	1
	60°C	1 minute	
	95°C	1 second	

Standard cycling mode using the QuantStudio™ Real-Time PCR System.

2.3.7 sncRNAseq and analysis

Whole-cell reactive and quiescent astrocyte and corresponding ADEV RNA samples were submitted to Novogene, UK for sncRNAseq and data analysis, with samples couriered on dry ice. Cellular RNA samples were sent in excess of the 2µg minimum, whereas all EV RNA was sent to achieve the ~10ng minimum. All whole-cell RNA samples had RNA integrity number (RIN) values of >9.8, whereas ADEV RIN values were 2.5-3.1.

The following methods were completed by Novogene upon receipt of RNA samples. Firstly, *in house* quality control was performed by removing reads containing poly-N, A, T, C or G, 5' adapter contaminants, low quality reads and reads without the insert tag or 3' adapter. The Q20, Q30 and GC-content of the data was then calculated.

Bowtie was then used to map small RNA tags to reference sequence without mismatch (Langmead *et al*, 2009). These mapped tags were then used to identify known miRNA, with miRBase20.0 as a reference, using mirdeep2 and sncRNA-tools-cli to obtain the miRNA and draw secondary structures (Friedländer *et al*, 2012). Custom scripts were then used to identify base bias at each miRNA position and obtain the miRNA counts. Small RNA tags were removed originating from protein-coding genes, rRNA, snRNA, tRNA, snoRNA and repeat sequences. 'Novel' miRNAs were also predicted using miREvo and mirdeep2, although these have not been analysed in this thesis. Since some RNAs can be mapped to more than one RNA type, the following priority rule was followed: known miRNA > rRNA > tRNA > snRNA > snoRNA > repeat > gene > NAT-siRNA > gene > novel miRNA > ta-siRNA. Differential expression analysis was performed using DESeq R package (1.8.3). The Benjamini & Hochberg method was used to adjust p values.

2.3.7.1 Motif analysis of astrocyte whole cell or ADEV enriched miRNAs

ADEV-enriched miRNAs were defined as those with significantly greater expression in ADEVs than their respective quiescent or reactive astrocyte whole-cell condition (Log2FC >0.5, padj<0.05), and *vice versa* for cell-enriched. Motif analysis of the ADEV or whole-cell-enriched miRNAs was performed

using the Catalog of Inferred Sequence Binding Preferences of RNA binding proteins (CISBP-RNA) database (Ray *et al*, 2013). The PWMs – LogOdds model with a threshold of 6 was used to identify human RBPs predicted to bind to the given miRNAs via motif sequences. Certain miRNAs contain multiple motifs corresponding to one RBP, therefore duplicates were removed from analysis.

2.3.7.2 Data integration using Ingenuity Pathway Analysis

Ingenuity Pathway Analysis (IPA) was used to integrate whole-cell astrocyte sncRNAseq (Chapter 4) and proteomic data (Chapter 3). A Target Filter Analysis of the sncRNAseq data was performed to identify the predicted gene targets of miRNAs differentially expressed ($\text{Log}_2\text{FC} > \pm 0.5$, $\text{padj} < 0.05$) between quiescent and reactive astrocytes. The proteomic data that had already been filtered using StatsPro was subjected to a core analysis in IPA to identify differentially expressed proteins ($\text{Log}_2\text{FC} > \pm 0.5$, $\text{padj} < 0.05$). The miRNA and protein analyses were then overlaid, to identify pairings where both a miRNA and a protein it is predicted to target were differentially expressed. Likely regulatory interactions were identified as those where a miRNA and its predicted protein target were differentially expressed in opposite directions, as miRNAs generally suppress gene expression.

2.4 Human astrocyte treatments and manipulations

Astrocytes have been treated with various types of compound in this thesis, including small molecules, plasmids and different types of miRNA inhibitors. Methods of delivering these compounds have included direct treatment in culture media, transfection, via ADEVs, lentiviruses, and electroporation (nucleofection). In some cases, HEK293T or HEK293S cells have also been treated as a comparison.

2.4.1 Small molecule treatments

tBHQ, edaravone and riluzole were resuspended in DMSO and stored at -20°C . Appropriate stock solutions were used so that the ratio of DMSO to media was always 1:1000 for astrocyte treatment, e.g. $2\mu\text{l}$ tBHQ (10mM) in 2ml medium to give a final concentration of $10\mu\text{M}$. Cells were typically treated the day after seeding, with exact times and densities provided in figure legends.

2.4.2 Transfection

2.4.2.1 Lipofectamine 2000

HEK293T cells were seeded at a density of 600,000 cells/well in a 6-well plate. Reactive human astrocytes were seeded at a density of 70,000 cells/well of a 6-well plate. The next day, transfections were performed by first incubating $5\mu\text{l}$ Lipofectamine 2000 with $125\mu\text{l}$ Opti-MEM™ for 20 minutes. All incubations were at room temperature. Separately, $1\mu\text{g}$ NURR1-expressing pcDNA3.1

plasmid (for HEK293T cells) or pmaxGFP (for astrocytes) and 125µl Opti-MEM™ were incubated for 5 minutes. The two reactions were then mixed and incubated for 30 minutes. This was then added dropwise to the cells in culture medium. HEK293T cells were lysed 24 hours later for western blot analysis. Astrocytes were imaged 24 hours later in brightfield and GFP channels of a widefield microscope. The pmaxGFP plasmid [Lonza] map is under licence, while the NURR1 plasmid map is shown in Appendix Figure 9.1.

2.4.2.2 Lipofectamine RNAiMAX

25µl Opti-MEM and 1µl RNAiMAX reagent were mixed and briefly incubated, then added to a suspension of 10,000 reactive astrocytes in a total volume of ~250µl and seeded in one well of an ibidi dish. After 24 hours, cells were imaged with a brightfield microscope.

2.4.3 miRNA power inhibitor treatment

miRCURY™ LNA miRNA Power Inhibitors (PIs) [Qiagen] were suspended in Hyclone water to produce a 50µM stock, aliquoted and stored at -20°C. The PIs purchased are presented in Table 2.13.

Table 2.13. miRNA PIs used in Chapter 5, with sequences and supplier details.

Power inhibitor	Sequence	Catalogue number
hsa-miR-340-5p	5' ATCAGTCTCATTGCTTTATA 3'	YI04106015
hsa-miR-27b-3p	5' GCAGAACTTAGCCACTGTGA 3'	YI04103307
Negative control	5' AGAGCTCCCTTCAATCCAA 3'	YI00199007

2.4.3.1 Direct treatment of miRNA power inhibitors

The day after seeding reactive human astrocytes, cells were treated with PI (100nM or 200nM as required) in fresh medium. In certain experiments (indicated in figure legends), serum-free astrocyte medium was used rather than complete astrocyte medium, to avoid possible interference of serum. After 48 hours (or 24 or 72 hours for a time course experiment), cells were harvested in TRIzol for RNA extraction and RT-qPCR.

Due to concerns of the PIs not working, successful resuspension of the original vial in Hyclone water was confirmed by the detection of nucleic acids on a 4% agarose gel (data not shown).

2.4.3.2 ADEV-mediated delivery of miRNA power inhibitors

Reactive ADEVs were isolated and counted using the Zetaview, following methods described in sections 2.1.3. ADEVs (10⁹ per condition in a total PBS volume of 300µl) were then incubated for 4 hours with 3µl control or miR-340-5p PI with rocking to facilitate passive inhibitor uptake into ADEVs, a method described in a pre-print study by our research group (Mesquita-Ribeiro *et al*, 2025). The total volume was then applied to reactive astrocytes in 6 well plates, giving a final inhibitor concentration of 100nM in 1.5ml medium. These treated astrocytes had been seeded 24 hours previously at a density of 150,000

cells/well, and were not the same culture that had been used for ADEV isolation. After 48 hours of miR-340-5p PI and ADEV treatment, RNA was extracted from cells for RT-qPCR.

2.4.4 Lentivirus-mediated delivery of miRNA inhibitors

Commercial miRNA inhibitor lentiviral vectors were purchased from Applied Biological Materials Inc. (Abm) [Canada], via the UK supplier NBS Biologicals Ltd. The vectors (500ng) were purchased pre-packaged in VSV-G pseudotyped, third generation lentivirus particles, at a titre of 10^7 IU/ml in a total volume of 2ml. All viruses were aliquoted upon arrival, then stored at -80°C .

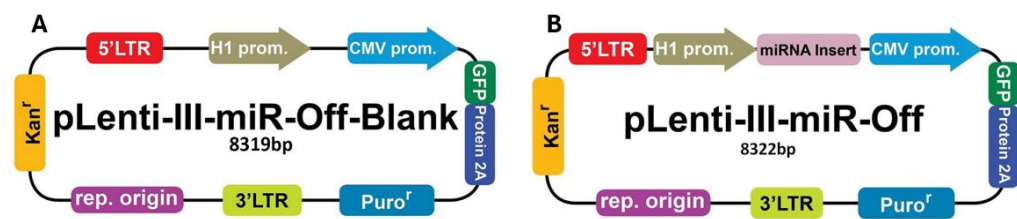


Figure 2.1. ABM lentivirus miRNA inhibitor vectors. Images are taken from Applied Biological Materials Inc. (abmgood.com). A) Control vector [m008]. B) miRNA inhibitor vector, as applicable for miR-340-5p [mh30561] and miR-27b-3p [mh30385].

Following optimisation experiments, reactive astrocytes were seeded at a density of 5,000 cells/well of a 96-well plate for imaging or immunocytochemistry, or 35,000 cells/well of a 12-well plate for RT-qPCR. After 24 hours, astrocytes were treated with lentivirus at a multiplicity of infection (MOI) of ~ 12.5 (12.5 μl) for immunocytochemistry and ~ 9.4 (65.6 μl) for RT-qPCR (lower MOI for RT-qPCR due to the greater virus volume and therefore cost required for a 12-well plate). MOIs are approximate as it was assumed that cell numbers double in the 24 hours after seeding, which may not always be the case, especially with older ($>p4$) astrocytes. The equation for calculation of MOI is:

$$MOI = \frac{\text{Virus titre} \times \text{Virus volume}}{\text{Total cell number}}$$

Lentivirus was resuspended with 8 $\mu\text{g/ml}$ polybrene transduction reagent [Merck, TR-1003-G] in a small volume of astrocyte medium (total volume of 60 μl in 96-well plates, 350 μl in 12-well plates) and cells were treated. After 24 hours the lentivirus was removed, followed by two washes with Advanced DMEM. Cells were replenished with fresh astrocyte medium and transduction efficiency was monitored by imaging in the brightfield and green channels of the widefield microscope. As shown in Figure 2.1, all vectors contained a GFP sequence, allowing fluorescent microscopic assessment of transduction efficiency. 24 hours after removing the lentivirus, cells were processed for further analysis (RT-qPCR or immunocytochemistry).

For spinoculation, astrocytes were lifted with trypsin and neutralised with astrocyte medium, as described in section 2.1.1.1. 24,000 cells were aliquoted

in a total volume of 500µl with polybrene (8µg/ml) and 30µl control lentivirus (MOI 12.5) in astrocyte medium. The suspension was gently mixed and incubated at room temperature for 20 minutes, followed by centrifugation at 800g for 30 minutes. The pelleted cells were then resuspended in the same suspension and the whole volume was then transferred to one well of a 24-well plate. As above, the virus was removed 24 hours later and GFP expression was monitored daily via fluorescence.

2.4.5 Nucleofection of human astrocytes with miRNA inhibitors

For each nucleofection, a mix of 84µl P3 Primary Cell Nucleofector® Solution and 16µl Supplement 1 was incubated at room temperature for 15 minutes, along with miRNA standard inhibitor or PI (as indicated in text) and pmaxGFP plasmid if required. For nucleofection of 500,000 cells, 0.7µg GFP plasmid and 10µl miRNA inhibitor/PI (50µM stock) was used, with volumes scaled accordingly if different cell numbers were required. The PI details were given in section 2.4.3, while standard inhibitor details are shown in Table 2.14. No GFP plasmid was added if the cells were to be used for calcium imaging, to prevent interference with Fluor 4. Astrocytes were pelleted by centrifugation at 400g for 4 minutes, then resuspended in the nucleofection reagent mix. After a 15-minute incubation, the cell suspension was then transferred to a 100µl Nucleocuvette® and nucleofected in the 4D-Nucleofector™ Core Unit [Lonza, AAF-1003B] using the CA 137 pulse code for P3 primary cell types. The suspension was immediately added to fresh medium then dishes were seeded.

Table 2.14. Standard (non-power) miRNA inhibitors used in Chapter 5, with sequences and supplier details.

Inhibitor	Sequence	Catalogue number
hsa-miR-340-5p	5' ATCAGTCTCATTGCTTTATA 3'	YI04106015
Negative control	5' TAACACGTCTATACGCCCA 3'	YI00199006

2.5 Functional cellular assays

Cell viability assays, ROS assays and calcium imaging have been used to assess the effects of different cell treatments on human astrocyte health, function and activity.

2.5.1 Resazurin cell viability assay

Resazurin assays were used to measure the effects of nucleofection or small molecules (tBHQ, edaravone and riluzole) on astrocyte viability. Specific treatment details, e.g. incubation times, are given in Figure legends. Reactive human astrocytes were plated in a black 96-well plate at a density of 5,000 cells/well in triplicate. The resazurin assay was performed at the end of the

specified treatment. All cells were washed with PBS and incubated with 100µl resazurin stock solution (0.1mg/ml in PBS) for 2 hours at 37°C, as well as resazurin-only wells (no cells). Fluorescence was measured at 540nm excitation/590-10nm emission, with a gain of 1044 using the FLUOstar® Omega spectrophotometer [BMG LABTECH]. Triplicate values were averaged before subtracting resazurin-only values. Percentage viability was calculated compared to the untreated control.

2.5.2 ROS assay

The DCFDA/H2DCFDA – Cellular ROS Assay Kit [Abcam, UK] was used to measure ROS in human astrocytes. Reactive astrocytes were plated in a black 96-well plate at a density of 5,000 per well. After 44 hours, cells were washed with 1x Dilution Buffer, then treated with 10µM DCFDA in 1x Dilution Buffer supplemented with 10% FBS (Supplemented Buffer) for 30 minutes at 37°C. Cells were washed again, then incubated for 4 hours with the desired compounds, DMSO (vehicle control), 200µM H₂O₂ (positive control) or left untreated. All treatments were performed in triplicate and were in Supplemented Buffer, including Supplemented Buffer-only controls (no cells). Fluorescence was measured at 485nm excitation/520nm emission, with a gain of 1444 using the FLUOstar® Omega spectrophotometer. Triplicate values were averaged before subtracting Supplemented Buffer-only values. Percentage viability was calculated compared to the untreated control.

2.5.3 Calcium imaging

Calcium imaging of reactive human astrocytes was performed 24 hours after nucleofection with a miR-340-5p standard inhibitor, or small molecule treatment (edaravone, riluzole and tBHQ) compared to controls. Nucleofection was performed as described in section 2.4.5 with no pmaxGFP plasmid, with 80,000 of the nucleofected cells seeded in Nunc dishes. The first two biological replicates consisted of one technical replicate each and were derived from the same nucleofection used for proteomic analysis and a resazurin assay, in which 600,000 cells were nucleofected with 12µl inhibitor. The third biological replicate consisted of 3 technical replicates (dishes) in which 325,000 cells were nucleofected with 6.5µl inhibitor, so the same inhibitor:cell ratio.

To investigate the effect of edaravone, riluzole and tBHQ on calcium signalling, reactive astrocytes were seeded in Nunc dishes at a density of 60,000 cells/well. Approximately 24 hours later they were treated with the desired compounds or DMSO, with two technical replicates per condition, and calcium imaging was performed 24 hours later.

For calcium imaging, cells were moved to a dark room, with all future work occurring under red light only. Medium was removed and cells were incubated for 30 minutes with Fluor-4 (1µM), diluted in imaging buffer (135mM NaCl, 3mM KCl, 10mM HEPES, 15mM glucose, 1mM MgSO₄, 2mM CaCl₂, pH 7.4). Fluor-4 was removed and cells were incubated in imaging buffer for 30 minutes

for de-esterification. The buffer was removed, with a few drops re-added to prevent cells from drying out. This protocol, although with Fluor-5 and a different stimulation regime, is published in (Arendt-Tranholm *et al*, 2025) (preprint).

Calcium imaging was performed using an Olympus IX70 Inverted tissue culture microscope attached to a CoolLED pE excitation system, PE-2 collimator, and an ORCA-R2 Hamamatsu Camera Controller C10600. Images were captured using the multi-acquisition function on ImageJ. Exposure was set at 100ms and binning at 2x2, with the number of positions and slices set to 1. Cells were imaged at 10x magnification using Channel LED Green 470 every 500 milliseconds for a total of 8 minutes, resulting in a total of 960 frames. The stimulation sequence is provided in Table 2.15.

Table 2.15. Sequence and timings of stimulations used for calcium imaging of human primary astrocytes.

Stimulation (with final concentration)	Length of stimulation (minutes)	Frame numbers (approximate)
Buffer	1	1-120
Glutamate (200 μ M)	2	121-360
ATP (300 μ M)	3	361-720
Ionomycin (1 μ M)	2	721-960

No additional buffer was added for the first minute, with only the few drops of buffer remaining. The three following simulants were added in 300 μ l volumes, without removal of the previous stimulant. Therefore, double the final concentration was added for ATP and triple the final concentration for ionomycin.

2.5.4 Calcium imaging data analysis

Images were analysed using FIJI. Cells were drawn around using the freehand selection tool in a frame of the ionomycin stimulation, which ensured all cells were visible. The multimeasure tool was used to measure mean grey value (intensity) for all 960 frames for each ROI (cell). The unit of measurement to describe changes in intracellular Ca^{2+} concentration (Ca^{2+} signal) upon each stimulation is $\Delta F/F$. While specific details are provided in the next section, $\Delta F/F$ values were calculated by quantifying the change in fluorescence intensity relative to resting (baseline) intensity (Macleod, 2012). For all data analysis methods, an average of the two technical replicates was calculated and presented as one biological replicate (one datapoint).

2.5.4.1 Threshold of a calcium response to neurotransmitter stimulation

For all subsequent analysis methods, it was necessary to identify cells which responded to glutamate and/or ATP. While this could be done subjectively by eye, instead numerical threshold for a response was determined. This threshold was determined as a maximum peak (highest $\Delta F/F$ value during

stimulation) of $>0.01 \Delta F/F$, which was calculated separately for the glutamate and ATP stimulation of each cell. The cells which met this threshold also visually appeared to respond, supporting that it was an appropriate threshold.

To calculate a normalised maximum peak value (Arendt-Tranholm *et al.*, 2025), the highest mean grey value during the stimulation was normalised to a glutamate or ATP baseline as required. The neurotransmitter-specific baselines were calculated as the average mean grey value of each cell 15 seconds (30 frames) before the stimulation. The following formula was then used to identify the normalised maximum (max.) peak for each cell. The example of ATP is given, with the same formula used for glutamate:

$$\frac{\Delta F}{F} \text{ max. ATP peak} = \frac{\text{max. ATP mean grey value} - \text{ATP baseline}}{\text{ATP baseline}}$$

Therefore, the cells with a $\Delta F/F$ max. peak value of >0.01 by the above method were determined to have responded to the corresponding neurotransmitter.

2.5.4.2 Presentation of calcium imaging data

A calcium trace was produced for all cells which responded to glutamate and/or ATP, which was determined by the method above. However, to produce the trace, for responding cells, all mean grey values were normalised to the overall baseline, which was calculated as the average mean grey value across the first 15 seconds (30 frames) of the experiment, rather than the neurotransmitter-specific baseline. This was to demonstrate that fluorescence did not return completely to baseline after stimulation with glutamate or ATP. Normalisation for each datapoint was done using the following formula, to produce a $\Delta F/F$ value:

$$\frac{\Delta F}{F} = \frac{(\text{mean grey value} - \text{baseline})}{\text{baseline}}$$

An average $\Delta F/F$ value for each frame was then calculated, across all cells which had been determined to respond to glutamate and/or ATP. These 960 average values were then plotted (y axis) against time (x axis) to produce a calcium trace. However, for this thesis the ionomycin stimulation was removed from the trace, as the strong calcium response to ionomycin would hide the more subtle responses to glutamate and ATP.

To calculate the percentage of cells which respond to each neurotransmitter, the maximum peaks for ATP and glutamate (normalised to a neurotransmitter-specific baseline as described in the previous section) were averaged across all responding cells for the given neurotransmitter. The number of cells which responded to glutamate or ATP were then counted, as two separate categories irrespective of whether each cell responds to one or both neurotransmitters. These numbers were presented as the percentage of cells responding to the respective neurotransmitter, out of the total number of cells measured.

Finally, area under the curve (AUC) analysis was performed to measure the overall intensity of calcium signal throughout a stimulation. Separately for glutamate and ATP, the $\Delta F/F$ values produced by normalisation to the original

baseline were averaged across all cells that responded to the given neurotransmitter. This original baseline was used as this had previously been used to produce the calcium trace, for which AUC was now being calculated. This produced an average trace consisting of 240 frames for the glutamate stimulation and 360 for ATP. For each neurotransmitter, AUC was calculated using the trapezium rule, i.e. splitting the area up frame by frame to calculate the area of many small sections using the following formula:

$$\frac{\Delta F}{F} \times s = \frac{(F_1 + F_2) \times (T_1 + T_2)}{2}$$

Where F_1 and F_2 are two adjacent average $\Delta F/F$ values in terms of time (e.g. at 0.5 seconds and 1 second). T refers to time in seconds, so in this example $T_1 = 0.5$ and $T_2 = 1$. The sum of the resultant $\Delta F/F.s$ ($\Delta F/F \times s$) values for each neurotransmitter was then calculated and presented.

2.6 Statistical analysis

Statistical analysis was completed using GraphPad Prism version 9.4.1 for Windows [Boston, USA], except for sncRNAseq (DESeq2, completed by Novogene) and proteomic analysis (limma test using StatsPro), which are discussed in sections 2.3.7 and 2.2.4.3. For experiments with 2 independent conditions, for example reactive and quiescent astrocytes or treated and untreated, a Welch's t-test was used. This was selected due to high variance in some experiments, and used throughout for consistency. For comparisons of more than two variables, one-way ANOVAs were used, with Dunnett's multiple comparisons test to compare each experimental value to the control. The ROUT test ($Q=1\%$) was used to identify outliers, however no outliers were identified in this thesis.

Bar graphs were plotted $\pm$ standard error of the mean (SEM), with individual datapoints representing biological replicates. When multiple technical replicates were completed, an average was calculated for each biological replicate, rather than plotting each technical replicate, as indicated in Figure legends. Statistical significance ($p < 0.05$) was illustrated as * on graphs. The number of asterisks signify the level of significance: $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****). If no * is present for a comparison, then it is non-significant ($p > 0.05$).

Chapter 3. Proteomic characterisation of a human primary astrocyte model for the study of Nrf2 pathway activation

3.1 Introduction

The main goal of this thesis was to inhibit specific miRNAs in human astrocytes, to work towards an astrocyte-targeted Nrf2-activating therapy for ALS. Use of an appropriate human astrocyte model and understanding its molecular and phenotypic background was crucial for these investigations, for several reasons. Firstly, species differences in miRNA sequence and expression, and astrocyte function and complexity mean that a human astrocyte model is most appropriate for translational research (Ason *et al*, 2006; Oberheim *et al.*, 2006). Furthermore, astrocytes predominantly have a persistent (chronic) reactive phenotype in ALS, likely as both a consequence and cause of motor neurone death, which must be reflected in the model (Reischauer *et al*, 2018; Taha *et al*, 2022; Vargas & Johnson, 2010). Modelling genetic forms of ALS requires astrocytes to have ALS-associated genetic variants, however this work will not address this, instead attempting to model general Nrf2 pathway dysfunction, that is likely a feature of ALS regardless of genetic influence. Findings from this thesis could then be taken forward in ALS-specific astrocyte models.

By molecular profiling of the selected human reactive astrocyte model and the associated quiescent astrocyte control model, I aimed to identify disease-associated phenotypes, especially those involving the Nrf2 pathway, to rescue with a miRNA-targeting treatment. This also facilitated rational selection of therapeutic targets. To achieve this, Chapter 3 presents a thorough whole-cell proteomic analysis of the human astrocyte model, while a corresponding sncRNAseq analysis will be discussed in Chapter 4.

3.1.1 Human astrocyte models

Primary astrocytes extracted from rodent tissue have long been used for astrocyte research, however they lack the complexity of human astrocytes and are not the most appropriate for studying human diseases (Oberheim *et al.*, 2006). Immortalised astrocyte cell lines of both human and rodent origin are also used as a cheaper and easier alternative to primary cells, however they often do not harbour classical astrocyte phenotypes such as GFAP expression and S100 β secretion. These cell lines are also often glioma-derived rather than healthy astrocytes, such as the C6 rat glioma cell line, limiting their utility for studying normal astrocyte physiology (Galland *et al*, 2019).

An arguably more physiological method of human astrocyte research is to generate astrocytes from human donor somatic cells, such as skin fibroblasts. Somatic cells can be converted to iPSCs which resemble embryonic stem cells that can be differentiated into any cell type, including astrocytes (Birger *et al.*,

2019; Santos *et al*, 2017; Takahashi *et al*, 2007; Takahashi & Yamanaka, 2006). Astrocytes can also be 'reprogrammed' directly from fibroblasts, with a benefit of this method being that astrocytes retain age-related transcriptional and functional differences, which is valuable for studying an age-related disease (Caiazzo *et al*, 2015; Gatto *et al*, 2021). In ALS research, fibroblasts are often taken from ALS patients, as disease-associated mutations and all other genetic information are retained (Birger *et al*, 2019; Juopperi *et al*, 2012). Alternatively, mutations can be engineered into iPSCs using CRISPR-Cas9 technology before differentiation to astrocytes, although appropriate isogenic controls are important (Canals *et al*, 2023).

ALS is a highly heterogeneous disease, which is likely to be reflected in these patient-derived astrocyte models (Su *et al*, 2014). While this heterogeneity is important to consider in research, it may prevent significant findings unless large sample sizes are used. Furthermore, culture and differentiation of iPSC-derived or fibroblast-derived astrocytes (iAstrocytes) is time consuming and expensive, meaning it is not always the best use of limited resources for preliminary studies. Here, a simple, cost-effective astrocyte model was required for exploratory analyses, the results of which could be explored further in other cell models in the future.

However, I still aimed to model ALS in a broad sense. To achieve this, a model of reactive astrocytes was required, along with an associated quiescent astrocyte model for comparison. Cytokines are frequently used to activate astrocytes, with IL-1 α , TNF α and C1q secretion by activated microglia shown to induce neurotoxic reactive (A1) astrocytes in mice (Liddelow *et al*, 2017). However, some studies use serum to culture or differentiate both 'reactive' and 'quiescent' astrocytes, which has been suggested to promote reactivity (Escartin *et al*, 2021; Perriot *et al*, 2018; Prah *et al*, 2019; Roybon *et al*, 2013). A recent study published by our research group presented a simple model of FBS-induced reactivity compared to a serum-free quiescent astrocyte model, which will be discussed further in section 3.2.1 (White *et al*, 2024). FBS was used instead of human serum as it is widely available and accessible, and is commonly used for cell culture, therefore investigation of its effects on astrocyte biology will be relevant to many other studies.

FBS is a medium supplement commonly used in astrocyte culture to promote survival and proliferation, even promoting differentiation of iPSC-astrocytes (Prah *et al*, 2019; Soubannier *et al*, 2020). However, astrocytes do not typically contact serum in the brain due to the BBB. Therefore, FBS in culture likely mimics exposure to serum caused by a disruption to the BBB, where astrocytes adopt a more reactive phenotype to minimise damage to the brain (Prah *et al*, 2019). BBB and blood spinal cord barrier (BSCB) damage has been demonstrated in several neurodegenerative diseases, including ALS, for which the evidence is reviewed in (Pan & Nicolazzo, 2022). This evidence includes detection of blood-derived IgG protein in patient spinal cord, motor cortex and CNS, suggesting barrier leakage (Donnenfeld *et al*, 1984; Leonardi *et al*, 1984). In summary, FBS-cultured astrocytes can be used to approximately model the

reactivity induced by BBB disruption in neurodegenerative disease, and in this thesis are used to mimic changes that are a pathogenic feature of ALS.

3.1.2 Mass spectrometry for proteomic analysis

As discussed, I aimed to conduct a mass spectrometry (MS)-based proteomic analysis of the selected astrocyte model. MS is a powerful tool for the simultaneous identification and quantification of thousands of proteins from biological samples. In the ALS field, it has potential for clinical biomarker discovery as well as basic research. Many studies have identified differentially expressed proteins in the CSF and postmortem tissue of ALS patients, with chitinase proteins among the candidates for a novel proteomic biomarker (Oh *et al*, 2023; Thompson *et al*, 2021). Recently, a panel of 33 plasma proteomic biomarkers of ALS was identified, including the previously established neurofilament light chain (NEFL) biomarker (Chia *et al*, 2025). Beyond ALS, large scale proteomic analyses are being conducted to identify diagnostic and predictive biomarkers for a range of diseases, for example 2920 plasma proteins were linked to diseases in a study of 53,026 adults (Deng *et al*, 2025). Protein MS has also been used to profile ALS cell models, including to investigate ALS astrocyte pathogenicity. For example, MS of the conditioned medium from C9orf72-ALS patient-derived iPSC-astrocytes revealed a deficiency in the secretion of antioxidant proteins, suggesting susceptibility to oxidative damage (Birger *et al*, 2019).

Briefly, in a typical MS profiling experiment proteins are digested with proteases to form short peptides before injection to the mass spectrometer, where they are first ionised to give them a charge. These ions then fragment and are directed into a mass analyser, where they separate based on their mass/charge (m/z) ratios. Finally, the separated ion fragments reach a detector which calculates their mass and abundance to create a mass spectrum, from which peptides and the original proteins can be identified (Garg & Zubair, 2024).

The proteomic method to be used in this study is SWATH-MS, a label-free proteomic method that uses data-independent acquisition (DIA) for the unbiased detection and relative quantification of thousands of proteins (Gillet *et al*, 2012). Since SWATH-MS is an LC-MS/MS method, peptides resulting from trypsin digestion are separated by liquid chromatography coupled to a tandem mass spectrometer, where ions are separated by one mass analyser (MS1) then fragmented and analysed by a second mass analyser (MS2), resulting in several fragment ion spectra derived from each precursor ion. For SWATH-MS, first one precursor ion (MS1) spectrum is recorded to measure every precursor ion, with no fragmentation. Then there is a series of fragmentation to record MS2 spectra, with the mass spectrometer moving sequentially through windows of typically $25m/z$ to cover the full m/z range. Repeated cycling through windows creates a comprehensive dataset of all precursor and fragment ions (Ludwig *et al*, 2018).

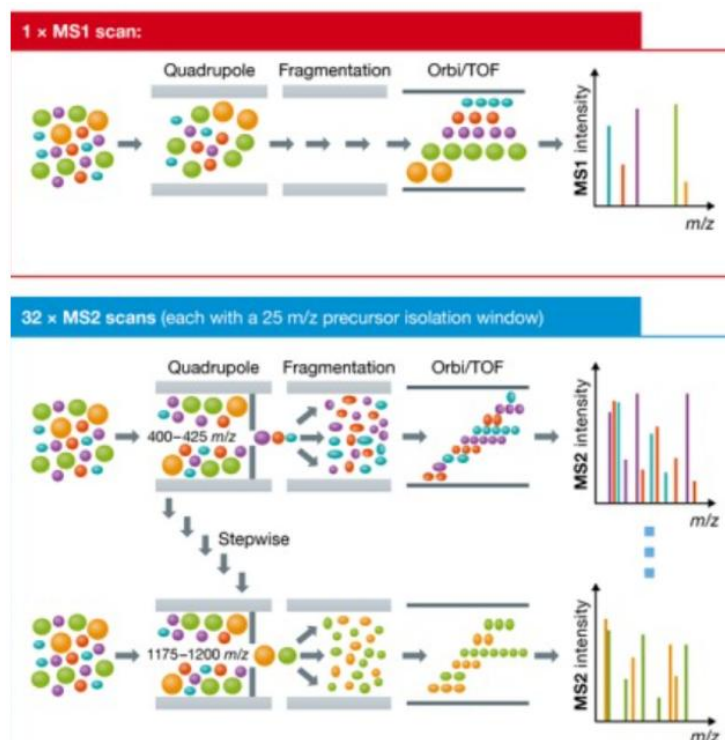


Figure 3.1. SWATH-MS method of precursor ion and fragment ion detection. Figure taken from (Ludwig et al, 2018). Precursor ions are generated from tryptic peptides. A quadrupole mass analyser then separates all precursor ions which progress to be separated by the Orbitrap or time of flight (Orbi/TOF) mass analyser, which generates a precursor ion (MS1) spectrum. In a series of 32 scans, precursor ions are then selectively fragmented in window increments of 25m/z, progressing from 400m/z to 1200m/z. The fragmented ions are separated and measured by the Orbi/TOF to create fragment ion (MS2) spectra (Ludwig et al., 2018).

The DIA method employed by SWATH-MS means that all ions in the specified m/z range are fragmented and detected in MS2. In contrast, for the more traditional data-dependent acquisition (DDA) method, only the most abundant ions in each window would be fragmented (Hu et al, 2016). Therefore, in DDA the mass spectrometer is biased towards precursor ions with the strongest signal and low-abundance peptides are often missed, favouring robust protein identification at the expense of quantitation. In summary, DIA is an unbiased method with better proteome coverage, sensitivity and reproducibility than DDA (Doerr, 2015).

SWATH-MS combines the DIA method of data acquisition with a targeted data extraction strategy. According to Gillet et al, fragment ion signals are mined “for constellations of signals that precisely correlate with the known coordinates of a targeted peptide, thus uniquely identifying the peptide in the map”. Briefly, this means that proteome-wide spectral libraries are used for grouping fragments as known peptides for protein identification and quantification (Gillet et al., 2012).

In summary, SWATH-MS is a highly effective, sensitive and reproducible proteomics method which will facilitate a deep exploration of whole-cell human astrocyte protein expression in this study.

3.1.3 Experimental aims

Our broad experimental aims of chapter 3 are as follows:

- Evaluate human primary FBS-induced ‘reactive’ astrocytes as a platform for future Nrf2 pathway manipulation, regarding evidence for chronic astrocyte reactivity and Nrf2 pathway differences compared to the corresponding ‘quiescent’ astrocyte model.
- Complete a comprehensive semi-quantitative whole-cell proteomic analysis of the human quiescent and reactive astrocyte models.
- Use the proteomic data to identify ALS-associated and Nrf2 pathway proteins expressed in human astrocytes, and those that are differentially expressed upon FBS-induced reactivity.

3.2 Results and Discussion

3.2.1 Evaluation of a ‘quiescent’ and ‘reactive’ astrocyte model for the study and manipulation of the Nrf2 pathway

All work in this thesis required a simple and accessible model of human astrocytes. The main thesis aim was to upregulate the Nrf2 pathway in reactive astrocytes via miRNA targeting. Therefore, it was preferable for the reactive astrocyte model to exhibit impaired Nrf2 signalling (compared to the associated quiescent astrocyte model), reflective of an ALS-associated phenotype which I aimed to therapeutically rescue. Evaluation of Nrf2 pathway components was therefore the first experimental aim. Before discussing this experiment, a general description of the human astrocyte models used in this thesis will be provided.

The quiescent and reactive astrocyte models used in this thesis were recently published by our research group, for which I was a contributing author (White *et al.*, 2024). My personal contribution to the publication was development of a quantitative morphological assay to distinguish between the quiescent and reactive astrocyte models, which will be discussed shortly. Both models use commercial human primary foetal cortical astrocytes [SC1800] and are relatively simple and cost-effective. Media compositions are described in section 2.1.1. Briefly, the quiescent model uses a serum-free medium containing G5 supplement, a serum-free supplement which supports astrocyte survival. For the reactive astrocyte model, astrocytes are continuously cultured in medium containing 2% FBS, since exposure to serum has previously been shown to cause irreversible reactive changes (Foo *et al.*, 2011; Zamanian *et al.*, 2012). Unlike the reactive astrocyte model, these quiescent astrocytes do not

proliferate. Representative images of the astrocyte models are shown in Figure 3.2.

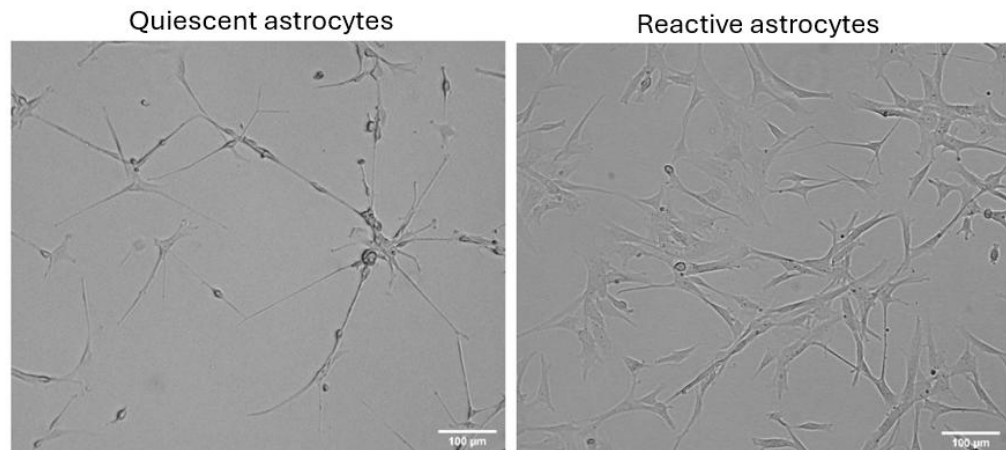


Figure 3.2. Brightfield images of human primary quiescent and reactive astrocytes. Quiescent astrocytes were cultured in G5 medium (serum-free). Reactive astrocytes were cultured in astrocyte medium containing 2% FBS.

These human astrocyte models have been analysed extensively in (White *et al.*, 2024). Transcriptomic evidence supporting the ‘quiescent’ phenotype of the quiescent model included the upregulation of supportive KEGG pathways, such as axon guidance and synapse-associated pathways, according to an RNAseq analysis. In comparison, the reactive astrocytes displayed upregulation of CNS disease-associated pathways. Inflammation in the reactive astrocyte model was demonstrated by increased expression of pro-inflammatory cytokine IL-1 β and anti-inflammatory IL-10, measured by RT-qPCR. By subjective visual assessment of their morphology, the reactive astrocytes did not appear to revert to their quiescent state upon serum removal, suggesting that FBS induces an irreversible change, in line with the sustained reactivity of ALS astrocytes (White *et al.*, 2024).

Morphological differences between the quiescent and reactive astrocytes were also quantified, with cellular hypertrophy of the reactive model (illustrated by an increased area/perimeter ratio) further evidence for their reactivity. Morphologically, the quiescent astrocytes have long, thin, branching processes with smaller cell bodies, therefore closely resembling *in vivo* astrocytes, as shown in Figure 3.2. In comparison, the reactive astrocytes have larger cell bodies and thick, short processes. A series of experiments were carried out (as shown in Appendix Figure 9.2) which demonstrate that FBS exposure significantly increased the area/perimeter ratio of human astrocytes. Although not used further in this thesis (e.g. as a functional readout of miRNA manipulations in Chapter 5), this morphological assay could be a valuable tool for future studies focused on modulating astrocyte reactivity, with area/perimeter ratio allowing quantitative comparisons of astrocyte reactivity states.

A limitation of this reactive astrocyte model is the reduced expression of astrocyte markers compared to quiescent astrocytes, including GFAP, contrary to the typical GFAP upregulation observed upon reactivity (Escartin *et al.*, 2021; White *et al.*, 2024). We previously hypothesised that this may be due to the maturing effect of FBS, supported by the increased nuclear size of the reactive astrocytes (White *et al.*, 2024).

So far, a general description of these human astrocyte models has been provided. To compare Nrf2 signalling between these quiescent and reactive astrocytes, RT-qPCR was performed to measure expression of *NRF2* in addition to three of its target genes (i.e. Nrf2 activates their expression): *HMOX1* (encoding HO-1), *NQO1* and *IDH1*. Figure 3.3 shows that there is significantly reduced expression of *NRF2* and *IDH1* in the reactive astrocyte model compared to quiescent astrocytes. This suggests the Nrf2 pathway may be downregulated in this model of reactive astrocytes, providing a cellular platform to investigate Nrf2 pathway ‘rescue’ in future experiments, for example using miRNA inhibitors. While *HMOX1* and *NQO1* expression did not significantly differ between quiescent and reactive astrocytes, I hypothesised that the decrease in *NRF2* expression would result in altered expression of other target genes. This hypothesis will be addressed by the proteomic analysis in the next section.

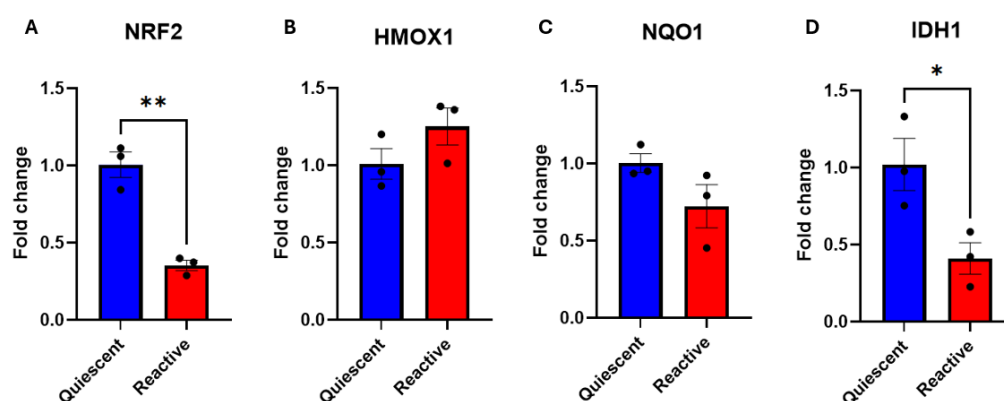


Figure 3.3. Expression of NRF2 and its target genes HMOX1, NQO1 and IDH1 in reactive astrocytes compared to quiescent astrocytes, as measured by RT-qPCR. Quiescent astrocytes had been cultured in serum-free medium, while reactive astrocytes were cultured in medium containing 2% FBS. A) NRF2. B) HMOX1. C) NQO1. D) IDH1. B2M and 18S were used as housekeepers and all data was normalised to the average control value for a given gene. Welch's *t*-test, *n*=3.

3.2.2 Semi-quantitative mass spectrometry analysis of human primary quiescent and reactive astrocytes

As discussed, a proteomic analysis was utilised to profile the Nrf2 pathway in reactive and quiescent astrocytes, further to the RT-qPCR presented in Figure 3.3. An additional aim of this analysis was to identify differentially expressed ALS-associated proteins. Not only would this improve our molecular

understanding of astrocyte reactivity, but it may suggest a possible neuroinflammatory role of any differentially expressed ALS-associated proteins, regardless of whether their change is a consequence or cause of reactivity. Differential expression of any less well characterised disease-associated proteins in this ALS-relevant cell type provides further insight into their possible ALS pathogenic mechanism.

A previous limited, non-quantitative proteomic analysis of this model was published in our study, using a DDA LC-MS/MS method at the Cambridge Center for Proteomics (White *et al.*, 2024). As discussed, there are limitations to the DDA method, resulting in a relatively low number of proteins detected, with an average of 1,088 in quiescent and 680 in reactive. For this thesis, the aim was to improve upon this proteomic analysis by moving to the unbiased, more sensitive DIA method and a more advanced method of MS. To achieve this, the semi-quantitative MS approach known as SWATH-MS was employed to profile the proteome of these astrocyte models. SWATH-MS was performed at Nottingham Trent University, in collaboration with Dr Clare Coveney and Dr David Boock.

Three batches of proteomic analyses were completed, where a batch refers to six samples (three quiescent, three reactive) being processed from an individual experiment and run on the mass spectrometer in the same session. Relative abundance of a protein in one sample can be compared to other samples within a batch, but not between batches. All data processing and statistical analysis was performed separately for each batch. The data analysis presented in this thesis primarily focuses on batch 1, which had the greatest proteome coverage (as shown in Table 3.1). Batches 2 and 3 had a slightly lower sequence coverage, therefore were used to validate results and identify the most robust protein expression changes upon reactivity.

Prior to any proteomic data processing or statistical analysis, all non-human contaminant proteins were removed, such as bovine or pig proteins from culture medium and trypsin, respectively. Common human contaminant proteins, such as those in the common Repository of Adventitious Proteins (cRAP) (<https://www.thegpm.org/crap/>) were not removed. This was because the cRAP database contains several proteins of interest to this study, such as the Nrf2 target genes NQO1 and PRDX1, inflammatory TNF α and IL-8 and neurodegeneration-associated TAU. While keratin proteins from human skin or hair are likely contaminants, they are not of biological interest so would not be highlighted or experimentally pursued because of this study.

Table 3.1 shows the number of protein groups identified in each condition, before and after stringent filtering using a web-based version of the R-package StatsPro (Yang *et al.*, 2022), with more details given in section 2.2.4.3. Protein groups consisted of unique proteins, where peptide sequences can be assigned to one protein with certainty, and ambiguous protein groups, where peptide sequences may belong to multiple proteins, often isoforms. In StatsPro, the missing values (NA) ratio was set at 0.4 (i.e. 0.33). This meant that if a protein group was detected in at least 4/6 samples, values were imputed for any

replicates with missing values. This decision was made because despite the sensitivity of DIA proteomics, low abundance proteins can fall below the threshold for identification. Any protein groups which did not meet the threshold were removed from the analysis. To remove protein groups which are highly variable between replicates, the threshold for the coefficient of variance was set at 0.3. This meant that protein groups with >30% variance between the three replicates were removed from the analysis.

Table 3.1. Number of protein groups detected by SWATH-MS analysis of human quiescent and reactive astrocytes.

	Protein groups detected in at least 1 astrocyte sample (before StatsPro)			Protein groups detected after StatsPro filtering		
	Total protein groups	Unique proteins	Ambiguous protein groups	Total protein groups	Unique proteins	Ambiguous protein groups
Batch 1	4858	4797	61	3952	3927	41
Batch 2	3134	3073	61	2431	2384	47
Batch 3	3952	3882	70	2480	2437	43

Batches 1 and 3 consisted of 6 samples (3 quiescent, 3 reactive). However, one quiescent sample had to be removed from batch 2 StatsPro analysis, as too much protein was loaded to the mass spectrometer due to an error during protein quantification. Protein groups consist of unique proteins, where peptide sequences can be assigned to one protein with certainty, and ambiguous protein groups, where peptide sequences may belong to multiple proteins. StatsPro filtering removed proteins with variance of >30% or detection in less than 4/6 samples.

Table 3.1 shows that >98% of all protein groups were unique proteins, before and after StatsPro analysis in all batches. Therefore, ambiguous protein groups were removed for future analysis, leaving only unique proteins for which measurement of their peptide sequences could be assigned to one protein with complete confidence. All following analysis refers to 'proteins' not 'protein groups', and only proteins which passed through StatsPro filtering are referred to from here. The Venn diagram in Figure 3.4 illustrates a relatively high degree of overlap between the three proteomics batches. While significantly more proteins were detected in batch 1, batches 2 and 3 share most of their proteins (>94%) with at least 1 other batch. This confirmed the suitability of batches 2 and 3 for validating proteins of interest from batch 1.

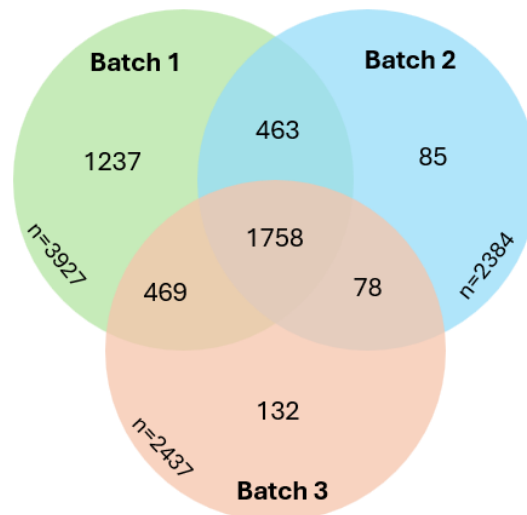


Figure 3.4. Venn diagram representing the degree of overlap between astrocyte proteomics batches. SWATH-MS was used for three batches of proteomic analysis of human primary quiescent and reactive astrocytes, with data analysed using StatsPro. All numbers represent the number of unique proteins remaining after StatsPro analysis. *n* = total number of proteins identified per batch.

The astrocyte identity of these samples was confirmed by comparing the proteomic data to a published human fibroblast iPSC-derived astrocyte dataset (Ravagnani *et al*, 2023) (data accessed via Mendeley Data DOI:10.17632/52vtypkjzd.1). Proteins detected (LFQ value>1) in at least 1/6 samples from healthy donors were compared to proteins detected in at least 1/6 samples (reactive or quiescent) in batch 1. 2557 of the total 2855 proteins (90%) detected in iPSC-astrocytes were also detected in batch 1, strongly supporting the astrocyte identity of the cells used in this thesis. The 10% difference is likely due to differences in proteomic methods and the older (non-fetal) identity of fibroblast-derived astrocytes.

To identify proteins that were differentially expressed between quiescent and reactive astrocytes, a threshold of adjusted p value (*padj*) <0.05 and Log2 fold change (FC) >±0.5 was set. The volcano plots in Figure 3.5 depict the spread of data and differentially expressed proteins for all three batches of proteomics. As well as having reduced proteome coverage, batches 2 and 3 had much lower numbers of differentially expressed proteins, as shown in Figure 3.5B and C. Again, these two batches were only used to validate the most robust protein changes identified in batch 1. Interestingly, there were consistently more proteins downregulated upon reactivity than upregulated in all three batches. A prediction may be that this general reduction in proteome complexity upon reactivity hinders physiological astrocyte function to some extent, as may be expected for a disease-associated astrocyte.

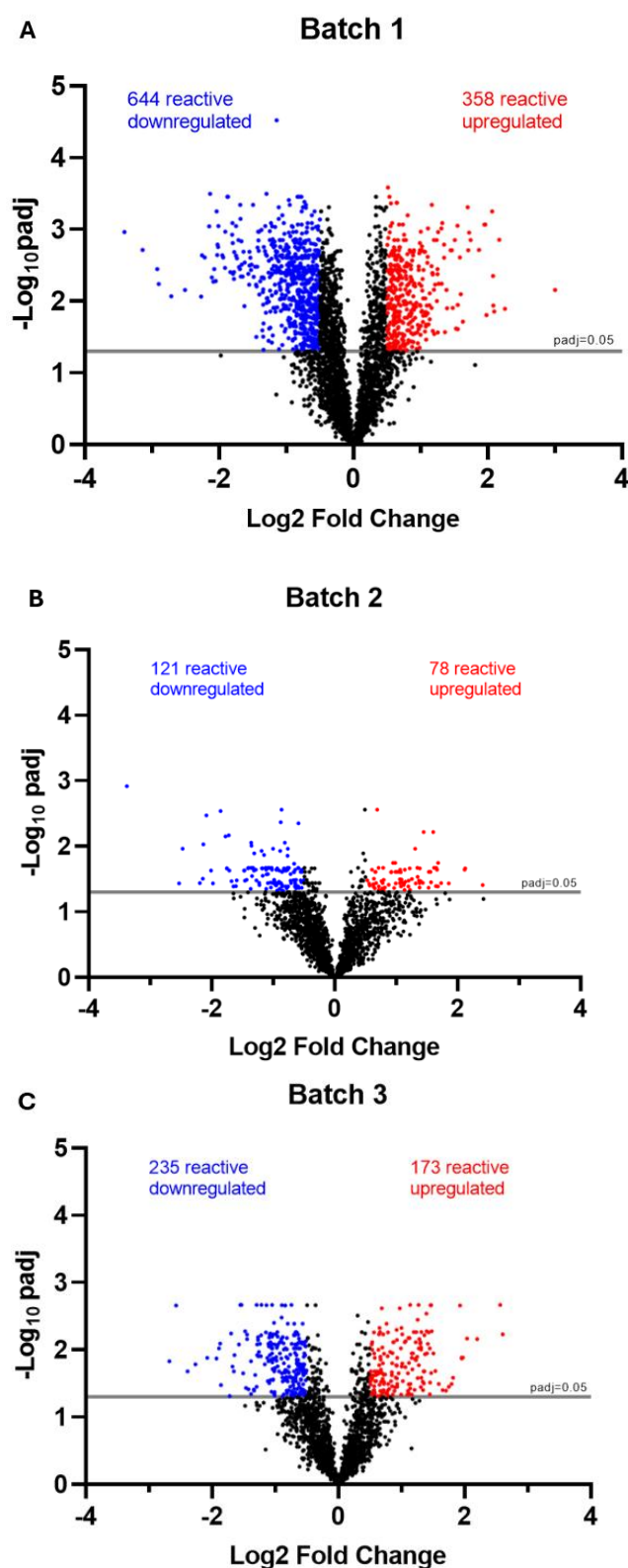


Figure 3.5. Volcano plots showing differential expression of proteins between reactive and quiescent astrocytes. SWATH-MS was used for proteomic analysis of human quiescent and reactive astrocytes, with data analysed using StatsPro and the limma test. Grey line is at $-\text{Log}_{10} \text{padj}$ 0.05. Colour change is at $\text{Log}_2 \text{FC} > \pm 0.5$ (for statistically significant proteins). A) Batch 1. B) Batch 2. C) Batch 3.

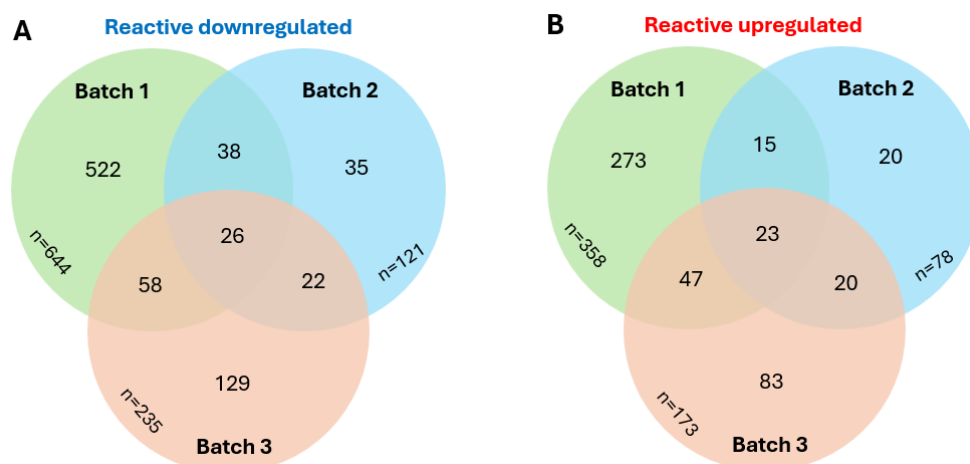


Figure 3.6. Venn diagrams representing the degree of overlap in differentially expressed proteins between astrocyte proteomics batches. SWATH-MS was used for proteomic analysis of human primary quiescent and reactive astrocytes, with data analysed using StatsPro and the limma test. A) Numbers of proteins downregulated in reactive astrocytes compared to quiescent ($p_{adj} < 0.05$, $\text{Log}_2\text{FC} < 0.5$). n = total number of downregulated proteins in each batch. B) Numbers of proteins upregulated in reactive astrocytes compared to quiescent ($p_{adj} < 0.05$, $\text{Log}_2\text{FC} > 0.5$). n = total number of upregulated proteins in each batch.

Figure 3.6 represents the degree of overlap in differentially expressed proteins between proteomics batches. Unlike the Venn diagram in Figure 3.4 showing total number of proteins detected, there were considerable differences in the differentially expressed proteins of each batch. In part this was due to the much larger number of differentially expressed proteins in batch 1, but it also highlights the variable nature of proteomic analysis. StatsPro may also be a contributing factor, due to the stringent filtering criteria.

Table 3.2 illustrates the top 10 upregulated proteins in reactive astrocytes (batch 1) in terms of Log_2FC value. A notable finding is the upregulation of ERO1A (ER oxidoreductin 1 α), which is known to produce ROS in the ER (Samtleben *et al*, 2022), therefore its upregulation may promote oxidative stress in reactive astrocytes. ERO1A upregulation has been demonstrated in astrocytes from multiple sclerosis patient brain tissue, a disease known to have a reactive astrocyte component (Colón Ortiz & Eroglu, 2024; Samtleben *et al.*, 2022).

Table 3.2. The top 10 most upregulated proteins in human reactive astrocytes compared to quiescent astrocytes.

Top 10 most upregulated proteins (by Log2FC) upon astrocyte reactivity	Average quiescent astrocyte value (MaxLFQ)	Average reactive astrocyte value (MaxLFQ)	Log2FC	padj
A2M	119	956	3.00	0.00700
AK4	77.1	369	2.26	0.0128
LDHA	6240	28000	2.17	0.00139
LMO7	141	602	2.10	0.0139
SP100	34.2	145	2.08	0.0115
ERO1A	145	612	2.08	0.00447
UAP1	95.0	397	2.06	0.000559
DNAJB4	168	663	1.98	0.0156
PLOD2	287	1120	1.96	0.000851
TFRC	129	499	1.95	0.000855

The order of proteins is in terms of Log2FC. Average MaxLFQ values are provided, with StatsPro calculated Log2FC and padj values (limma test). Values rounded to 3 s.f.

Table 3.3 shows the top 10 downregulated proteins in reactive astrocytes (batch 1). TNC (tenascin-C) has been shown to restrict astrogliosis in mouse brains after ischemic stroke (Dzyubenko *et al*, 2022), supporting the data in Table 3.3 that TNC levels are higher in the quiescent astrocyte model. Reduced CALB1 (calbindin-1) expression correlated with persistent astrogliosis in the mouse hippocampus following hypoxia-ischemia (Goffigan-Holmes *et al*, 2019), supporting its downregulation in reactive astrocytes in Table 3.3. However, MAP2 (microtubule-associated protein 2) downregulation appears to conflict previous reports, as reactive astrocytes induced by brain injury in mice were shown to have increased expression of this typically neurone-associated protein (Geisert *et al*, 1990).

Table 3.3. The top 10 most downregulated proteins in human reactive astrocytes compared to quiescent astrocytes.

Top 10 most downregulated proteins (by Log2FC) upon astrocyte reactivity	Average quiescent astrocyte value (MaxLFQ)	Average reactive astrocyte value (MaxLFQ)	Log2FC	padj
TNC	2990	281	-3.41	0.00108
PRPH	603	68.5	-3.14	0.00193
DCLK1	619	81.8	-2.92	0.00356
TMT1B	415	55.6	-2.90	0.00576
CALB1	417	63.6	-2.71	0.00854
CKB	1920	337	-2.51	0.00699
RTN1	579	120	-2.27	0.00862
MAP2	367	76.9	-2.26	0.00229
CRMP1	582	125	-2.21	0.00246
ASS1	220	49.5	-2.15	0.000903

The order of proteins is in terms of Log2FC. Average MaxLFQ values are provided, with StatsPro calculated Log2FC and padj values (limma test). Values rounded to 3 s.f.

Pathway analysis has not been presented in this thesis as it did not inform experimental direction, with the Nrf2 pathway and ALS-associated proteins determined to be the focus from the project outset. Briefly, by Metascape pathway analysis of reactive upregulated proteins (batch 1), the top 3 enriched pathways were “rRNA processing in the nucleus and cytosol”, “ribosome biogenesis” and “VEGFA VEGFR2 signalling”. In downregulated proteins, the top pathways were “nucleobase-containing small molecule metabolic process”, “carboxylic acid metabolic process” and “metabolism of lipids” (Zhou *et al*, 2019).

3.2.2.1 Proteomic analysis confirms downregulation of Nrf2 pathway components in reactive astrocytes

So far, a general description of the proteomic data has been given. The following aim was to investigate expression of Nrf2 pathway components. As detailed in section 3.2.1, RT-qPCR revealed reduced expression of *NRF2* and its target gene *IDH1* in reactive compared to quiescent astrocytes. Proteomic analysis allowed investigation of whether these mRNA changes were reflected at the protein level, and whether expression of any other Nrf2 target genes were altered. Nrf2 itself was unfortunately not detected in any single proteomic run, even when the data were searched for proteins with post-translational modifications. Alongside batch 1, an additional two reactive astrocyte samples were analysed that had been lysed in urea buffer, rather than RIPA buffer, in case Nrf2 was RIPA-insoluble. However again, Nrf2 was not detected. Anecdotally, colleagues who have performed SWATH-MS on other

cell types such as iPSC-derived motor neurones have also not detected Nrf2 (personal communications).

Despite this, many of the downstream Nrf2 target proteins, i.e. genes that Nrf2 promotes transcription of, were detected. A manual list of 47 Nrf2 target genes had been collated from the literature, as shown in Table 1.2, Chapter 1. In the batch 1 raw data, 32 Nrf2 target genes were detected, which was reduced to 26 after StatsPro analysis, as listed in Appendix Table 9.2. Figure 3.7 shows that in batch 1 there were 12 differentially expressed Nrf2 target proteins between reactive and quiescent astrocytes. Strikingly, all 12 were downregulated upon astrocyte reactivity. This strongly suggests that Nrf2 activity or expression is downregulated in this model of reactive astrocytes.

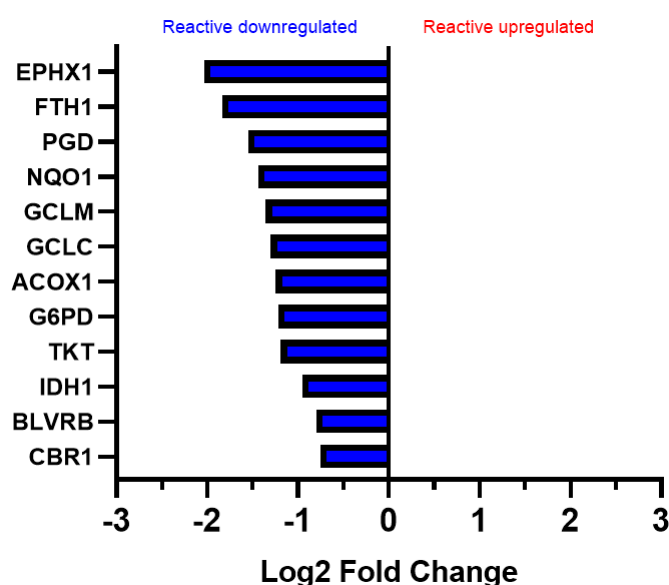


Figure 3.7. Nrf2 target gene products differentially expressed between human quiescent and reactive astrocytes, determined by proteomic analysis. SWATH-MS was used for proteomic analysis of human quiescent and reactive astrocytes, with data analysed using StatsPro. Nrf2 target gene products differentially expressed ($p_{adj} < 0.05$, $\text{Log}_2\text{FC} > \pm 0.5$) are presented, for batch 1 of the proteomic analysis.

To identify the most robust changes in Nrf2 target genes, the same analysis was completed for batches 2 and 3. Table 3.4 shows that three proteins were downregulated upon reactivity in all three batches – CBR1, IDH1 and PGD. FTH1 was decreased in two batches. Most importantly, there were no upregulated Nrf2 target genes in any batch, strongly supporting a considerable downregulation of the Nrf2 pathway in reactive astrocytes. Overall, this data supports the use of this reactive astrocyte model for modelling Nrf2 pathway dysfunction, and to attempt ‘rescue’ of the Nrf2 pathway using miRNA inhibitors.

Table 3.4. Validation of differentially expressed Nrf2 target gene products by additional proteomic analyses of human quiescent and reactive astrocytes.

	Nrf2 target proteins downregulated in reactive astrocytes	Nrf2 target proteins upregulated in reactive astrocytes
Batch 1	ACOX1, BLVRB, CBR1, EPHX1, FTH1, G6PD, GCLC, GCLM, IDH1, NQO1, PGD, TKT	
Batch 2	CBR1, FTH1, IDH1, PGD	
Batch 3	CBR1, IDH1, PGD	

Three SWATH-MS proteomic analyses of reactive and quiescent human astrocytes were completed (batches 1, 2 and 3). Nrf2 target gene products differentially expressed ($p_{adj} < 0.05$, $\log_2 FC > \pm 0.5$) between quiescent and reactive astrocytes are shown, with proteins in red (batches 2 and 3) validating the results of batch 1.

While Nrf2 protein could not be detected by SWATH-MS, in Figure 3.3, section 3.2.1, downregulation of *NRF2* mRNA was demonstrated in reactive astrocytes. The decreased expression of its target gene *IDH1* was demonstrated at the mRNA (RT-qPCR) and protein level (SWATH-MS). Despite being a well-validated Nrf2 target gene, *HMOX1* expression did not differ by RT-qPCR, and the HO-1 (encoded by *HMOX1*) protein did not pass through StatsPro filtering. While SWATH-MS measured downregulated NQO1 protein upon reactivity, this was not reflected by RT-qPCR. It is not unexpected that some Nrf2 target genes were unaffected Nrf2 downregulation. For example, transcription of *NQO1* is also activated by Nrf1, perhaps compensating for the Nrf2 loss (Jaiswal, 2000). Nrf1 did not pass StatsPro analysis, although its raw values appeared similar between reactive and quiescent astrocytes. Overall, the downregulation of many Nrf2 target genes in Figure 3.7 and the downregulation of Nrf2 itself in Figure 3.3 demonstrates that the Nrf2 pathway is dampened in this model of reactive astrocytes.

Attempts were made to validate the proteomic data by western blot, as shown in Figure 3.8, particularly to further validate the dampened Nrf2 pathway. Two antibodies were trialled to measure Nrf2 by western blot. Figure 3.8A shows very poor staining using a Santa Cruz antibody [sc-13032], which did not improve (with specificity) upon longer exposure. Therefore a ProteinTech antibody [16396-1-AP] was trialled in Figure 3.8B. Nrf2 antibodies are known to produce bands at multiple molecular weights, with many historic studies likely misinterpreting their results due to confusion about which molecular weight band to measure (Kemmerer *et al*, 2015; Lau *et al*, 2013). According to Lau *et al*, the Nrf2 species at ~95-110 KDa is more biologically relevant than the ~55-65 KDa species representing Nrf2's predicted molecular weight. The unexpected migration of Nrf2 to ~95-110 KDa may be due to the abundance of acidic residues (Lau *et al*, 2013).

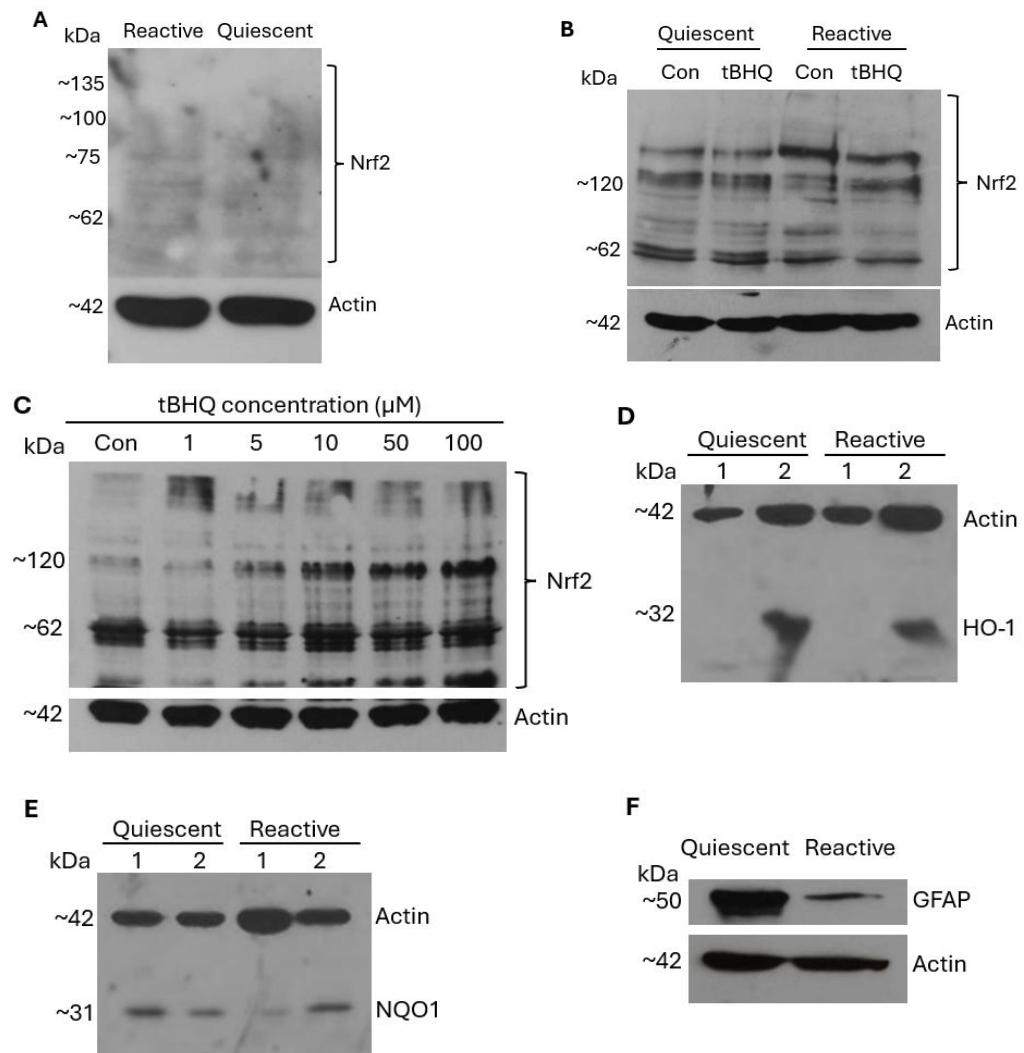


Figure 3.8. Western blot validation of proteomic changes in human quiescent and reactive astrocytes. It was not always possible to perform validation in the same samples as for proteomics. Actin was used as a housekeeper in all figures. No quantitative analysis or statistical tests performed. A) Nrf2 [Santa Cruz Biotechnology, sc-13032] in reactive and quiescent astrocytes ($n=1$). 20μg protein loaded. B) Nrf2 [Proteintech, 16396-1-AP] in quiescent and reactive astrocytes treated with control (con) DMSO or tBHQ (10μM) for 24 hours ($n=1$). 8μg protein loaded. C) Nrf2 [Proteintech] in HEK293S cells treated for 24 hours with increasing concentrations of tBHQ or DMSO (1%, corresponding to the highest tBHQ concentration) ($n=1$). 20μg protein loaded. D) HO-1 in quiescent and reactive astrocytes ($n=2$). 5μg protein loaded. E) NQO1 in quiescent and reactive astrocytes ($n=2$). 5μg protein loaded. F) GFAP in quiescent and reactive astrocytes ($n=1$). 7μg protein loaded.

To aid interpretation of the Nrf2 staining, quiescent and reactive astrocytes were treated with the commercial Nrf2 activator tBHQ (10μM) for 24 hours. tBHQ is an FDA-approved food additive which blocks the KEAP1-Nrf2 interaction by modifying KEAP1 cysteine residues, therefore reducing proteasomal degradation of Nrf2 (Abiko *et al*, 2011; Khezerlou *et al*, 2022). Western blots have detected tBHQ-induced Nrf2 increases in many studies

(including at 10 μ M), and tBHQ has been shown to be functional in astrocytes, resulting in neuroprotection (Abiko *et al.*, 2011; Jessen *et al.*, 2020; Kraft *et al.*, 2004). Despite stronger staining, the ProteinTech antibody did not detect an increase in Nrf2 expression upon tBHQ treatment of quiescent or reactive astrocytes, at either molecular weight (n=1). The same antibody did detect a progressive increase in Nrf2 expression (120 kDa species) in HEK293S cells upon increasing tBHQ treatment (n=1), including at 10 μ M as shown in Figure 3.8C. This suggests that western blot using the ProteinTech antibody is not an appropriate measure of Nrf2 expression in this astrocyte model, although it is unclear why that is.

I then sought to validate the relative expression of two Nrf2 target genes, HO-1 and NQO1, by western blot. In the proteomic data, HO-1 did not pass through the StatsPro proteomic data filtering so was not statistically analysed, however the mean raw values suggest perhaps a slight downregulation upon reactivity (68.9 in quiescent, 41.0 in reactive). NQO1 was downregulated upon reactivity, as shown in Figure 3.7 and validated in Table 3.4. Figure 3.8D shows that in two replicates of quiescent and reactive astrocytes, HO-1 expression varied greatly, with high detection in one replicate but not the other. Although not as extreme, NQO1 expression also varied between the two replicates in Figure 3.8E, therefore no clear expression change was observed.

Although western blots were not successful for validating Nrf2 pathway changes, it was still important to be able to validate other differentially expressed proteins to validate the proteomic method. GFAP was selected as a candidate protein for validation, largely because it is a commonly used marker of astrocytes and astrocyte reactivity (Escartin *et al.*, 2021). While GFAP also did not pass StatsPro analysis, its mean raw values suggest it is strongly downregulated in reactive astrocytes (17766 in quiescent, 809 in reactive). This was validated by the western blot in Figure 3.8F, as well as the western blots and immunofluorescence we previously published (White *et al.*, 2024). While this may be a limitation of our astrocyte model, perhaps suggesting these cells become less 'astrocyte-like' upon serum exposure, the model is sufficient for the subsequent studies on Nrf2 pathway dysregulation.

Immunocytochemistry was also performed (n=2) to compare Nrf2, HO-1 and NQO1 expression between reactive and quiescent astrocytes, as shown in Figure 3.9. HO-1 and NQO1 protein expression was quantified in the whole of each cell, while Nrf2 was measured in the whole-cell, nucleus and cytoplasm compartments separately due to its activity-dependent shuttling between the nucleus and cytoplasm (Cullinan *et al.*, 2004). This allowed a nucleus/cytoplasm ratio to be calculated for each cell, as shown in Figure 3.9E. Only two replicates were performed due to limited antibody, therefore no statistical tests were performed. However, there appeared to be a slight trend towards decreased fluorescence in all proteins in reactive astrocytes, except cytoplasmic Nrf2.

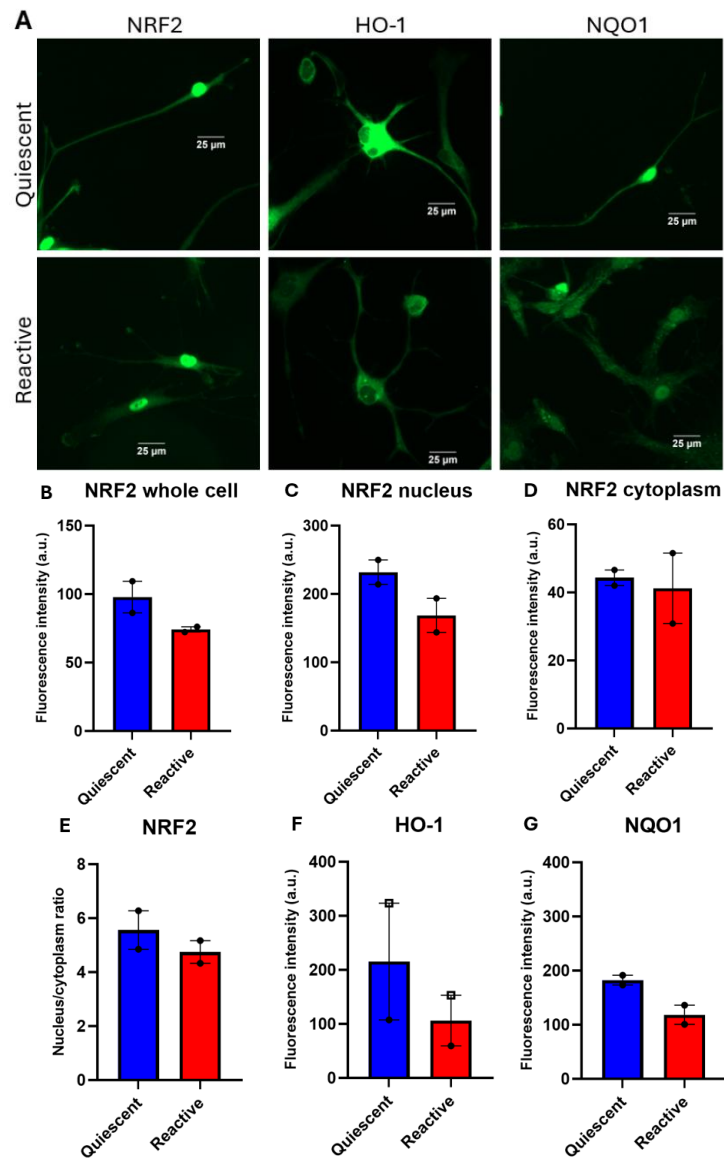


Figure 3.9. Immunofluorescence validation of Nrf2 pathway protein expression in human quiescent and reactive astrocytes (n=2). Quiescent and reactive astrocytes were seeded at 5,000 cells/well in ibidi dishes and fixed 2 days later. Immunocytochemistry was performed using the following primary antibodies: Nrf2 (Santa-Cruz, 1:100), HO-1 (1:200) or NQO1 (1:200). Alexa Fluor™ 488 goat anti-rabbit secondary antibody (1:400, green) was used in combination with DAPI (100nM) and phalloidin (1:500, red, not shown). Cells were imaged at 20x magnification (red and blue channels 20%/150ms, green channel 30%/300ms). Green fluorescence intensity (arbitrary units (a.u.)) was measured for each cell using Fiji, then the average background intensity of each image was subtracted. A) Quiescent and reactive astrocytes stained for Nrf2, HO-1 or NQO1. Phalloidin and DAPI not shown. B-D) Nrf2 fluorescence in the B) whole cell, C) nucleus and D) cytoplasm of quiescent and reactive astrocytes. E) Ratio of Nrf2 fluorescence in the nucleus versus the cytoplasm. Ratios were calculated for each cell individually. F) HO-1 fluorescence in the whole cell. Datapoint symbols illustrate variability between the two replicates. G) NQO1 fluorescence in the whole cell. Minimum 20 cells from 4 images analysed. No statistical test used (n=2).

Unlike Nrf2 and NQO1, HO-1 fluorescence varied greatly between biological replicates, as illustrated by the square and circle datapoints in Figure 3.9F. This reflected the varied western blot results of Figure 3.8D. Anecdotally, HO-1 expression also appeared to vary greatly between cells even within the cell type and biological replicate (by visual assessment of stained cells), suggesting rather heterogeneous expression irrespective of treatment or cell type.

While immunocytochemistry may be a useful tool for measuring Nrf2 pathway components within one cell model in future experiments, it is perhaps not the most appropriate for comparing quiescent and reactive astrocytes due to their drastic morphology differences. Quiescent astrocytes have a reduced area/perimeter ratio, suggesting that proteins may be ‘condensed’ into a smaller space, therefore appearing brighter. Additionally, we previously reported that the reactive astrocytes have increased nuclear size compared to quiescent astrocytes (White *et al.*, 2024), which may be predicted to result in a ‘dispersing’ of nuclear proteins and reduction in nuclear immunofluorescence.

In conclusion, while western blot was effective at validating the GFAP expression change indicated by SWATH-MS, neither western blot or immunocytochemistry appeared to be reliable methods for comparing expression of Nrf2 pathway components between quiescent and reactive astrocytes. Despite these challenges, the proteomic results in Figure 3.7 and RT-qPCR in Figure 3.3 strongly suggest a dampening of the Nrf2 pathway in this model of reactive astrocytes. RT-qPCR was therefore used for most future analyses of the Nrf2 pathway, except for a single-cell analysis presented in Chapter 5.

3.2.2.2 Differential expression of ALS-associated proteins upon astrocyte reactivity

After the investigation of Nrf2 pathway proteins, I aimed to identify any ALS-associated proteins differentially expressed between quiescent and reactive astrocytes. Any altered expression may be a contributing factor or consequence of astrocyte reactivity, but either way it may be predicted to impact astrocyte function. It should be noted that for many ALS-associated proteins, it is not known or is not as simple as the protein is upregulated or downregulated in ALS. Variants in some genes, such as *SOD1* and *C9orf72*, likely have deleterious effects due to both gain and loss-of-function mechanisms (Mejzini *et al.*, 2019). Even so, identifying differential expression of a protein upon astrocyte reactivity would be a first step towards understanding whether it may be involved in neuroinflammation in ALS.

ALSoD was used to find ALS-associated proteins (Abel *et al.*, 2012). ALSoD contained 154 genes as of August 2024, which are assigned different terms according to the strength of evidence for their ALS-association – from definitive ALS gene down to tenuous. After StatsPro analysis, there was robust detection of 55/154 (36%) ALSoD proteins in batch 1 (i.e. detected in $\geq 4/6$ samples), as listed in Appendix Table 9.3. Of note, the only ALS-associated protein which

was also in the list of Nrf2 target gene products in Table 1.2 and detected in batch 1 was SQSTM1, however it was not differentially expressed. Of the 55 ALS-associated proteins detected, 18 were differentially expressed. Fourteen were downregulated in reactive astrocytes, while only 4 were upregulated, as shown in Figure 3.10A. Validation of differentially expressed ALS-associated proteins in batches 2 and 3 will be discussed shortly. Although proteins such as SMN1 and NEFL are typically described as neuronal, the human protein atlas does support some astrocytic expression of these proteins (Karlsson *et al*, 2021).

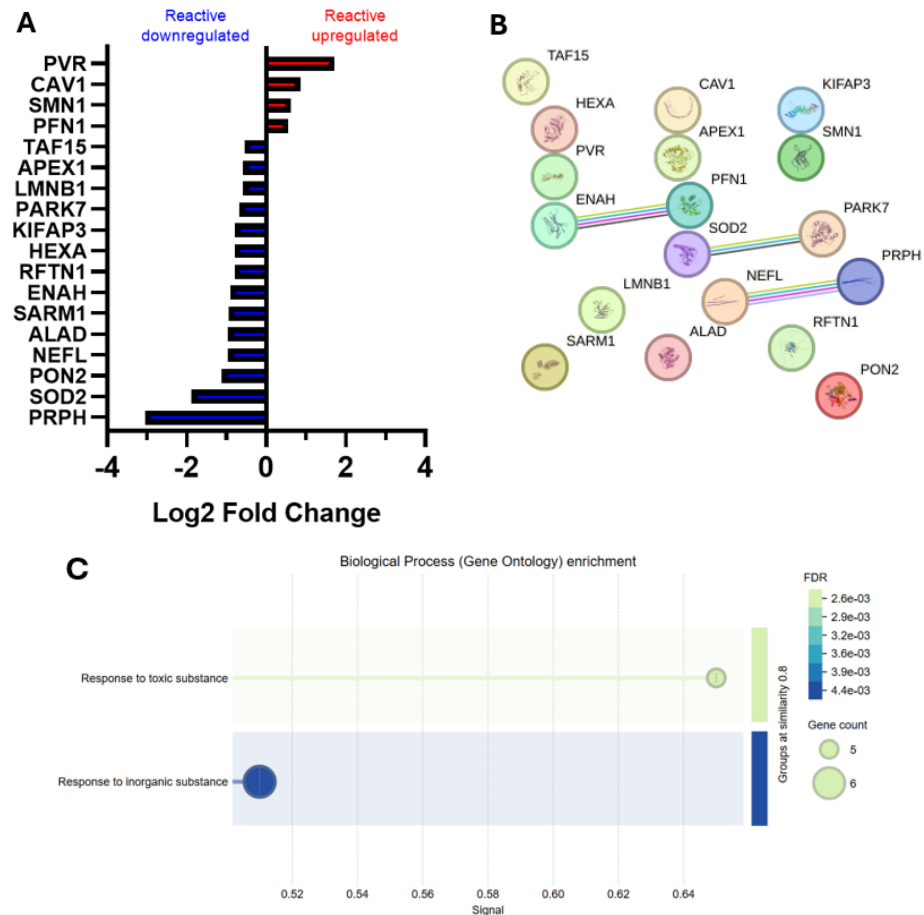


Figure 3.10. ALS-associated proteins differentially expressed between human quiescent and reactive astrocytes, determined by proteomic analysis. A) ALS-associated proteins differentially expressed ($p_{adj} < 0.05$, $\text{Log}_2\text{FC} > \pm 0.5$) between quiescent and reactive astrocytes in batch 1 of the SWATH-MS proteomic analysis. B) STRING functional interaction network of the differentially expressed ALS-associated proteins. The line colour corresponds to the source of interaction information. Pink= experimentally determined interactions. Pale blue= known interactions from curated databases. Green= gene neighbourhood predicted interactions. Red= gene fusion predicted interactions. Dark blue= gene co-occurrence predicted interactions. Yellow= text mining. Black= co-expression. Lilac= protein homology. Only high confidence (> 0.7 interaction score) interactions are included. C) Biological processes enriched in the differentially expressed ALS-associated proteins. B and C generated in STRING.

Protein-protein interactions between the 18 differentially expressed ALS-associated proteins were identified using the STRING (Search Tool for the Retrieval of Interacting Genes/proteins; string-db.org) online bioinformatics tool. STRING collates information on both physical interactions and functional associations between proteins, with evidence including published literature and computational predictions (Szkarczyk *et al*, 2023). Only high confidence interactions (>0.7 interaction score) were selected. There were only 3 interaction pairs between these 18 proteins, as represented in Figure 3.10B. It therefore appears unlikely that these differentially expressed proteins are in a regulatory functional network, instead perhaps being differentially expressed upon reactivity independent of each other.

STRING Gene Ontology analysis was then used to identify enriched biological functions of the differentially expressed ALS-associated proteins. Although limited confidence should be put in a pathway analysis of only 18 proteins, Figure 3.10C suggests enrichment of proteins involved in the response to toxic and inorganic substances. This may further suggest dysregulated stress responses in reactive astrocytes, which may be exacerbated by ALS variants in the associated genes.

PFN1 was the only definitive ALS-associated protein (according to ALSod) differentially expressed between quiescent and reactive astrocytes in batch 1. While PFN1 variants have been shown to cause ALS (Wu *et al*, 2012), there have not been any studies focussed on identifying an astrocytic role of PFN1 in ALS pathogenesis. Some of the non-definitive ALS-associated proteins in Figure 3.10A have been previously linked to astrocyte reactivity, although generally not in the context of ALS. In a mouse stroke model, caveolin 1 (CAV1) expression was increased in reactive astrocytes in areas surrounding the lesion. Knockout of CAV1 resulted prevented proper glial scar formation around the lesion, and altered the reactive morphology of astrocytes (shorter processes) (Blochet *et al*, 2020). In a SOD2 knockout mouse model, astrogliosis in the hippocampus was indicated by increased astrocyte density (Baier *et al*, 2022). These studies support the findings in Figure 3.10A that reduced SOD2 and increased CAV1 are associated with astrocyte reactivity. However, astrocytic SARM1 (sterile alpha and TIR motif containing 1) was found to promote neuroinflammation in a mouse model of multiple sclerosis by inhibiting GDNF expression (Jin *et al*, 2022), contrasting the reduction upon reactivity in Figure 3.10A. Furthermore, PARK7 (parkinsonism associated deglycase) expression has been shown to be upregulated in reactive astrocytes from PD patients (Neumann *et al*, 2004), however it was downregulated in our reactive astrocytes.

It may be hypothesised that some of the changes in Figure 3.10A could contribute to changes seen in ALS patients. For example, a large-scale analysis of postmortem ALS brain tissue RNAseq found a consistent increase in CAV1 expression compared to controls (Adey *et al*, 2023). Perhaps this increase could be in part attributed to the increased CAV1 expression in reactive astrocytes shown in Figure 3.10A, although validation studies would be required to confirm this. Of course, there will also be many protein changes in ALS patients

that cannot be explained by astrocyte reactivity. For example, serum peripherin (PRPH, an intermediate filament) was reported to be increased in MND patients (Sabbatini *et al*, 2021), contrasting the decreased expression identified in reactive astrocytes.

One additional ALS-associated protein of interest is Apolipoprotein E (APOE), which did not pass the StatsPro analysis as it was in only 3/6 samples. However interestingly, it was detected in all three quiescent samples but no reactive samples, suggesting there may be a complete loss of APOE upon reactivity, at least below the detection limit. This is the only ALSod protein to be detected in 3/3 samples of one cell model, and no samples of the other. The effect of APOE on astrocyte reactivity may depend on its isoform, as the AD risk allele *APOE4* may promote inflammation and astrocyte reactivity more than the protective *APOE2* allele (de Leeuw *et al*, 2022; Parhizkar & Holtzman, 2022). APOE was not detected in proteomics batches 2 and 3.

To identify the most robust ALS-associated (ALSod) protein changes upon reactivity, batches 2 and 3 of the astrocyte proteomics were then analysed. In Table 3.5, the proteins in red validated the results of batch 1. The only result to be the same in all three batches was downregulation of Lamin B1 (LMNB1) upon reactivity. LMNB1 is assigned ‘moderate evidence’ in ALSod, as a linkage analysis found an LMNB1 polymorphism that segregated with ALS in a European cohort (Johnson *et al*, 2014). LMNB1 is essential for brain development, but its levels then drop during aging, leading to its implication in neurodegenerative diseases such as AD and PD (Koufi *et al*, 2023). We previously suggested in (White *et al*, 2024) that the serum culture of this reactive astrocyte model promotes cellular maturation, which may explain the downregulation of LMNB1.

Table 3.5. Validation of differentially expressed ALS-associated proteins by additional proteomic analyses of human quiescent and reactive astrocytes.

	ALSod proteins downregulated in reactive astrocytes	ALSod proteins upregulated in reactive astrocytes
Batch 1	ALAD, APEX1, ENAH, HEXA, KIFAP3, LMNB1, NEFL, PARK7, PON2, PRPH, RFTN1, SARM1, SOD2, TAF15	CAV1, PFN1, PVR, SMN1
Batch 2	HNRNPA2B1, LMNB1, PON2, PRPH, RFTN1	CAV1
Batch 3	APEX1, LMNB1 , MATR3, SOD1	ANXA11, PVR

Three SWATH-MS proteomic analyses of reactive and quiescent human astrocytes were completed (batches 1, 2 and 3). ALSod proteins differentially expressed ($p_{adj} < 0.05$, $\text{Log}_2\text{FC} > \pm 0.5$) between quiescent and reactive astrocytes are shown, with proteins in red (batches 2 and 3) validating the results of batch 1.

ALSoD protein changes validated in one additional proteomics batch were the upregulation of CAV1 and PVR and downregulation of PON2, PRPH, RFTN1 and APEX1 upon reactivity. CAV1 and PRPH have already been discussed, while little is known about the tenuously ALS-associated RFTN1 (raftlin) regarding ALS or astrocyte reactivity. Expression of the CD155 poliovirus receptor encoded by *PVR* was shown to be upregulated in U87MG glioblastoma cells compared to normal human astrocytes (Sloan *et al*, 2004). While unrelated to neurodegeneration, this is an example of disease-associated upregulation of PVR in astrocyte-like cells, in a cancer type where reactive astrocytes in the tumour microenvironment promote cancer progression (Guan *et al*, 2018), perhaps reflecting the upregulation of PVR upon reactivity in Table 3.5.

Moving to the downregulated proteins of Table 3.5, paraoxonase 2 (PON2) is an antioxidant enzyme of the inner mitochondrial membrane. Its expression was reported as significantly higher in mouse astrocytes than neurones, likely due to the important role of astrocytes protecting neurones from oxidative stress (Giordano *et al*, 2011). Therefore, its downregulation upon reactivity is further evidence for a dysregulated antioxidant system in this model of reactive astrocytes. Astrocyte antioxidant system dysregulation may therefore be a driving disease mechanism in ALS patients with *PON2* mutations, meaning *PON2*-ALS patients may particularly benefit from an astrocyte Nrf2-activating therapy. Similarly, the DNA repair protein Apurinic/apyrimidinic endonuclease 1 (APEX1) has been shown to protect against oxidative stress in mouse neurones and zebrafish (Pei *et al*, 2019; Vasko *et al*, 2005).

To summarise, several of the reactivity-associated changes in expression of ALS proteins can be rationalised based on existing knowledge. However, for many proteins, especially those with only very limited association with ALS, their possible involvement in astrocyte reactivity has been demonstrated, a process known to be pathogenic in ALS. Further investigation of these proteins in ALS astrocytes is therefore warranted, to work towards an astrocyte-targeted therapy for ALS.

3.3 Conclusions

In Chapter 3, the simple and cost effective models of human primary quiescent and reactive astrocytes recently published by our group were presented (White *et al*, 2024). Briefly, the chronic FBS-induced model of reactive astrocytes reflects the persistent nature of reactivity in ALS and exhibits reduced expression of Nrf2 pathway components (measured by RT-qPCR), mimicking Nrf2 pathway impairment in ALS. A proteomic analysis of both astrocyte models was conducted, with two additional proteomic analyses performed for validation. This confirmed dampening of the Nrf2 pathway in the reactive astrocyte model, indicated by downregulation of 12 Nrf2 target gene products in reactive astrocytes (batch 1), while none were upregulated. It should be noted that this downregulation could in part be due to the increased age of the reactive astrocytes (P4) compared to quiescent astrocytes (P2). As mentioned

in Chapter 1, expression of Nrf2, HO-1 and NQO1 was shown to severely decline in mouse astrocytes after 60 days in culture (Duan *et al.*, 2009).

A recent study published after the completion of this work (Elsharkasi *et al.*, 2025) suggests that this downregulation of the Nrf2 pathway may also be driven by the difference in culture medium between the two astrocyte models. Human primary astrocytes (SC1800, the same as our model) were cultured in either reactivity-inducing astrocyte medium or an Advanced DMEM/F12-based neuronal differentiation medium, similar to our serum-free G5 medium but with different supplements. The Advanced DMEM/F12-based medium strongly induced the Nrf2 pathway (measured by NQO1 activity), likely due to Advanced DMEM/F12 itself, not the supplements. Commercial Nrf2 activators were ineffective in this medium, perhaps suggesting that the medium disrupted KEAP1/Nrf2 binding. This may offer an alternate interpretation of my findings, suggesting that Nrf2 activation occurs in the quiescent astrocyte model rather than indicating Nrf2 dysfunction in the reactive model.

However, irrespective of the mechanism underlying the different Nrf2 phenotypes of these astrocyte models, I have presented a reactive astrocyte model in which I aimed to 'rescue' the Nrf2 pathway with miRNA inhibitors in Chapter 5, towards the level of quiescent astrocytes. Therefore, this model is an appropriate platform for this experimental purpose.

Further to the analysis of the Nrf2 pathway, the first proteomic analysis (batch 1) achieved robust detection of 55/154 ALS-associated proteins (ALSoD) in our astrocyte models. Differential expression of 18 of these proteins suggests their possible involvement in astrocyte reactivity, providing further insight into their pathogenic mechanisms in ALS. Future work would be required to elucidate these precise mechanisms and to validate the changes in an ALS-specific astrocyte model, for example in patient-derived iPSC-astrocytes. This data also facilitated predictions that ALS patients with certain ALS subtypes may particularly benefit from an astrocyte Nrf2-targeted therapy, for example patients with mutations in the antioxidant PON2 protein. PON2 protein was downregulated in the reactive astrocyte model, perhaps contributing to oxidative stress which may be exacerbated by a mutation.

In this chapter, the proteomic background of the selected model of human reactive and quiescent astrocytes was explored. However, it was not known which miRNAs were co-expressed, and to what extent. To make rational choices of miRNA therapeutic targets to be inhibited in Chapter 5 with the aim of rescuing Nrf2 signalling, a sncRNAseq analysis of the astrocyte models was therefore conducted, which will be presented in Chapter 4.

Chapter 4. MiRNA expression changes in human astrocytes upon FBS-induced reactivity

4.1 Introduction

There are currently limited studies profiling miRNA expression in human astrocytes in a broad, unbiased manner, especially investigating the potential interplay between miRNA expression and astrocyte reactivity. Much of what is known about the involvement of miRNAs in astrocyte reactivity has been inferred from studies examining the effects of inflammatory stimuli or neurodegenerative disease phenotypes such as amyloid β accumulation or SOD1^{G93A} mutation, on astrocyte miRNA expression (Chu & Williams, 2021).

To address this knowledge gap, here I investigate miRNA expression in our models of human astrocytes (presented in Chapter 3) and their associated ADEVs using sncRNAseq. Identifying miRNAs which are differentially expressed between quiescent and reactive astrocytes will allow hypotheses of how and which miRNAs may be involved in astrocyte reactivity, and how this may affect communication with other cell types such as motor neurones via their released ADEVs. This data will also allow indirect investigation of astrocytic miRNAs in ALS pathogenesis, with motif analysis elucidating how ALS-associated RBPs may be involved in miRNA sorting and loading into ADEVs, and how this may be altered upon astrocyte reactivity or RBP pathology. Integration of proteomic (Chapter 3) and sncRNAseq data (this Chapter) will facilitate predictions of the extent to which astrocyte proteomes may be regulated by miRNAs. Furthermore, it will be elucidated whether the Nrf2 pathway deficit described in Chapter 3 is likely to be caused by, or independent of, augmented miRNA targeting of Nrf2.

Astrocyte sncRNAseq will also aid selection of potential miRNA therapeutic targets, as well as being a useful resource for other researchers who wish to target miRNAs in astrocytes to investigate different hypotheses. As a cautionary note, this data will be most useful for others when using the same human primary astrocyte model (SC1800), as subtle changes are anticipated between models. For example, miRNA expression is known to change upon astrocyte age (and aging in general), therefore expression profiles are likely to differ between this foetal astrocyte model and those which are derived from adult donor fibroblasts (Chu & Williams, 2021; Huan *et al*, 2018; Raihan *et al*, 2018; Rao *et al*, 2016). Astrocyte miRNA expression also varies between adult brain regions. For example, expression varies between the interlaminar astrocytes of cortex layer 1 and those deeper in the cortical layers, while there are more pro-inflammatory miRNAs in white matter than grey matter (Rao *et al*, 2016). Therefore, the global miRNA expression profile of this cortical astrocyte model may differ from non-cortical and spinal cord models.

4.1.1 miRNA expression in astrocytes and ADEVs

MiRNA expression in rodent astrocytes and/or their ADEVs has been analysed in several studies including upon various stimuli (Chaudhuri *et al.*, 2018; Lin *et al.*, 2015; Mor *et al.*, 2011). For example, the nCounter® miRNA expression assay detected 131 miRNAs in rat cortical ADEVs, with differential expression of 7 miRNAs in response to ATP stimulation of the astrocytes, 10 in response to IL-1 β and 15 due to TNF α . Interestingly these miRNA changes were not detected at the whole-cell level in the astrocytes themselves, suggesting ADEV-specific miRNA changes in response to different proinflammatory cytokine (likely reactivity-inducing) or neurotransmitter stimuli (Chaudhuri *et al.*, 2018). While rodent studies are less useful for identifying miRNA targets for human studies, general mechanisms such as this may be conserved.

One example of a human astrocyte model profiled by sncRNAseq is a schizophrenia patient-derived iPSC astrocyte model, where the authors found reduced expression of four inflammation-related miRNAs compared to healthy controls (miR-127-5p, miR-206, miR-337-3p and miR-1185-1-3p), perhaps an opposite finding to what one might expect in an ALS model with an inflammatory component (Akkouh *et al.*, 2021).

The reactive model of human primary astrocytes (SC1800) used in this thesis has been sequenced at the level of sncRNAs in previous studies, but always without a serum-free quiescent astrocyte control (Casselli *et al.*, 2017; Chen *et al.*, 2023; Gordillo-Sampedro *et al.*, 2024). One study used this to investigate miRNA changes in astrocyte-to-neurone reprogramming, a procedure which shows promise in the field of regenerative medicine. It should be noted that slightly different culture conditions were employed for reactive astrocyte culture, including 10% FBS (as opposed to 2% FBS used herein). Astrocytes were converted by overexpression of the NeuroD1 transcription factor, with authors finding that the observed upregulation of miR-375 had a neuroprotective role during the process (Chen *et al.*, 2023). Another study used the SC1800 astrocyte model to investigate miRNA changes associated with *Borrelia burgdorferi* infection, the bacterium which causes Lyme disease. This allowed authors to predict gene expression changes which may underlie the neurological symptoms often associated with Lyme disease (Casselli *et al.*, 2017).

The most relevant study to this thesis was published by Gordillo-Sampedro *et al.* in 2024, reported after completion of this analysis. Here the authors applied whole-cell sncRNAseq to the SC1800 primary reactive astrocyte model alongside human iPSC-astrocytes, and to ADEVs from both models. The authors speculated that some miRNAs are passively loaded into ADEVs, while other miRNAs are intentionally sorted and enriched in ADEVs to target neuronally expressed genes. In line with this prediction, sequence motif analysis of these EV-enriched miRNAs was also performed, with identification of short sequences known to represent binding motifs of different RBPs, including the ALS-associated protein FUS (Gordillo-Sampedro *et al.*, 2024). Wild-type FUS

had previously been demonstrated to have a role in miRNA loading into EVs from several non-CNS mouse cell lines, with preferential loading of miRNAs containing the CGGGAG sequence, termed an 'EXOmotif' (Garcia-Martin *et al.*, 2022). While the CGGGAG sequence may be mouse-specific, approximately 15% of human ADEV miRNAs were found to contain other sequences recognised by FUS, suggesting a role of FUS in ADEV miRNA sorting (Gordillo-Sampedro *et al.*, 2024). A separate hypothesis which our group is now testing is that FUS dysfunction may display dysregulated miRNA sorting in astrocytes, resulting in alterations to astrocyte-motor neurone communication mediated via ADEVs.

In another study investigating miRNA secretion via ADEVs, Shanthi *et al* sequenced miRNAs from primary human astrocytes and ADEVs from a single young adult male donor. Similarly, they found enrichment of nervous system development GO terms from targets of miRNAs preferentially secreted in ADEVs, more so than in the astrocytes themselves, suggesting an intentional packaging of miRNAs for neuronal communication (Shanthi *et al*, 2023).

In summary, while limited studies have profiled miRNA expression in human astrocytes and their ADEVs, this has not been with the aim of understanding how miRNAs are involved in regulation of astrocyte reactivity. Furthermore, astrocyte sncRNAseq data has not yet been used to investigate how astrocytic miRNAs may directly contribute to ALS pathogenesis or biomarker signatures seen in those living with ALS.

4.1.2 Experimental aims

The broad experimental aims of Chapter 4 are as follows:

- Conduct whole-cell sncRNAseq of our models of 'quiescent' and 'reactive' human primary astrocytes and their purified ADEVs, to identify miRNA expression changes associated with FBS-induced astrocyte reactivity
- Identify candidate miRNAs with potential roles in astrocyte reactivity
- Evaluate whether miRNA sorting and loading into ADEVs by ALS-associated RBPs is affected by astrocyte reactivity.
- Determine through integration with proteomic data any connections between miRNA expression and Nrf2 pathway changes
- Select candidate Nrf2-targeting miRNAs for specific inhibition in astrocytes

4.2 sncRNAseq analysis of human quiescent and reactive astrocytes and their extracellular vesicles

Chapter 3 detailed the proteomic changes in human astrocytes that occur upon FBS-induced reactivity. This led to the discovery that there is reduced expression of Nrf2 and its downstream target genes in our reactive astrocyte model, relative to quiescent cells grown in the absence of FBS. Therefore, the reactive astrocyte model is an appropriate platform to investigate whether therapeutic elevation of Nrf2 via miRNA targeting in astrocytes represents a possible neuroprotection strategy. Since miRNA targeting was the overall aim, it was therefore also important to know the miRNA landscape of human astrocytes, and whether this changes upon reactive remodelling in this model.

To achieve this, a comprehensive sncRNAseq analysis of quiescent and reactive astrocytes was completed at the whole-cell level, using a commercial RNA sequencing service (Novogene). In parallel, RNA content in ADEVs (isolated via SEC of conditioned medium) derived from these cells was also profiled, to provide insight into how ADEV miRNA-mediated communication from astrocytes to motor neurones may be influenced by astrocyte phenotype.

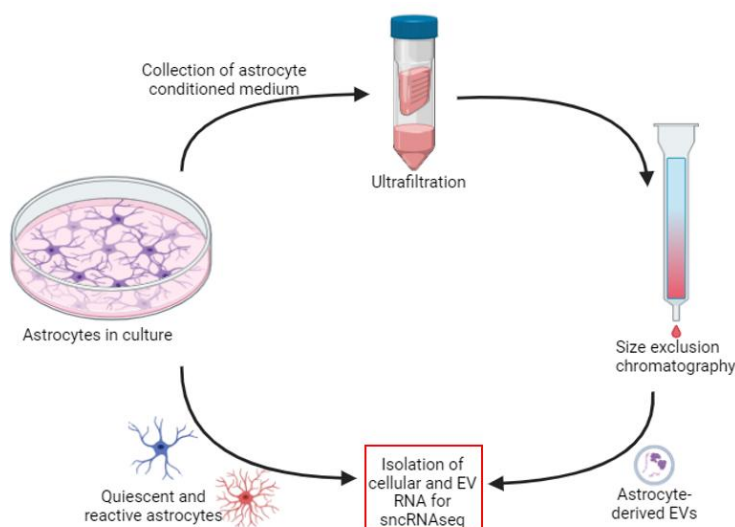


Figure 4.1. Schematic demonstrating the method of astrocyte and ADEV sample collection for sncRNAseq. Quiescent astrocytes were cultured in serum-free G5 medium which was collected every 3-4 days. Reactive astrocytes were first cultured in astrocyte medium containing 2% FBS, then switched to serum-free astrocyte medium, which was collected after 3 days. After conditioned medium collection, cellular RNA was isolated. Due to low EV RNA yield, ~80ml conditioned medium was required for EV purification, not the standard ~40ml. Ultrafiltration then size exclusion chromatography (SEC) of the conditioned medium was performed in two 40ml batches to isolate purified EVs. The two EV batches were then combined and concentrated for each sample. EV RNA was extracted, then whole-cell and ADEV total RNA samples were submitted to Novogene for sncRNAseq. Figure created using BioRender.

For both reactive and quiescent astrocytes, three independent replicates of whole-cell RNA and corresponding ADEV-associated RNA were collected and submitted to Novogene for sncRNAseq (covering miRNA sequences) and data processing, including readcount normalisation using DESeq2. It should be noted that in this case, the reactive astrocytes were grown in serum-free astrocyte medium for three days (to avoid contaminating with EVs from FBS) allowing isolation of ADEVs that could directly be compared to their cells of origin. Until this point, including 3 passages and approximately 3 weeks in culture, the reactive astrocytes had been continually cultured in astrocyte medium containing 2% FBS, therefore modelling chronic astrocyte reactivity. Astrocyte proliferation and morphology were unaffected by the switch to serum-free astrocyte medium (by subjective visual assessment), and minimal proteomic impact was confirmed by an exploratory SWATH-MS analysis of whole-cell lysates that came from the exact same quiescent and reactive astrocyte samples as the RNA for sncRNAseq (data not shown). StatsPro analysis of quiescent vs reactive (in this case serum-starved) astrocytes was completed, as described in Chapter 3. After StatsPro analysis, a total of 2012 protein groups were detected, consisting of 1790 unique proteins and 252 ambiguous proteins. The differentially expressed unique proteins were then compared to those from batch 1 of the astrocyte proteomics presented in Chapter 3, where reactive astrocytes were maintained in FBS throughout:

- 32/131 (24%) of reactive astrocyte upregulated proteins (serum-starved sncRNAseq samples) were the same as reactive upregulated proteins in batch 1 of the astrocyte proteomics (serum-maintained).
- 69/124 (56%) of reactive astrocyte downregulated proteins (serum-starved sncRNAseq samples) were the same as reactive downregulated proteins in batch 1 of the astrocyte proteomics (serum-maintained).

Considering the variable nature of different SWATH-MS runs, (evidenced from direct comparisons of proteomic data in Chapter 3), this level of overlap is relatively high. This suggests minimal proteomic impact of three days of serum-starvation in reactive astrocytes. Importantly, the decreased Nrf2 phenotype in FBS-cultured reactive astrocytes (Chapter 3) was also detected in the serum-starved reactive astrocytes. Specifically, expression of the Nrf2 target genes EPHX1, PGD, ACOX1 and FTH1 was significantly downregulated upon astrocyte reactivity, with no upregulated Nrf2 target genes (data not shown). Therefore, these serum-starved reactive astrocyte sncRNAseq samples will continue to be called “reactive astrocytes”, despite subtleties in their culture.

4.2.1 ADEV characterisation

After ultrafiltration and SEC of astrocyte conditioned medium, nanoparticle tracking analysis (NTA) was performed using the Zetaview® to confirm EV presence in purified fractions. As shown in Appendix Figure 9.3, ADEVs were in the expected size range (~50-300nm) although concentration varied between replicates. Characterisation of the ADEVs from these astrocyte models, isolated using the same procedure, was performed by Dr Katherine White and was

recently published in (White *et al.*, 2024). This included Transmission Electron Microscopy (TEM), with ADEVs typically displaying the characteristic cup-shaped morphology that occurs under vacuum, as shown in Figure 4.2A. Figure 4.2 was sourced and adapted from (White *et al.*, 2024). Protein markers of EVs were identified using LC-MS/MS as well as a lack of contaminant or cell-based proteins, with data shown in Figure 4.2B, as is stipulated in the MISEV guidelines. Most of the proteins (526 out of a total of 562 ADEV proteins) were also found in the Vesiclepedia database, a repository of proteins previously identified in EVs (Chitti *et al.*, 2023; White *et al.*, 2024). Overall, this provides evidence that the EVs isolated from our astrocyte model, using the specified method, can confidently be described as EVs, with minimal contamination.

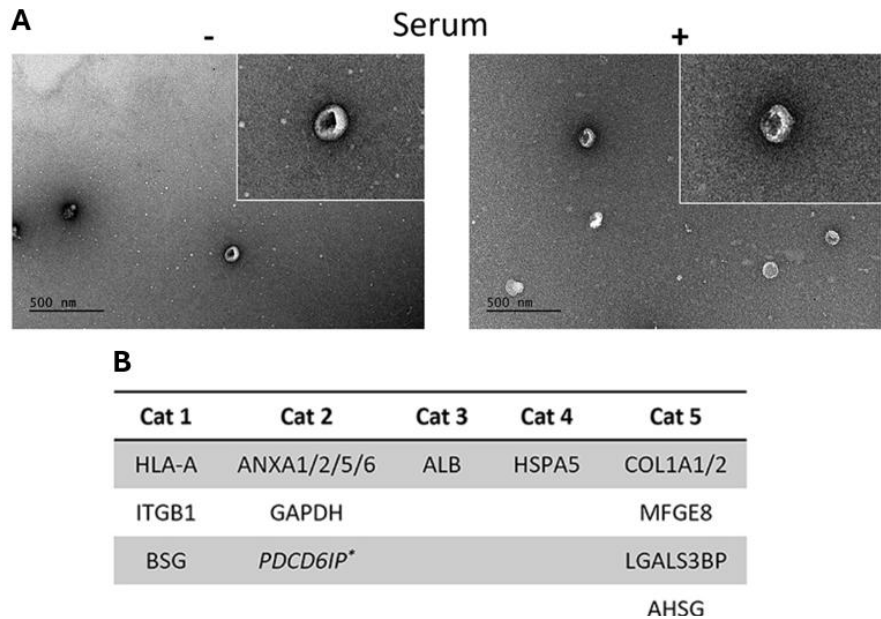


Figure 4.2. Characterisation of EVs isolated from quiescent and reactive astrocytes. Figure adapted from (White *et al.*, 2024). Reactive astrocytes were grown in serum-containing astrocyte medium (+), while quiescent astrocytes were cultured in serum-free G5 medium (-). A) TEM images of quiescent and reactive ADEVs. B) LC-MS/MS proteomic analysis of quiescent and reactive ADEVs identified EV-associated proteins (Category 1 and 2) and secreted proteins (Category 5) and a lack of contaminant (Category 3) or cell-based (Category 4) proteins, as stipulated by the MISEV guidelines. *Also named Alix.

Total RNA was extracted from cultured astrocytes using TRIzol®, then RNA quality and quantity was measured by Nanodrop and RNA Screentape® assay [DeepSeq, University of Nottingham]. All RNA 260/230 and 260/280 purity ratios were >1.8 and RIN values >9.8, illustrating high RNA purity and quality. Total RNA quantity was in significant excess of the minimum 2µg required by Novogene.

After NTA to confirm EV presence and the expected size range (Appendix Figure 9.3) all remaining isolated ADEVs were used for RNA extraction to achieve the maximum yield, regardless of the EV concentration determined by NTA. Total EV RNA was extracted using TRIzol® LS Reagent and quantified by a High Sensitivity RNA Screentape® assay [DeepSeq]. One of the reactive ADEV

samples (“Reactive ADEV 1” in Table 4.1) was the combination of two biological replicates due to low EV RNA yield, with the respective whole-cell RNA samples also combined. Novogene required 10ng EV RNA for each sample, a criterion which was only just met. ADEV RIN values were low (2.5-3.1), which is typical for EV-associated RNA due to the low levels of large ribosomal RNAs (Li *et al*, 2023).

4.2.2 sncRNA profile of human quiescent and reactive astrocytes whole-cell extracts and purified ADEVs

Whole-cell and ADEV RNA was submitted to Novogene for sncRNAseq. First, Novogene completed its own quality control, including taking their own concentration measurements which were used to determine the volumes of RNA required for sncRNAseq. All whole-cell RNA samples passed quality control, while the ADEV RNA samples failed due to low yield, however they were still used for sncRNAseq.

Novogene mapped miRNAs and other sncRNAs to reference sequences as described in Chapter 2. Raw and processed sncRNAseq data was returned by file transfer, including various bioinformatic analyses which will be discussed in the following section. The first step with assessing the sncRNAseq data was to determine the data quality and to describe the overall sncRNA landscape of the samples before focussing on the miRNA fraction. Table 4.1 demonstrates the high sequencing accuracy of the sncRNAseq data. The average number of reads per sample mapped to the genome was 89.45% (96.23% for cells and 82.67% for EVs). Typically 70-90% of RNAseq reads (all RNA species) are expected to map to the human genome, with lower percentages indicating low sequencing accuracy or contaminating DNA (Conesa *et al*, 2016). Table 4.1 also shows that the total numbers of raw reads were comparable between samples, ranging from $\sim 1.8 \times 10^7$ to $\sim 5.4 \times 10^7$.

Table 4.1. Total number of raw reads (total sncRNA) and percentage of sncRNAs mapped to the human genome are high and relatively consistent between samples.

Sample	Total sncRNA	Mapped sncRNA
Reactive astrocyte 1	23505684	22078447 (93.93%)
Reactive astrocyte 2	21960302	20756537 (94.52%)
Reactive astrocyte 3	17889890	16912801 (94.54%)
Quiescent astrocyte 1	19212792	18774878 (97.72%)
Quiescent astrocyte 2	21561242	21200475 (98.33%)
Quiescent astrocyte 3	19394065	19070062 (98.33%)
Reactive ADEV 1	28414310	25076840 (88.25%)
Reactive ADEV 2	36421009	27052235 (74.28%)
Reactive ADEV 3	25943792	24781505 (95.52%)
Quiescent ADEV 1	38247942	28726572 (75.11%)
Quiescent ADEV 2	28700195	23688793 (82.54%)
Quiescent ADEV 3	54329636	43640285 (80.33%)

Table adapted from Novogene.

Figure 4.3 shows the Pearson correlation between samples at the level of total sncRNA. Notably there was a high degree of similarity between replicates of the whole-cell samples, demonstrating the repeatability of the experiment. According to Novogene, similarity is indicated by a Pearson correlation R^2 value of >0.8 . R^2 values averaged around 0.950 for reactive astrocytes and 0.990 for quiescent astrocytes. Values were also relatively similar between reactive and quiescent astrocytes, with an average R^2 value of 0.813 and no values below 0.789. As expected, cellular and EV samples showed a much lower degree of similarity, with R^2 values ranging from 0.586 to 0.759.

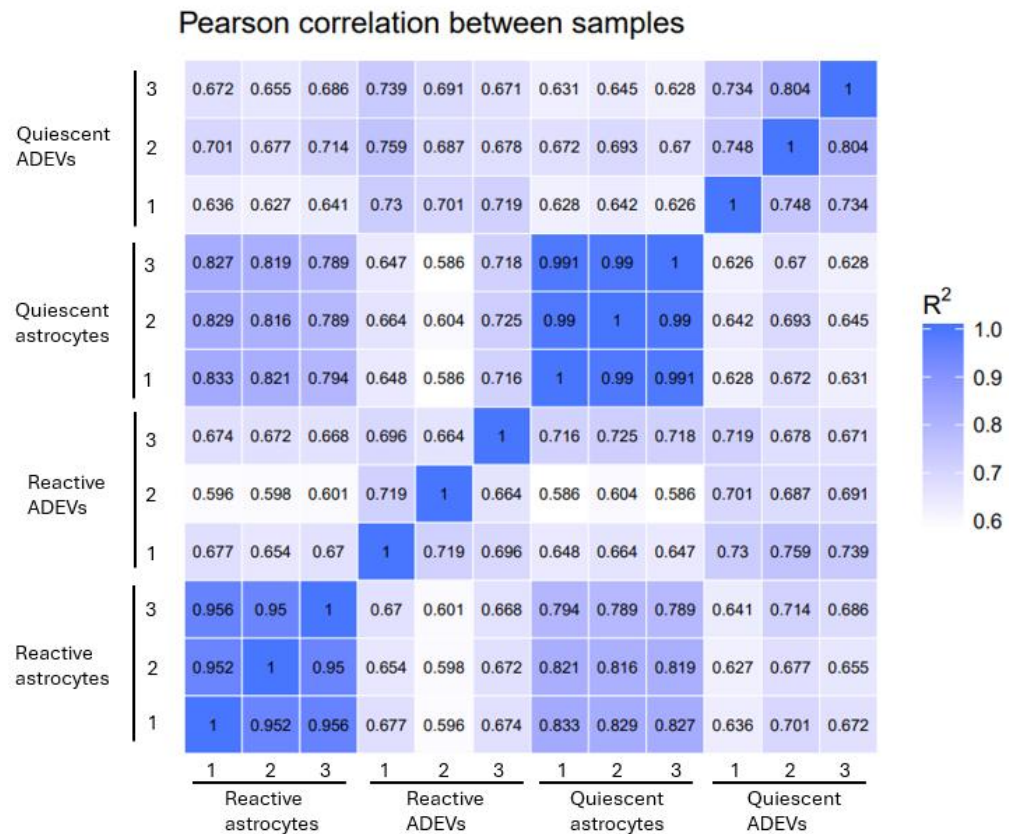


Figure 4.3. *sncRNAs from human reactive and quiescent astrocytes are highly similar between replicates, while ADEVs display more variation.* Figure adapted from Novogene showing the Pearson correlation between samples. The closer an R^2 value is to 1, the more similar two samples are.

For the ADEV samples there was a higher degree of variability between replicates, with average R^2 values of 0.762 for quiescent ADEVs and 0.693 for reactive ADEVs. This may suggest that the ADEV sncRNAseq is less reproducible, perhaps due to lower RNA yield and quality. When considering the variation between reactive and quiescent ADEVs, the average R^2 value was 0.708. This variation may in part be due to biological differences between reactive and quiescent ADEVs, however as demonstrated the ADEV samples were highly variable even between biological replicates of the same condition. To summarise, the most variable sample group were reactive ADEVs, while the least variable samples were quiescent astrocytes. The overall average R^2 value was 0.719, which is lower than the ideal value due to high levels of variation in ADEV samples.

Sequence length distribution of sncRNAs has previously been reported to be very different in EVs compared to whole cells, in a study of mouse cortical neurones (Mesquita-Ribeiro *et al*, 2021). The authors found that 22nt sequences had the highest abundance in neuronal cell bodies, whereas 30nt sequences were most abundant in EVs. This corresponded to miRNAs being the most abundant sncRNAs in neuronal cell bodies, while tRNA-derived small RNAs (tsRNAs) were the most abundant in EVs, sncRNAs which are formed by cleavage of tRNA that can also regulate gene expression (Mesquita-Ribeiro *et*

al., 2021; Zhang *et al.*, 2023). The results of Figure 4.4A-F reflect this, as in astrocyte cellular samples the most common read length was 22nt in all but one quiescent astrocyte sample. Whereas in ADEV samples, read length varied more between samples, but most were between 32nt and 36nt, as illustrated in Figure 4.4G-L.

This can be explained by the proportions of sncRNA types detected in different sample types, as shown in Table 4.2. MiRNAs were by far the most common sncRNAs in whole-cell astrocyte samples, especially quiescent astrocytes. The slightly lower percentage of miRNAs in reactive astrocytes (49.26% compared to 76.01% in quiescent astrocytes) is reflected in the greater proportion of intronic reads (23.17% in reactive astrocytes, 2.21% in quiescent astrocytes). In ADEVs, the largest number of reads mapped to intronic regions. Exon and intron regions detected are likely to be degraded fragments of mRNA. However, additional bioinformatic methods were not employed to identify tsRNAs in this dataset unlike in (Mesquita-Ribeiro *et al.*, 2021), therefore these may be the major ADEV sncRNA species, like in cortical neurone EVs. Typically to identify tsRNAs, sequences must be mapped to a tsRNA reference database, rather than the whole genome (Wang *et al.*, 2022), which was not completed for this data. Therefore, in the future further analysis of this sncRNAseq dataset could be performed to identify tsRNAs, which may have an overlooked role in astrocyte and ADEV-mediated gene regulation.

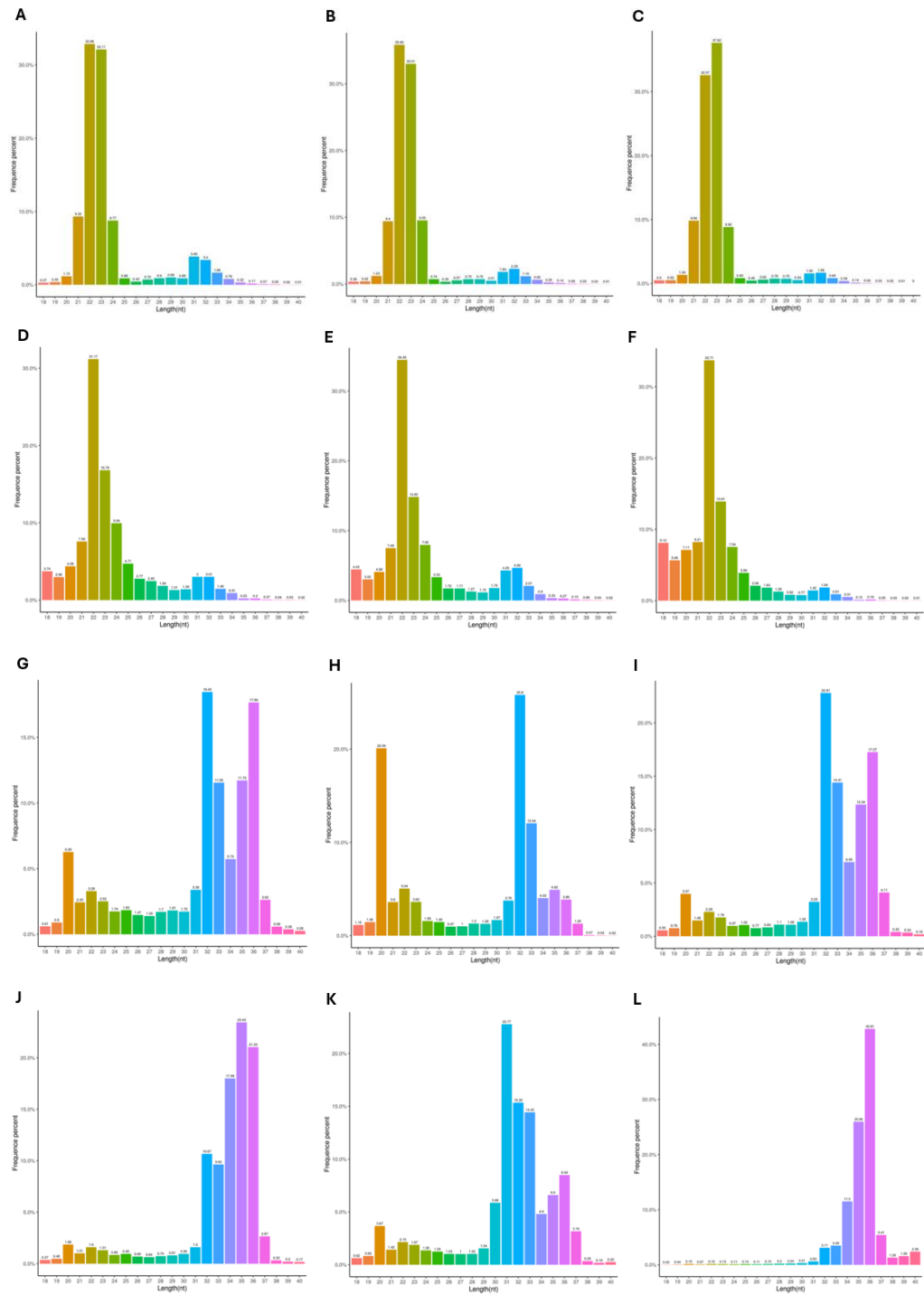


Figure 4.4. Sequence length distribution of snRNAseq samples. Distributions for each biological replicate are shown. A-C) Quiescent astrocytes, D-F) Reactive astrocytes, G-I) Quiescent ADEVs, J-L) Reactive ADEVs. Figures produced by Novogene.

Table 4.2. Average percentage distribution of sncRNA types across whole-cell astrocyte and purified ADEV samples.

	Quiescent astrocytes	Reactive astrocytes	Quiescent ADEVs	Reactive ADEVs
miRNA	76.01%	49.26%	3.47%	1.08%
rRNA	0.13%	3.59%	0.54%	0.30%
tRNA	0.07%	0.17%	0.01%	0.02%
snRNA	0.10%	0.43%	0.05%	0.02%
snoRNA	1.39%	1.86%	0.09%	0.01%
Repeat region	2.07%	3.54%	10.63%	14.45%
Exon	4.94%	5.97%	21.38%	5.13%
Intron	2.21%	23.17%	37.85%	58.07%
Other	13.07%	12.77%	25.98%	20.92%

Percentages are averages across triplicates out of the total number of reads that map to the genome. “Other” refers to reads that could not be mapped to any of the other RNA types. Highest percentages for each sample type in bold.

4.2.3 miRNA changes in human astrocytes upon FBS-induced reactivity

So far, a general description of the sncRNAseq data has been provided in this chapter. Since I aimed to understand how the astrocyte and corresponding ADEV-associated miRNA landscape changes upon reactivity, the focus now will be placed specifically on miRNA analysis. A total of 1649 miRNAs were detected across the 12 samples (whole-cell and purified ADEVs). 122 predicted “novel” miRNAs were also detected, however these have not been included in the following analysis as they cannot be confirmed as functional miRNAs and their target genes and biological functions are unknown. Figure 4.5 shows the number of the 1649 miRNAs detected in each sample type and the overlap between conditions. As expected, fewer miRNAs were detected in ADEVs than in astrocytes themselves, consistent with miRNA compartmentalisation.

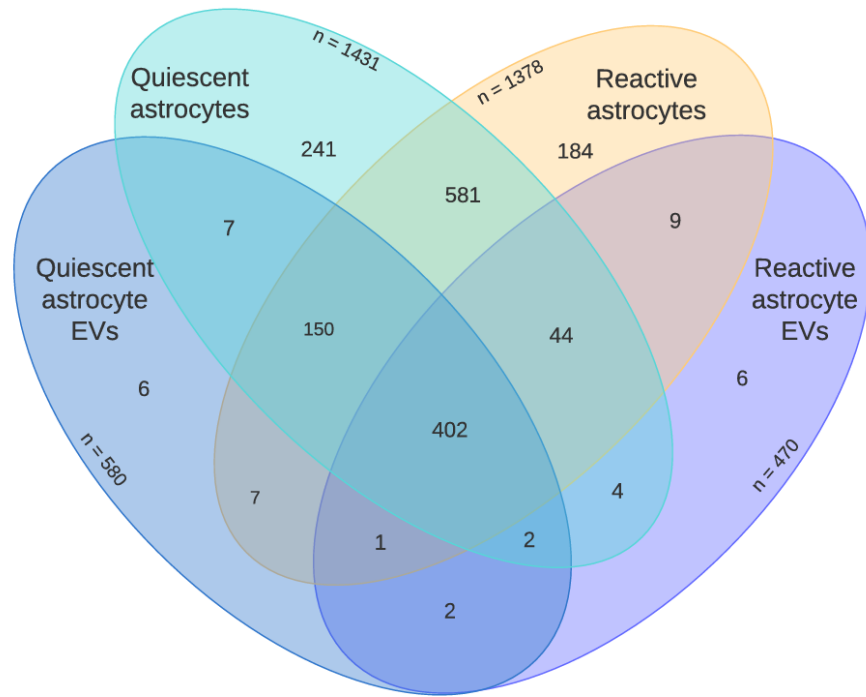


Figure 4.5. Total number of miRNAs detected by sncRNAseq of human reactive and quiescent astrocytes and their purified EVs. MiRNAs included were detected a minimum of once in 1/3 replicates of the respective condition. *N* = total number of miRNAs in the respective condition. Degree of overlap between conditions is illustrated by the numbers (of miRNA) in the Venn diagram. Figure produced using Lucidchart.

4.2.3.1 miRNA expression in ADEVs compared to their cells of origin

As mentioned, ADEVs were isolated from the same cells that were used for the whole-cell RNA, therefore the ADEV data can be directly compared against their cells of origin. As expected, the identity of miRNAs in EVs largely reflected their cells of origin, with an average of 96% of ADEV miRNAs being detected in the corresponding whole-cell sample, as illustrated in Figure 4.6.

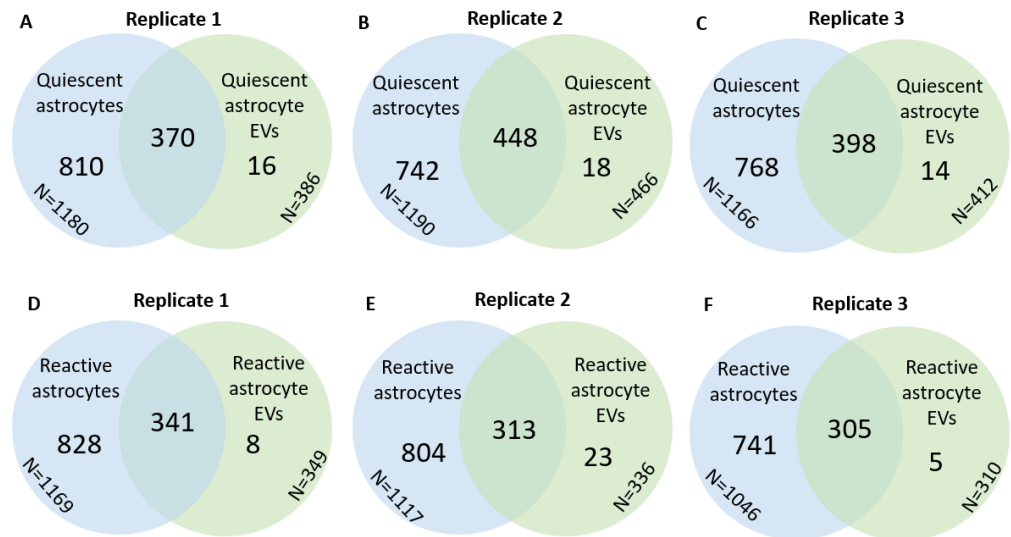


Figure 4.6. Overlap in detected miRNAs between astrocytes and their associated EVs. EVs were isolated from the conditioned medium of the associated cellular astrocyte sample. No data filtering was performed, so detection was defined as a minimum of 1 readcount in a single sample. A-C) Total numbers of miRNAs detected in three replicates of quiescent astrocyte whole-cell extracts and their corresponding EVs. D-F) Total numbers of miRNAs detected in three replicates of reactive astrocyte whole-cell extracts and their corresponding EVs.

Regarding miRNAs which were detected exclusively in an ADEV sample but not its associated cellular sample, there was limited consistency between replicates. The only miRNAs which were detected exclusively in ADEVs in at least two biological replicates were miR-320e, miR-516b-5p, miR-1298-3p and miR-4429 in quiescent ADEVs and miR-508-3p in reactive ADEVs. Most of these miRNAs had very low abundances (readcounts) in ADEVs so their detection in ADEVs but not whole-cells is likely due to chance. However, miR-516b-5p showed noticeably high abundance in ADEVs compared to cells, with average raw readcounts of 251 and 184.3 in quiescent and reactive ADEVs respectively, but only 17 and 1 in quiescent and reactive astrocytes, since it was not detected in 1/3 reactive and 2/3 quiescent astrocyte whole-cell samples. Therefore, upon differential expression analysis between cells and ADEVs, which will be discussed in section 4.4, miR-516b-5p was found to be significantly EV-enriched in both astrocyte types (Log2FC 2.15, padj $1.32 \cdot 10^{-17}$ in quiescent, Log2FC 8.69, padj $1.00 \cdot 10^{-6}$ in reactive). Overall, this may suggest that astrocytes preferentially package miR-516b-5p into EVs to exert a specific function. Little is reported about a CNS role of miR-516b-5p, therefore pathway analysis of its predicted targets (microT-CDS score >0.8) was performed using miRPath (DIANA tools) and PANTHER to elucidate the biological significance of its enrichment in ADEVs (Aleksander *et al*, 2023; Ashburner *et al*, 2000; Tastsoglou *et al.*, 2023). Unlike elsewhere in this thesis, TargetScan was not used for target prediction due to the very large number of gene targets (5175) predicted to be targeted by miR-516b-5p. However, there were no significantly enriched KEGG pathways using miRPath or GO terms using PANTHER, meaning it is difficult to predict the

biological impact of increased release of miR-516b-5p from astrocytes via ADEVs.

To identify miRNAs that were differentially expressed between reactive and quiescent astrocytes and ADEVs, the DESeq2 normalisation and analysis performed by Novogene was used. A significance threshold of $\text{padj} < 0.05$ and $\text{Log}_2\text{FC} > \pm 0.5$ was selected, as this was the same threshold as for the StatsPro proteomics analysis in Chapter 3. However, unlike StatsPro, DESeq2 calculates a fold change for all detected miRNAs, even if they are only detected in one sample, although detection in multiple samples (3/3 of one condition or 2/3 of both conditions) is required to calculate an adjusted p value. The DESeq2 method may therefore be advantageous over StatsPro, as miRNAs which are present in all replicates of one cell type and none of the other (which may be a biologically important finding) are not missed from the analysis. For example, let-7f-2-3p was not detected in any reactive astrocyte whole-cell samples, but detection in all three quiescent astrocyte samples gives a normalised quiescent readcount of 8.41. Using DESeq2, the Log_2FC was calculated to be -5.56, with an adjusted p value of < 0.05 . Therefore, these significant values demonstrate that this miRNA differs significantly in expression between reactive and quiescent astrocytes.

Figure 4.7 illustrates the differential expression of miRNAs between reactive and quiescent astrocytes. Interestingly, 555 miRNAs were differentially expressed out of the total 1632 miRNAs detected in either cell type. This very large percentage (34%) of differentially expressed miRNAs suggests that miRNAs may have an important role in astrocyte reactivity that should be investigated further. This percentage is slightly more than the number of differentially expressed proteins (proteomics batch 1 in Chapter 3) where 26% of proteins were differentially expressed, further indicating an important role of miRNAs. This difference between miRNA and protein does not appear to be due to reduced variance between sncRNAseq replicates. In fact, the average coefficient of variance (CV) values (calculated by dividing standard deviation by the average using raw values) of the sncRNAseq samples were 35% for quiescent astrocytes and 52% for reactive astrocytes, compared to only 9% and 12% respectively for the proteomic samples, suggesting greater variation between sncRNAseq samples.

Of course, some miRNA changes may be a consequence, rather than a cause, of reactivity. Furthermore, unlike the proteomic analysis where all Log_2FC values were less than ± 4 , there are many miRNAs with Log_2FC values from ± 5 to ± 10 . Many miRNA changes were also highly statistically significant, illustrated in the volcano plot of Figure 4.7 where the highest datapoint on the y axis (miR-542-3p) has a $-\text{Log}_{10}\text{padj}$ value of 51.7. In comparison, the most significant protein expression change in batch 1 of the astrocyte proteomics in Chapter 3 (DECR1) had a $-\text{Log}_{10}\text{padj}$ value of 4.52. These differences in the degree of differential expression could be due to differences between SWATH-MS and RNAseq, or DESeq2 and the limma test. It may also suggest that the proteome is more static than the miRNAome, or illustrate the importance of miRNA changes in regulating astrocyte reactivity.

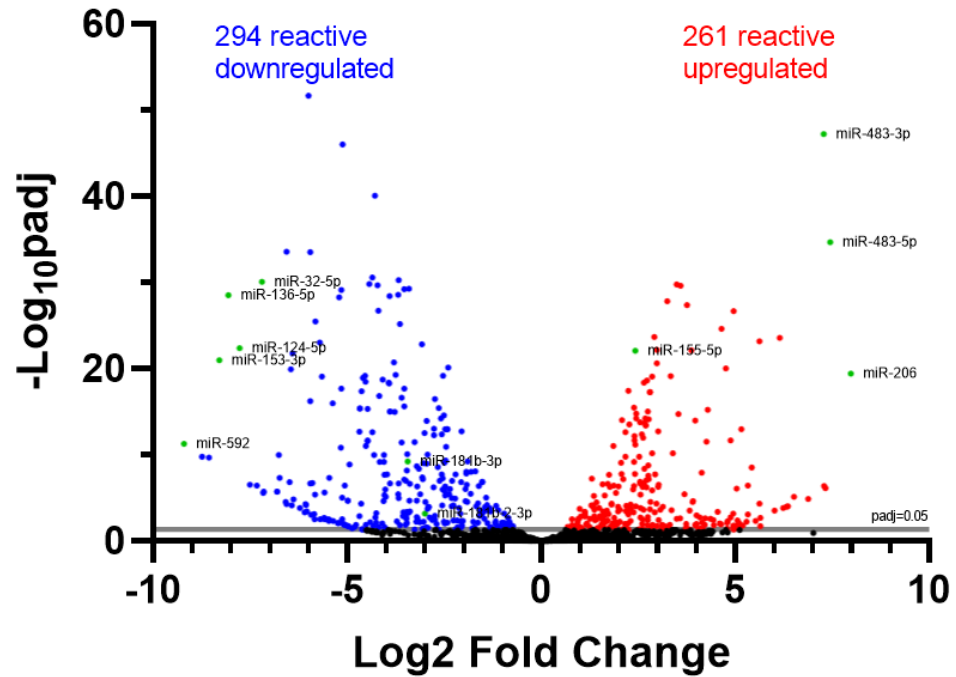


Figure 4.7. Volcano plot showing differential expression of miRNAs between human quiescent and reactive astrocytes (whole-cells). Differential expression analysis of sncRNAseq data was performed by Novogene using DESeq2. Colour change is at $\text{Log}_2\text{FC} > \pm 0.5$ (blue= downregulated in reactive astrocytes, red= upregulated in reactive astrocytes). Grey line is at $-\text{Log}_{10}\text{padj} = 1.301$ ($\text{padj} = 0.05$). Labeled miRNAs with green datapoints were investigated by literature search as potential astrocyte reactivity-regulating miRNAs.

Whilst little is known about miRNA-mediated regulation of astrocyte reactivity, some differentially expressed miRNAs indicated in Figure 4.7 have previously been linked to reactive or ALS astrocytes. For example, miR-181b has previously been described as a negative regulator of astrogliosis and was decreased in iAstrocytes derived from an sALS patient and an mSOD1-ALS patient (Gomes *et al.*, 2022; Hutchison *et al.*, 2013). My data reflects these findings, as both miR-181b-2-3p and miR-181b-3p were significantly downregulated in reactive astrocytes compared to quiescent ($\text{Log}_2\text{FC} -3.00$, $\text{padj} 0.00069$ and $\text{Log}_2\text{FC} -3.44$, $\text{padj} 6.29 \times 10^{-10}$, respectively).

MiR-155-5p, which is significantly upregulated in reactive astrocytes ($\text{Log}_2\text{FC} 2.43$, $\text{padj} 8.45 \times 10^{-23}$) has been linked extensively to inflammation, including in the context of reactive and ALS astrocytes (Hu *et al.*, 2022; Mahesh & Biswas, 2019). The pro-inflammatory stimuli IL-1 β , TNF α and pro-inflammatory macrophage conditioned medium have all been shown to upregulate miR-155-5p in human astrocytes (Junker *et al.*, 2009; Korotkov *et al.*, 2020; van Scheppingen *et al.*, 2016). MiR-155-5p is known to augment inflammation by reducing expression of the anti-inflammatory protein SOCS1 (Cardoso *et al.*, 2012). MiR-155-5p overexpression was shown to promote activation of rat astrocytes via targeting of Ndfip1, a neuroprotective ubiquitination-regulating protein, illustrated by increased proliferation and invasion. MiR-155-5p

overexpression also promoted glial scar formation in a rat model of spinal cord injury (He *et al*, 2023).

Regarding ALS astrocytes, miR-155-5p upregulation was reported in mature (13 days *in vitro*) spinal cord astrocytes from mSOD1 mice compared to controls, reflecting the upregulation reported in whole spinal cords from mSOD1 mice and ALS patients (Gomes *et al*, 2020; Koval *et al.*, 2013). The contribution of ADEV-associated miR-155-5p in ALS pathogenesis is debated, since Gomes *et al* demonstrated depletion of miR-155-5p in small EVs from mSOD1 mouse cortical and spinal cord astrocytes compared to controls, while Marton *et al* showed that EVs from mSOD1 astrocytes induce motor neurone death in part due to increased ADEV-miR-155-5p (Gomes *et al.*, 2020; Marton *et al.*, 2023).

The 10 miRNAs which were upregulated and downregulated to the greatest extent (in terms of Log2FC) upon astrocyte reactivity in our model were then manually researched in detail in the literature. These are likely candidates for miRNAs involved in astrocyte reactive remodelling, since the significant activation or repression of their expression may contribute to gene expression changes controlling reactivity.

4.2.3.2 Identification of the most upregulated miRNAs upon FBS-induced human astrocyte reactivity

Table 4.3 shows the top 10 most upregulated miRNAs upon FBS-induced astrocyte reactivity, in terms of Log2FC. All miRNA expression changes were highly statistically significant, with padj values all below 1×10^{-5} . Log2FC values were a minimum of 6.24, representing at least a 75x increase in DESeq2 normalised readcounts upon reactivity.

Table 4.3. The top 10 most upregulated miRNAs in human reactive astrocytes compared to quiescent astrocytes.

Top 10 most upregulated miRNAs (by Log2FC) upon astrocyte reactivity	Average quiescent astrocyte raw readcount	Average reactive astrocyte raw readcount	Log2FC	padj
miR-206	9.33	2290	7.98	1.46E-21
miR-483-5p	3.33	599	7.45	9.34E-38
miR-10526-3p	0.00	30.0	7.33	1.41E-07
miR-4728-5p	0.00	30.7	7.30	6.89E-08
miR-483-3p	12.7	1990	7.28	1.00E-50
miR-1193	0.00	21.0	6.88	2.70E-06
miR-4449	0.33	33.0	6.52	1.57E-06
miR-6858-5p	0.00	15.3	6.36	2.35E-05
miR-1260a	0.00	14.7	6.32	2.63E-05
miR-4706	0.00	15.0	6.24	3.87E-05

The order of miRNAs is in terms of Log2FC. Average raw readcounts are provided, with DESeq2 calculated Log2FC and padj values. Note that DESeq2 calculates fold changes even when the average of one condition is 0. MiRNAs in red were further investigated by literature search since they have average reactive astrocyte raw readcounts of >100. Values rounded to 3 s.f.

To predict which miRNA expression changes in Table 4.3 are most likely to have significant biological impact, abundance must be considered. The raw readcounts of all 10 miRNAs are extremely low in quiescent astrocytes, with 6 miRNAs not detected in even a single biological replicate. 7 of the miRNAs also showed relatively low expression in reactive astrocytes, with an average raw readcount of <100. In these cases, the reactive upregulation may therefore be due to chance and may not have a significant impact on gene regulation. This contrasts with the remaining three miRNAs, miR-206, miR-483-5p and miR-483-3p, which all undergo a dramatic upregulation upon reactivity, with average raw readcounts in reactive astrocytes of 2290, 599 and 1990, respectively. In terms of abundance in reactive astrocytes, these miRNAs rank 135th, 201st and 141st respectively out of a total of 1378 detected miRNAs.

Two miRNAs of note in Table 4.3 are miR-483-5p and miR-483-3p. Unlike many miRNAs, there is not a clear dominant miR-483 arm, with a similar number of reads of both arms across 101 experiments in miRbase (Kozomara *et al.*, 2018). This means that either the 5' or 3' end of pre-miR-483 may become the guide strand and result in a functional mature miRNA. The data in Table 4.3 suggests that miR-483-5p and miR-483-3p expression is likely correlated, which was previously shown in a study of adrenocortical carcinoma (Patterson *et al.*, 2011). While miR-483 has been extensively studied in contexts such as cancer, metabolic and cardiovascular diseases, there are limited reports of a neurological or neurodegenerative disease role (Asadi *et al.*, 2023).

The most notable report of an astrocytic role for miR-483 was published by Gordillo-Sampedro *et al*, where miR-483-5p was the most significantly EV-enriched miRNA in EVs released by human iPSC-derived astrocytes. Notably, miR-483-5p regulates the expression of methyl CpG-binding protein 2 (MeCP2) in neurones, an epigenetic regulator important for neurological function, which when mutated causes the neurodevelopmental disorder Rett syndrome (Gordillo-Sampedro *et al*, 2024). This suggests that the astrocytic EV-mediated release of miR-483-5p may be important for regulating neurological function. While miR-483 has not been clearly linked to ALS, elevated plasma miR-483-5p has been suggested as a possible Alzheimer's disease biomarker. Circulating miR-483-5p levels also correlated with age, perhaps indicating a role in age-related cellular processes or neurodegenerative diseases (Nagaraj *et al*, 2017; Sabry *et al*, 2020).

The most upregulated miRNA in reactive astrocytes compared to quiescent astrocytes was miR-206, as illustrated in Table 4.3 and Figure 4.7. MiR-206 has been described as a skeletal muscle specific miRNA, or “myomiR” (McCarthy, 2008). However, this may be a reductive description, with miR-206 readily detected in iPSC-derived astrocytes and the U251 human astrocytoma cell line (Akkouh *et al*, 2021; Duan *et al*, 2015). Gene ontology (GO) term analysis of miR-206 target genes (according to TargetScan) also supports a nervous system role for miR-206. Top 20 enriched pathways include ‘retrograde axonal protein transport’, ‘dorsal root ganglion morphogenesis’, ‘trigeminal nerve structural organisation’ and ‘neural crest cell fate commitment’ (data not shown).

MiR-206 has previously been linked to inflammation in U251 astrocytoma cells, a finding which may be predicted to translate to *bona fide* human astrocytes. Duan *et al* found that stimulation of U251 cells with LPS induced miR-206 expression. In these LPS-stimulated U251 cells, plasmid-mediated overexpression of miR-206 led to an increase in pro-inflammatory cytokines (IL-1 β , IL-6 and CCL5), while miR-206 knockdown had the opposite effect. MiR-206 directly targets the transcription factor *NR4A2/NURR1* (Nuclear receptor-related 1 protein), which contributes to the modulation of LPS-induced inflammation by activating downstream NF- κ B activity (Duan *et al*, 2015). NURR1 is also critical for dopaminergic neurone survival, with NURR1 dysfunction implicated in PD pathogenesis (Decressac *et al*, 2013).

MiR-206 upregulation has also been reported in several ALS patient biomarker studies, including in serum, skeletal muscle and plasma (de Andrade *et al*, 2016; Waller *et al*, 2017). MiR-206 may serve as a prognostic biomarker, as higher levels predicted a slower clinical decline over 12 months (de Andrade *et al*, 2016). I hypothesise that the increased miR-206 observed in ALS patients could in part be due to increased release of miR-206 from reactive astrocytes. MiR-206 is not differentially expressed between reactive ADEVs and quiescent ADEVs, but perhaps there is increased release of ‘free’ miR-206, with free miRNAs typically in complex with Argonaute proteins or high-density lipoproteins (Makarova *et al*, 2021).

This may be an example of ‘source-mapping’, where circulating miRNA biomarkers are mapped back to their cells of origin, in this case by correlating clinical and experimental cell culture data. A previous example of source-mapping includes the identification of neurone or microglia-derived miRNAs in the plasma of an epilepsy mouse model (Brindley *et al*, 2023). A wide range of cellular and tissue samples from ALS and healthy patients would be required for a comprehensive source-mapping study. However, in the future such studies may provide powerful insight into disease pathology. For example, if we understood which miRNAs are associated with processes such as astrocyte reactivity, motor neurone degeneration or muscle wasting, we may be able to decipher the order of these pathological events by monitoring these miRNAs in accessible body fluids throughout the disease course. Furthermore, source-mapping may aid precision medicine, for example, if a particular abundance of ‘reactive astrocyte’ miRNAs is detected in a patient’s blood, they may benefit more from an astrocyte-targeted therapy.

From my data alone, it is not possible to confirm that miR-206 upregulation in ALS patient blood and spinal cord is due in part to reactive astrocytes. Patil *et al* reported that only 0.89% of plasma miRNAs were known to be astrocyte-enriched, therefore CSF may be a more significant source of astrocyte-derived miRNAs (Patil *et al*, 2022). Furthermore, since miR-206 has been described as a myomiR, it has been hypothesised that miR-206 is increased in ALS patients as it is released from skeletal muscle when it degenerates (de Andrade *et al*, 2016). While this likely accounts for much of the increased miR-206 in ALS patients, given the amount of skeletal muscle in the body, it does not rule out a small contribution of reactive astrocytes.

To summarise, miR-206 is the most upregulated miRNA upon human astrocyte FBS-induced reactivity in this sncRNAseq analysis, and it has previously been associated with a pro-inflammatory response in astrocyte-like cells. MiR-206 upregulation has been reported widely in ALS patients, which may be predicted to be in part due to increased release from reactive astrocytes, in addition to release from degenerating muscle. I therefore decided to experimentally investigate whether miR-206 plays a role in human astrocyte reactivity, and specifically whether this involves regulation of its target gene *NURR1*. This work, including a more thorough discussion of miR-206 and *NURR1*, will be presented later in section 4.3.

4.2.3.3 Identification of the most downregulated miRNAs upon FBS-induced human astrocyte reactivity

To identify miRNAs which may be repressed during FBS-induced astrocyte reactivity, the top 10 most downregulated miRNAs upon reactivity were examined, illustrated in Table 4.4. As with the upregulated miRNAs, all 10 miRNAs had extremely low or no expression in reactive astrocytes, suggesting near complete repression upon astrocyte reactivity. To identify miRNAs for which their downregulation is likely to have the greatest biological significance, those with average quiescent astrocyte readcounts of >100 were selected. These miRNAs were miR-592, miR-153-3p, miR-136-5p, miR-124-5p and miR-

32-5p, which were then explored for possible links to astrocyte reactivity. There is little relevant research on miR-124-5p, as it is the clear passenger or non-dominant arm according to miRbase (Kozomara *et al.*, 2018).

Table 4.4. The top 10 most downregulated miRNAs in human reactive astrocytes compared to quiescent astrocytes.

Top 10 most downregulated miRNAs (Log2FC) upon astrocyte reactivity	Average quiescent astrocyte raw readcount	Average reactive astrocyte raw readcount	Log2FC	padj
miR-592	104	0.00	-9.21	5.32E-13
miR-4705	75.3	0.00	-8.73	1.92E-11
miR-7978	67.0	0.00	-8.56	2.53E-11
miR-153-3p	387	1.33	-8.30	3.64E-23
miR-136-5p	506	2.00	-8.06	4.80E-31
miR-124-5p	394	2.00	-7.77	1.16E-24
miR-219a-5p	32.0	0.00	-7.50	4.67E-08
miR-301a-3p	28.7	0.00	-7.33	6.55E-08
miR-32-5p	706	5.33	-7.19	6.88E-33
miR-1252-5p	25.33	0	-7.16	4.91E-07

Order is in terms of Log2FC. Average raw readcounts are provided, with DESeq2 calculated Log2FC and padj values. Note that DESeq2 calculates fold changes even when the average of one condition is 0. MiRNAs in blue were investigated by literature search since they have average quiescent astrocyte raw readcounts of >100. Values rounded to 3 s.f.

Interestingly, miR-592 was shown to inhibit the Nrf2 pathway in astrocytes isolated from AD rats, via targeting of the *KIAA0319* gene (encoding the KIAA0319 protein which has no clear links to oxidative stress) (Wu *et al.*, 2020). This contrasts the finding that the Nrf2 pathway is reduced in these reactive astrocytes compared to quiescent. MiR-592 is unlikely to be the cause of the Nrf2 pathway phenotype in our astrocyte model, as if so, it would logically be predicted to be upregulated upon astrocyte reactivity.

MiR-153-3p was previously shown to protect astrocytes, microglia and neurones from LPS-induced neuroinflammation, via targeting of Neurogenic differentiation 1 (*NeuroD1*). For example, in LPS-induced microglia miR-153-3p suppressed pro-inflammatory cytokines, ROS and multiple factors involved in the innate immune response. While in LPS-induced astrocytes there was repression of the STAT3 pathway, an important regulator of astrocyte reactivity (Choi *et al.*, 2022; Herrmann *et al.*, 2008). The data in Table 4.4 supports these findings, as miR-153-3p may be protecting quiescent astrocytes from neuroinflammatory changes. A strong downregulation of miR-153-3p may therefore promote inflammatory signalling, contributing to the reactivity state of the FBS-induced astrocytes.

Expression of miR-136-5p in mouse astrocytes was shown to be downregulated by stimulation with IL-17, a pro-inflammatory cytokine released by Th17 (T helper) cells which has previously been shown to initiate astrogliosis (Peng *et al*, 2017; You *et al*, 2017). This supports the finding in Table 4.4, that astrocyte reactivity in our model is associated with decreased miR-136-5p expression, and suggests that the effects observed by Peng *et al* are not specific to IL-17 stimulation, but perhaps a broader range of pro-inflammatory stimuli such as FBS.

MiR-32-5p has been shown to promote neuroinflammation, for example by inducing production of pro-inflammatory cytokines in LPS-treated rat spinal microglia (Yan *et al*, 2018). Importantly, miR-32-5p knockout in mice inhibited LPS-induced astrocyte activation, quantified by the number of GFAP-positive cells, suggesting that miR-32-5p promotes astrocyte reactivity. LPS also increased miR-32-5p expression in mouse prefrontal cortex and hippocampus, suggesting miR-32-5p may be activated by pro-inflammatory stimuli to augment the pro-inflammatory response further (Zhong *et al*, 2020). These studies contrast the data in Table 4.4 where miR-32-5p is *downregulated* upon FBS-induced human astrocyte reactivity, perhaps due to different pro-inflammatory stimuli. Furthermore, although miR-32-5p is a conserved miRNA, species differences may still exist between gene targets and therefore effects on biological processes.

In conclusion, I have identified several miRNAs which may have a functional role in regulation of astrocyte reactivity and which show often rational expression changes in our FBS-induced reactive astrocyte model. Considering the published literature in combination with this sncRNAseq data, strong candidates for further investigation include miR-206 as a promoter of astrocyte reactivity, while miR-153-3p and miR-136-5p may protect quiescent astrocytes against reactive remodelling. More experimental work would be required to validate these findings, which are presented in section 4.3 for miR-206.

4.2.3.4 RT-qPCR validation of miRNA expression changes upon FBS-induced human astrocyte reactivity

RT-qPCR was attempted to validate selected results of the sncRNAseq analysis. Whilst validation of the candidate miRNAs chosen for functional inhibition will be presented in section 4.6, an initial experiment aimed to validate a selection of miRNAs detected by sncRNAseq using RT-qPCR. Let-7i-5p and miR-151a-3p were selected as their expression does not significantly differ between reactive and quiescent astrocytes in the sncRNAseq, therefore they were predicted to be suitable housekeepers for normalisation. MiR-99a-5p was selected as it is significantly downregulated upon astrocyte reactivity (whole-cell), while miR-134-5p is upregulated. After sncRNAseq, there was only sufficient RNA from 4/6 samples remaining for RT-qPCR. Therefore as well as these samples (2 quiescent, 2 reactive), an additional new replicate of each condition was included. As noted earlier, the FBS-induced reactive astrocyte samples used for sncRNAseq were grown without serum for 3 days to collect ADEVs, unlike the new reactive sample, although this had minimal impact on RT-qPCR results. RT-

qPCR validation was not attempted using ADEV samples, since all RNA had been consumed for sncRNAseq and obtaining sufficient RNA from six new samples would be a considerable technical challenge.

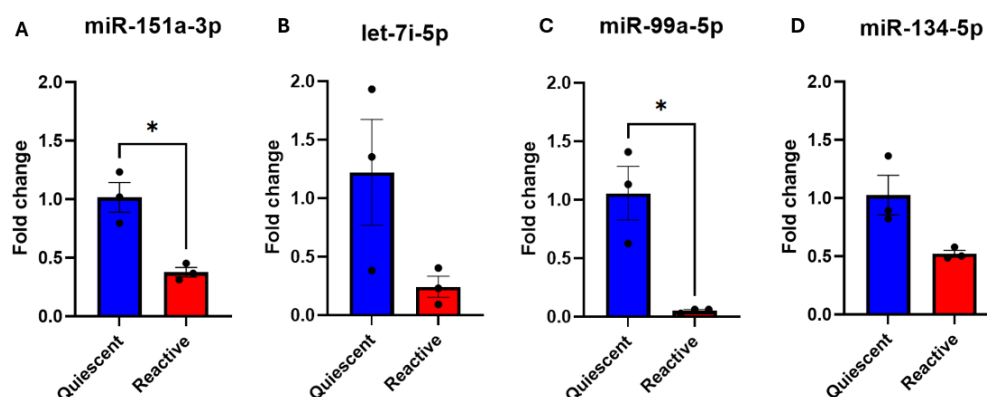


Figure 4.8. RT-qPCR validation of miRNA expression changes between quiescent and reactive human astrocytes. No housekeepers were used as all Ct values were >1. For each miRNA, all Ct values were normalised to the average of the three quiescent astrocyte replicates. Welch's t-test, n=3.

Firstly, despite a trend towards downregulation upon reactivity, Figure 4.8B shows that let-7i-5p expression does not differ significantly between reactive and quiescent astrocytes when measured by RT-qPCR, validating the results of the sncRNAseq. However, the maximum difference in Ct values between samples was >1, so let-7i-5p was not used as a housekeeper as it did not fulfil the stringent criteria preferable for miRNA RT-qPCR. Furthermore, this data should be interpreted with caution, as non-significance is likely due to variation between the quiescent astrocyte samples (although no outliers were detected by ROUT test). MiR-134-5p was not differentially expressed, although there was a trend towards reduced expression in reactive astrocytes, contrasting the results of the sncRNAseq. Expression of miR-151a-3p and miR-99a-5p was significantly reduced in reactive astrocytes compared to quiescent astrocytes when measured by RT-qPCR, while only miR-99a-5p was significantly downregulated in the sncRNAseq data. This may in part be because the RT-qPCR data was not normalised using housekeepers. Despite this, the observation that of the four miRNAs, miR-99a-5p was most downregulated upon reactivity of the four miRNAs broadly supports the sncRNAseq data, although this data should be interpreted with caution.

Differences in data obtained by RNAseq and RT-qPCR are not uncommon. A study by Everaert *et al* found that for an RNAseq analysis of protein coding genes, typically 15-20% will be non-concordant with RT-qPCR validation (Coenye, 2021; Everaert *et al*, 2017). Furthermore, the necessity of RT-qPCR for validating RNAseq is often debated due to the reliability of modern RNAseq methods. RT-qPCR validation is likely a relic from early microarray studies, which were more likely to have issues with reproducibility and bias (Coenye, 2021).

4.2.4 MiRNA changes in human astrocyte-derived EVs upon FBS-induced reactivity

So far, miRNA expression analysis of the astrocyte whole-cell samples has been presented. Unlike the large number of miRNAs that were differentially expressed between reactive and quiescent astrocytes, there were only 8 differentially expressed miRNAs between reactive and quiescent ADEVs identified by sncRNAseq analysis (using DESeq2), as illustrated in Figure 4.9. These 8 miRNAs are from a total of 643 miRNAs detected in at least a single EV sample (1.2%), which is a much lower degree of differential expression than the 34% between cellular samples. This may in part be due to higher levels of variation between EV samples, as previously shown in Figure 4.3, which may have prevented some miRNAs reaching statistical significance. It may also mean that FBS-induced reactivity does not have a major impact on which miRNAs are loaded into ADEVs, perhaps suggesting that the previously reported effects of astrocyte reactivity on neurones may not be caused by miRNA cargo changes in ADEVs. Another way to approach this hypothesis is to examine for a given astrocyte type (quiescent or reactive) which miRNAs are EV-enriched or cell-enriched, and whether this changes between quiescent and reactive astrocytes. This approach will be presented in section 4.4 (Figure 4.11).

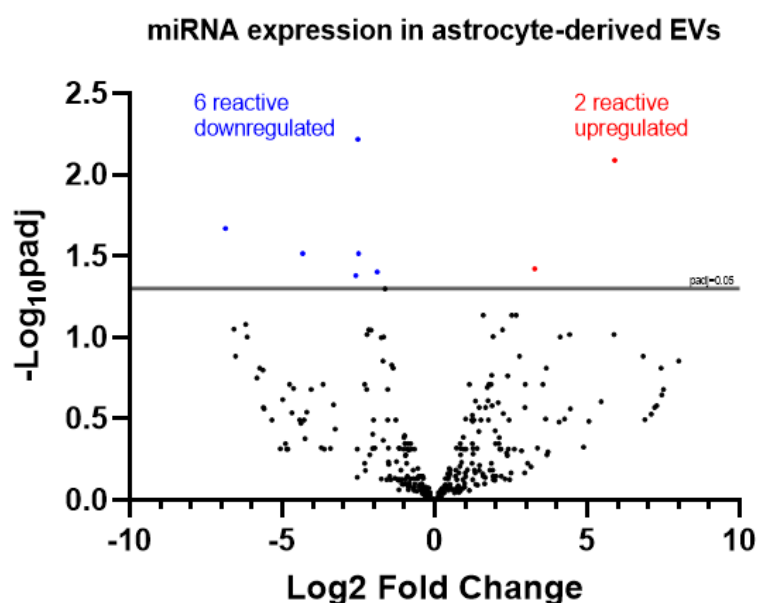


Figure 4.9. Volcano plot showing differential expression of miRNAs between human quiescent and reactive ADEVs. Differential expression analysis performed by Novogene using DESeq2. Colour change is at Log2FC >±0.5 (blue = downregulated in reactive astrocytes, red = upregulated in reactive astrocytes). Grey line is at -Log₁₀padj = 1.301 (padj = 0.05).

The most upregulated miRNA in reactive ADEVs (relative to quiescent ADEVs) was miR-378c, which interestingly was shown to be decreased upon Nrf2 ablation in the mouse heart (Quiles *et al*, 2017). While this is the opposite

direction as in our astrocyte model, where reduced Nrf2 in reactive astrocytes is associated with increased EV-associated miR-378c, it may still suggest a possible link between Nrf2 and miRNA changes upon astrocyte reactivity. The other reactive ADEV-upregulated miRNA, miR-184, has also been linked to Nrf2 signalling. In Neuro-2a cells, treatment with a miR-184 mimic decreased Nrf2 expression, while a miR-184 inhibitor increased Nrf2 expression (Zhang *et al*, 2019b). As well as reactive ADEVs, miR-184 was also upregulated in reactive astrocytes at the whole-cell level (Log2FC 4.14, padj 1.16×10^{-8}). Therefore, Nrf2 may be hypothesised as a target of miR-184 in astrocytes, with increased miR-184 levels upon reactivity contributing to the decreased Nrf2 expression. This phenotype may be propagated between cells due to increased EV-loading of miR-184.

Regarding the miRNAs downregulated in reactive ADEVs compared to quiescent ADEVs, let-7c-5p has previously been shown to suppress neuroinflammation and prevent microglial activation in a mouse model of ischemic stroke (Ni *et al*, 2015). Downregulation of let-7c-5p in reactive ADEVs, and a trend towards downregulation in reactive astrocytes (Log2FC -0.59, padj 0.13), supports this anti-neuroinflammatory role of let-7c-5p. Another miRNA downregulated in reactive ADEVs was miR-320c, which was found to be downregulated in the serum of ALS patients, compared to healthy controls, AD and multiple sclerosis patients (Raheja *et al*, 2018). Different expression compared to these “mimic” diseases further supports the potential of miR-320c as an ALS biomarker. A hypothesis may be that downregulation of miR-320c in serum is in part due to less miR-320c being secreted in ADEVs upon reactive remodelling. Similarly, another miRNA downregulated in reactive ADEVs, let-7b-5p, was reported to be downregulated in the plasma of a family with TARDBP^{G376D}-ALS, compared to both healthy controls and asymptomatic carriers (Ruffo *et al*, 2023).

Despite the limited findings above, the fact that only 8 miRNAs were differentially expressed between reactive and quiescent ADEVs may suggest that reactivity does not have a significant impact on the miRNAs loaded into ADEVs. Since reactive and quiescent astrocytes have majorly different whole-cell miRNA profiles, one might still suspect an important role of miRNAs in astrocyte reactivity. As discussed in section 4.2.3.2, the whole-cell sncRNAseq analysis pointed towards miR-206 as a possible astrocyte reactivity-regulating miRNA, which was taken forward for experimental investigation in the following section.

4.3 Is miR-206 involved in regulation of human astrocyte reactivity?

MiR-206 was the most upregulated miRNA in FBS-induced human reactive astrocytes in terms of fold change, as illustrated in Figure 4.7. A Log2FC value of 7.98 means that miR-206 is ~255x more abundant in reactive astrocytes than quiescent astrocytes, with average raw readcount values of 2294 and 9,

respectively. As discussed in section 4.2.3.2, miR-206 has previously been linked to inflammation in U251 astrocytoma cells, for example miR-206 overexpression increased inflammatory cytokine production (Duan *et al.*, 2015). MiR-206 upregulation has also been widely reported as upregulated in ALS patient tissues and biofluids, which I hypothesised to perhaps be in part due to release from reactive astrocytes.

In addition to a possible role in ALS and astrocyte reactivity, I hypothesised that miR-206 and Nrf2 may be linked (i.e. dampened Nrf2 signalling is linked to increased miR-206 in reactive astrocytes). Nrf2 has previously been shown to downregulate miR-206 indirectly via HDAC4 in A549 lung cancer cells (Singh *et al.*, 2013). Therefore, I hypothesised that the 255x increase in miR-206 upon FBS-induced astrocyte reactivity may be at least in part due to the decrease in Nrf2 levels. Furthermore, since Duan *et al* showed that miR-206 targets *NURR1* in LPS-treated astrocytoma cells, I hypothesised that the increased miR-206 in reactive astrocytes may result in a decrease in *NURR1* expression, irrespective of whether the miR-206 increase is due to Nrf2.

NURR1, also known as *NR4A2*, is a nuclear receptor best known for its function as a transcription factor and in development and maintenance of midbrain dopaminergic neurones (García-Yagüe Á & Cuadrado, 2023). *NURR1* has been shown to be neuroprotective and anti-inflammatory in mouse astrocytes, repressing NF-κB-p65-mediated expression of inflammatory mediators such as *iNOS* and *TNFα* (Saijo *et al*, 2009). Astrocyte reactivity induced by methamphetamine in mice was associated with decreased *NURR1* expression, with astrocyte-specific overexpression of *NURR1* shown to decrease neuroinflammation and damage to the BBB (Huang *et al*, 2022). These studies led to the hypothesis that *NURR1* would also be downregulated in our model of reactive astrocytes. *NURR1* was not detected in the proteomic analyses presented in Chapter 3, therefore alternate methods were required to investigate this hypothesis. Overall, the pathway predicted to be occurring in reactive astrocytes is shown in Figure 4.10A, which may be a contributing mechanism to astrocyte-mediated neurotoxicity in ALS. It should be noted that even if the whole pathway is not connected, repressed Nrf2 may still lead to increased miR-206, and miR-206 may still cause *NURR1* repression.

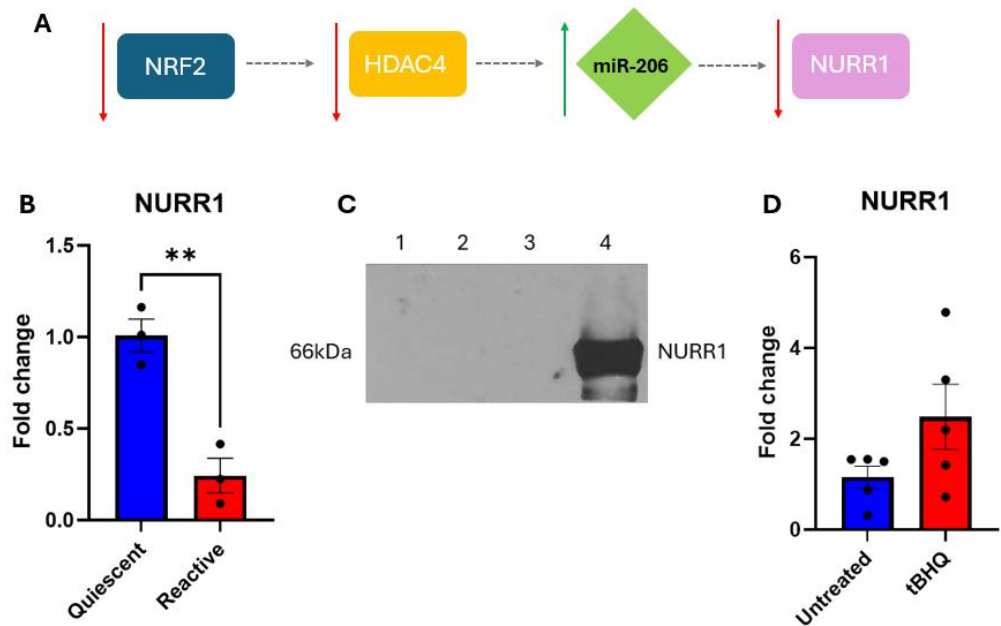


Figure 4.10. Investigating the possibility of a Nrf2/miR-206/NURR1 axis in human astrocytes. A) Schematic of the proposed hypothesis in reactive astrocytes. B) RT-qPCR of *NURR1* in quiescent and reactive human astrocytes ($n=3$). *B2M* and *18S* genes were used as housekeepers. Welch's *t*-test, $n=3$. C) Optimisation of human *NURR1* protein detection by western blot. 1- pre-macrophages ($30\mu\text{g}$), 2- iMacrophage ($30\mu\text{g}$), 3- reactive astrocytes treated with $10\mu\text{M}$ tBHQ for 24 hours ($10\mu\text{g}$), 4- HEK293T cells transfected with $1\mu\text{g}$ *NURR1*-encoding plasmid ($100\mu\text{g}$). Since relative quantification was not required, the maximum possible amount of protein was loaded for each sample. D) RT-qPCR of *NURR1* in tBHQ-treated reactive astrocytes. 5 replicates of reactive astrocytes were treated for 24 hours with $10\mu\text{M}$ tBHQ. *GAPDH*, *B2M* and *18S* genes were used as housekeepers and all data was normalised to the average control value for a given gene. Welch's *t*-test, $n=5$.

To investigate the proposed pathway in Figure 4.10A, firstly *NURR1* expression was measured by RT-qPCR in quiescent and reactive astrocytes. *NURR1* expression was predicted to be decreased upon FBS-induced reactivity, due to increased targeting by elevated miR-206. Figure 4.10B illustrates that *NURR1* expression is significantly reduced in reactive astrocytes compared to quiescent. This data supports the notion that reduced *NURR1* expression is a general feature of astrocyte reactivity, whether induced by FBS or other stimuli such as LPS or methamphetamine (Huang *et al.*, 2022; Saijo *et al.*, 2009). Therefore, it also may be predicted that *NURR1* expression is decreased in astrocytes of ALS patients, which would need to be validated in postmortem tissue. MiR-206 mimics or inhibitors would be required to investigate whether this is due increased miR-206, which will be discussed shortly.

In addition to a protective role against astrocyte reactivity and neuroinflammation, *NURR1* has been implicated in ALS pathogenesis, with upregulation detected in ALS patient peripheral blood (Valsecchi *et al.*, 2020). Valsecchi *et al.* hypothesised that *NURR1* is activated during early ALS as a

protective anti-inflammatory mechanism, however this is not sufficient to prevent disease progression. This was based on observation that *NURR1* expression was upregulated in the spinal cord of mSOD1 mice in both the asymptomatic and early symptomatic phases, but not at late stage. The mechanism of NURR1-mediated repression of NF- κ B signalling identified by Saijo *et al* was also identified in the asymptomatic mSOD1 mouse spinal cord. Perhaps consequently, *iNos* levels were downregulated in the asymptomatic stage compared to early symptomatic mice, followed by a strong upregulation in late-stage mice (Valsecchi *et al.*, 2020). Since the human reactive astrocytes used for Figure 4.10B were chronically reactive (following ~3-week culture in medium containing FBS) and the NURR1 phenotype observed matches late-stage ALS, perhaps our reactive astrocytes may be more appropriate for modelling late-stage ALS. An acutely reactive astrocyte model (e.g. short-term cytokine or FBS exposure) may be predicted to more accurately represent the phenotypes of early-stage ALS, including protective anti-inflammatory mechanisms such as *NURR1* upregulation, although this would require validation.

I sought to confirm whether the differential expression of *NURR1* mRNA in Figure 4.10B was also reflected at the protein level. NURR1 protein was not detected by the SWATH-MS analyses of Chapter 3, therefore a western blot was performed to measure NURR1 in 3 replicates of quiescent and reactive astrocytes (20 μ g protein). However, no NURR1 was detected even after a 20-minute exposure of the blot to film (data not shown). For antibody optimisation, samples expected to contain high levels of NURR1 protein were used for the western blot in Figure 4.10C. According to the human protein atlas, macrophages have relatively high expression of NURR1 (Karlsson *et al.*, 2021; Solís-Barbosa *et al*, 2024). Therefore, the first two samples in Figure 4.10C are whole-cell lysates from pre-macrophages and iPSC-derived macrophages (iMacrophages), which were a gift from Dr Daniel Scott [University of Nottingham]. Macrophage precursors (pre-macrophages) are an intermediate cell type formed after the differentiation of iPSCs to monocytes, which are further differentiated to macrophages (Klepikova *et al*, 2022). Since increased Nrf2 was predicted to result in increased NURR1 protein via the proposed pathway in Figure 4.10A, the third sample was from reactive astrocytes treated with the Nrf2 activator tBHQ. To produce the fourth sample for Figure 4.10C, HEK293T cells were transfected for 24 hours with a NURR1-encoding pcDNA3.1 plasmid (a gift from Nikki de Vivo, University of Nottingham) to induce exogenous NURR1 overexpression.

Firstly, Figure 4.10C shows that the NURR1 antibody can detect NURR1, as there was very strong detection in HEK293T cells transfected with NURR1 plasmid, even at short exposure times (<1 minute). However, endogenous NURR1 protein was not detected in any other sample after a 20-minute exposure. Although three to five times more protein was loaded of the HEK293T sample, the extreme difference in detection is unlikely to be due to this. Overall, this suggests that detection of endogenous NURR1 by western blot is not practical, likely due to low endogenous expression of this

transcription factor. It therefore cannot be confirmed that the reduction in *NURR1* mRNA upon astrocyte reactivity is reflected at the protein level, remaining with RT-qPCR for subsequent experiments.

To investigate whether Nrf2 affects *NURR1* expression, whether directly or indirectly such as via the pathway depicted in Figure 4.10A, reactive astrocytes were treated with the commercial Nrf2 activator tBHQ (10 μ M, as in Figure 3.8) for 24 hours before RT-qPCR. While *NURR1* expression increased upon tBHQ treatment in 3/5 replicates this was not statistically significant, as shown in Figure 4.10D. Overall it therefore appears unlikely that the hypothesis depicted in Figure 4.10A is valid, although sections of the pathway may occur in isolation. As mentioned, an example of this may be that miR-206 targeting contributes to the *NURR1* downregulation in reactive astrocytes. I sought to investigate this by inhibiting miR-206, with the additional aims of investigating the role of miR-206 in astrocyte reactivity, and to assess viability of miR-206 inhibition as a therapeutic strategy.

Before this, I sought to validate the dramatic increase in miR-206 upon astrocyte reactivity shown in Table 4.3 by RT-qPCR. Unfortunately, RT-qPCR was unsuccessful at reliably detecting any miR-206 expression in astrocytes. In initial experiments, miR-206 was detected sporadically across astrocyte samples and even no template control (NTC) samples, often with detection in only 1 of 2 technical replicates and no clear pattern between reactive and quiescent samples. In samples where miR-206 was detected, the levels were relatively low (Ct >35). To attempt optimisation, increased concentrations of cDNA were added to the RT-qPCR reaction. However, again miR-206 detection was inconsistent, with no clear correlation between cDNA concentration and miR-206 Ct value.

Since this RT-qPCR assay uses pre-designed and optimised primers supplied by Qiagen, with only one possible design for each miRNA, this was not a possible avenue for optimisation. Using a new batch of the same miR-206 primers appeared to resolve the issues of NTC detection, although miR-206 was not detected in either reactive or quiescent astrocyte sample tested (n=1). In the same assay, the human myoblast KM1421 cell line, a gift from Dr Sophie Joannis [University of Nottingham], served as a positive control for miR-206 detection. As expected, miR-206 expression was high in these muscle cells (n=1), with an average Ct value of 25.5 and clean amplification and melt curves, illustrating that measurement of miR-206 can reliably be measured by this method (optimisation data not shown).

Therefore, unfortunately it was not possible to confirm the upregulation of miR-206 in FBS-induced reactive astrocytes by RT-qPCR. Further experiments or perhaps interrogation of raw sncRNAseq data would be required to elucidate whether the RT-qPCR or sncRNAseq data is more reliable, although as discussed, today RNAseq is sufficiently reliable that RT-qPCR validation may not be warranted (Coenye, 2021). Since miR-206 levels could not be confirmed to be sufficiently high in reactive astrocytes for therapeutic manipulation, it could not be determined whether miR-206 targeting of *NURR1* occurred in reactive

astrocytes, and miR-206 was not selected as a candidate for further study in Chapter 5.

4.4 Motif analysis predicts a loss of ALS-associated RNA-binding protein-mediated miRNA sorting upon FBS-induced astrocyte reactivity

As discussed in section 4.1.1, Gordillo-Sampedro *et al* recently demonstrated that EVs purified from human primary and iPSC-derived astrocytes contain miRNAs that appear to have been selectively loaded into ADEVs, including by the ALS-associated RBP FUS. Here, I sought to expand this analysis, including determining if other ALS-associated RBPs may play a role in sorting astrocyte miRNAs into ADEVs, and whether FBS-induced astrocyte reactivity affects these processes. This will further understanding of how ADEV miRNAs are likely to be altered in ALS, including certain genetic forms, and the resultant effects on astrocyte-neurone communication. It should be noted that motif analysis is a bioinformatic process, therefore experimental work would in the future be required to validate these findings.

Motif analysis of ADEV-enriched and whole-cell-enriched miRNAs (Log2FC $>\pm 0.05$, padj <0.05 between cells and ADEVs) was performed using the sncRNAseq data for both quiescent and reactive astrocytes, separately. A schematic illustration of these comparisons between whole-cells and ADEVs is shown in green in Figure 4.11, while the comparisons in black were explored in section 4.2, to investigate the effects of FBS-induced astrocyte reactivity on miRNA expression. Regarding the comparisons (in green) between whole-cell and corresponding ADEV miRNA expression, some miRNAs are likely to be simply a sample of the intracellular *milieu*, resulting in similar expression between whole cells and ADEVs. In contrast, ADEV-enriched miRNAs may have been rationally loaded into EVs to mediate cell-cell communication. Whole cell-enriched miRNAs may have been retained in the cell to regulate homeostatic cellular functions, with any loading into EVs likely passive. In summary, exploring whole-cell or ADEV-enriched miRNA motifs can facilitate predictions of how RBPs may have mediated intentional miRNA sorting.

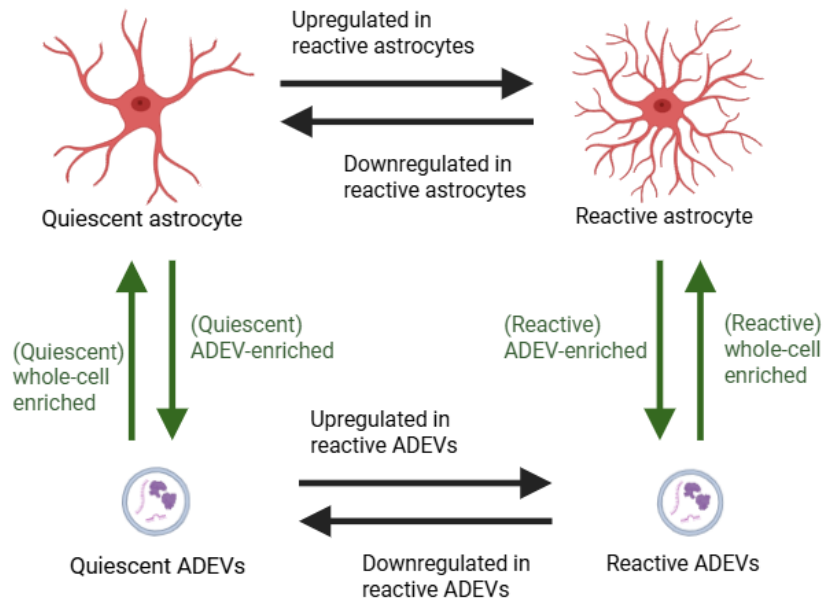


Figure 4.11. Schematic representation of different comparisons of miRNA expression in human quiescent and reactive astrocytes and ADEVs. The direction of an arrowhead indicates the sample with higher expression of a miRNA. For all comparisons, differential expression was defined as $\text{Log}_2\text{FC} > \pm 0.05$, $\text{padj} < 0.05$. Comparisons in green were used for motif analysis. Comparisons in black were presented in previous sections. Created using BioRender.

The CISBP-RNA database was used for motif analysis, which involved identification of human RBPs predicted to bind to given miRNAs via sequence motifs within the miRNAs (Ray *et al.*, 2013). For each miRNA in each dataset (e.g. quiescent ADEV-enriched miRNAs), CISBP produced a list of RBPs known to bind to motif sequences in the miRNA. Duplicate RBPs were removed for each miRNA, as certain miRNAs contain multiple motifs for the same RBP. The numbers of ADEV and whole-cell enriched miRNAs ($\text{Log}_2\text{FC} > \pm 0.05$, $\text{padj} < 0.05$) are represented in Table 4.5, along with the total number of RBPs with binding motifs corresponding to at least one of the miRNAs.

Table 4.5. Numbers of ADEV and whole cell-enriched miRNAs in human quiescent and reactive astrocytes and the numbers of RBPs predicted to bind.

	Enriched in astrocytes (whole-cells) vs ADEVs - <i>retained</i>		Enriched in ADEVs vs astrocytes (whole-cells) - <i>secreted</i>	
	Quiescent	Reactive	Quiescent	Reactive
Number of miRNAs	205	92	148	94
Number of RBPs known to bind to ≥ 1 motif sequence in ≥ 1 miRNA	252	220	255	248

ADEV-enriched miRNAs had significantly higher expression in ADEVs than whole-cells ($\text{Log}_2\text{FC} > 0.05$, $\text{padj} < 0.05$), and vice versa for whole-cell enriched miRNAs. Motif analysis was performed using the CISBP-RNA database.

Based on total miRNA numbers in Table 4.5, reactive astrocytes had less miRNAs enriched in either their ADEVs (relative to whole-cells) or whole-cells (relative to ADEVs), suggesting less overall miRNA sorting activity compared to in quiescent astrocytes. Total numbers of detected miRNAs were relatively similar between reactive and quiescent astrocytes, and the same for ADEVs, as shown in Figure 4.5 (section 4.2.3), supporting that the difference is due to miRNA sorting. Selective ADEV loading or cellular retention of miRNAs may be more significant in quiescent astrocytes, whereas there may be more passive loading of miRNAs from reactive astrocytes. A hypothesis may be that miRNA sorting and selective miRNA loading to ADEVs is another example of an important astrocyte function that is lost upon reactivity and neurotoxicity.

The RBPs predicted to bind to miRNAs were relatively similar between the 4 conditions in Table 4.5. 206 RBPs were predicted to bind to miRNAs enriched in all 4 conditions, whereas only 55 RBPs were predicted to bind to miRNAs enriched in 3 or fewer conditions. When RBPs were ranked in terms of the numbers of miRNAs they were predicted to bind to in each condition, the following RBPs were in the top 10 of all 4 conditions: MBNL3, MBNL2, MBLN1, SRSF9, SRSF1, SRSF2, SRSF8, RBM4B and RBM4. These RBPs are therefore top candidates for further investigation as mediators of miRNA sorting in astrocytes, regardless of reactivity status.

The lists of RBPs with ADEV and whole-cell enriched miRNA motifs were then cross-referenced with proteins in the ALSod database. A total of 9 ALSod proteins were identified, as shown in Table 4.6, including those encoded by the definitive ALS genes *FUS*, *HNRNPA1* and *TARDBP*. Notably, binding motifs for these 9 proteins were represented in the lists of quiescent ADEV and whole-cell-enriched miRNAs, reactive ADEV-enriched miRNAs, but none in reactive whole-cell-enriched miRNAs. Based on the number of miRNAs they were predicted to bind, RBMS1 ranked among the top ten RBPs targeting quiescent whole-cell-enriched miRNAs, while FUS and TAF15 were among the top ten

RBPs predicted to target quiescent ADEV-enriched miRNAs. For all other ALS-RBPs, they ranked in the bottom 55%, suggesting they may not be the major regulators of miRNA sorting in astrocytes.

Table 4.6. Numbers of reactive or quiescent astrocyte (whole-cell) or ADEV-enriched miRNAs containing motifs for an ALS-associated RBP.

ALS-associated RBP	Number of miRNAs containing an associated RBP motif				Percentage of RBP motif-containing miRNAs out of the total number of whole-cell or ADEV-enriched miRNAs			
	Whole cell-enriched		ADEV-enriched		Whole cell-enriched		ADEV-enriched	
	Qui.	Rea.	Qui.	Rea.	Qui.	Rea.	Qui.	Rea.
RBMS1	44	0	13	12	21.5	0.0	8.8	12.8
FUS	19	0	44	11	9.3	0.0	29.7	11.7
TIA1	14	0	5	11	6.8	0.0	3.4	11.7
TAF15	18	0	42	10	8.8	0.0	28.4	10.6
HNRNPA2B1	17	0	21	8	8.3	0.0	14.2	8.5
HNRNPA1	17	0	21	8	8.3	0.0	14.2	8.5
TARDBP (TDP-43)	9	0	21	6	4.4	0.0	14.2	6.4
EWSR1	5	0	20	1	2.4	0.0	13.5	1.1
MATR3	5	0	2	1	2.4	0.0	1.4	1.1

Values are also given as a percentage of the total number of miRNAs in the respective category. For example, 29.7% of miRNAs enriched in quiescent ADEVs (relative to quiescent astrocyte whole-cell) contain FUS-binding motifs, compared to only 11.7% of miRNAs enriched in reactive ADEVs. RBPs are from the ALSod database. Qui. = quiescent, Rea. = reactive. Data for 'definitive' ALS-associated RBPs (ALSod) are in red.

The striking absence of reactive astrocyte-enriched miRNAs with binding motifs for ALS-associated RBPs in Table 4.6 (zeros in column 3) may at first suggest a lack of expression of these proteins in reactive astrocytes, however the proteomic data in Chapter 3 indicates that this is not the case. Except RBMS1, all proteins were detected in batch 1 of the proteomic analyses, with detection in all 3 replicates of both reactive and quiescent astrocytes. The only differentially expressed protein was TAF15, which was downregulated in reactive astrocytes compared to quiescent (Log2FC -0.54, padj 0.044). Excluding RBMS1 and TAF15, where abundance may be a confounding factor in the analysis, Table 4.6 therefore suggests that the other seven ALS-associated RBPs may be failing to bind to and retain miRNAs in reactive astrocytes, relative to quiescent astrocytes. This may implicate loss of control of miRNA sorting in astrocytes as a general ALS pathomechanism associated with astrocyte reactivity, which may have unknown consequences on gene expression. Furthermore, this dysregulation may be exacerbated in ALS patients with a mutation in (and dysfunction of) one of the RBPs. TDP-43 or

FUS pathology would also be predicted to impact miRNA binding activity, therefore this may be a general mechanism in most ALS patients.

In summary, FBS-induced astrocyte reactivity appears to disrupt RBP-mediated miRNA selection for EV loading or cellular retention. This includes 9 ALS-associated RBPs, most of which appear to be more important for miRNA EV loading than cellular retention, as more motif-containing miRNAs were generally found to be ADEV-enriched in Table 4.6. However, this miRNA sorting activity may be disrupted for several RBPs upon reactivity, perhaps illustrating a novel pathogenic effect of astrocyte reactivity. Perhaps alterations to miRNA regulation by RBPs upon astrocyte reactivity, mutation in an ALS-associated RBP or TDP-43/FUS pathology would therefore alter the ADEV miRNA profile. This may alter EV-mediated communication and gene expression in recipient cells, therefore possibly contributing to motor neurone degeneration, although more studies would be needed to confirm this. Furthermore, while this study is focused on astrocytes, similar mechanisms may be occurring in other disease-relevant cell types, which warrants further investigation.

In this section, proteomic data of Chapter 3 was used to provide explanations for certain results obtained by motif analysis of the sncRNAseq data. In the next section this approach will be taken further, to interrogate whether there is a connection between miRNA and protein expression changes in our astrocyte models at the whole-cell level. This includes establishing whether miRNA expression changes may drive the Nrf2 pathway downregulation in reactive astrocytes, which was identified by proteomic analysis in Chapter 3.

4.5 Integration of proteomic and sncRNAseq data: miRNA-mediated gene targeting is unlikely to cause FBS-induced Nrf2 downregulation

So far, both proteomic and sncRNAseq analyses of FBS-induced reactive and quiescent astrocytes have been presented. Unlike the sncRNAseq, proteomics was not completed for ADEVs, in part because a method had not yet been optimised to quantify the very low concentrations of protein in ADEVs. However, integrative analysis of whole-cell proteomic and sncRNAseq data can be performed to highlight possible miRNA-mediated protein expression changes upon astrocyte reactivity.

Ingenuity Pathway Analysis (IPA) was used to integrate astrocyte whole-cell sncRNAseq data with batch 1 of the proteomic analysis presented in Chapter 3. A Target Filter Analysis of the miRNA data was performed to identify the predicted gene targets of all the miRNAs differentially expressed between quiescent and reactive astrocytes. The proteomic data that had already been filtered using StatsPro was subjected to a core analysis in IPA to identify differentially expressed proteins. The same criteria were used for differential expression analysis as throughout this thesis ($\text{Log}_2\text{FC} > \pm 0.5$, $\text{padj} < 0.05$), resulting in 1002 differentially expressed proteins and 555 differentially

expressed miRNAs, as reported in Chapters 3 and 4, respectively. Three significant proteins were missed from analysis (SLC14A1, HLA-DRB3 and MICOS10) due to a subtle change in StatsPro criteria after IPA was used, which could not be retrospectively altered.

The miRNA and protein analyses were then overlaid, to identify pairings where both a miRNA and a gene product it is predicted to target were differentially expressed, the numbers of which are represented in Table 4.7. In total, 2186 miRNA-protein interactions corresponding to 457 miRNAs and 698 proteins (genes) were identified using IPA where the miRNA and protein expression changed in opposite directions upon reactivity, as expected for a regulatory miRNA. Some miRNAs were predicted to target multiple proteins, and some proteins were predicted to be targeted by multiple miRNAs. Thus, 70% of protein changes (698/1002) could potentially be explained by miRNA changes. However, there is the caveat that many miRNAs also interacted with gene products that changed in the same direction. Specifically, there were 1,819 total interactions, corresponding to 456 miRNAs and 701 predicted protein targets, again representing 70% of the total protein changes.

Almost an identical number of differentially expressed proteins were predicted to be targeted by miRNAs changing in the same direction as the protein upon reactivity, as those changing in the opposite direction. Therefore, it is not possible to suggest from this data alone whether alterations in miRNA targeting are a major reason for the altered proteomic profile of quiescent and reactive astrocytes. Accordingly, the overall rationale of therapeutic manipulation of Nrf2-targeting miRNAs was not discounted.

Table 4.7. Numbers of predicted interactions between differentially expressed miRNAs and proteins upon FBS-induced astrocyte reactivity.

Directionality of regulation upon reactivity	Total miRNA-protein predicted interactions	Number of miRNAs	Number of proteins
miRNA down, protein up	546	215	225
miRNA up, protein down	1640	241	473
miRNA down, protein down	974	244	444
miRNA up, protein up	845	212	257

IPA was used to integrate sncRNAseq and proteomic data of quiescent and reactive astrocytes. Differential expression was defined as $\text{padj} < 0.05$, $\text{Log}_2\text{FC} \geq \pm 0.5$. The total number of predicted interactions is larger than the sum of miRNAs and proteins for each category, as several miRNAs can target one protein and several proteins can be targeted by one miRNA. The data in red represents predicted interactions which may correspond to miRNA-mediated

*regulation of protein expression (miRNA and protein regulated in **opposite** directions).*

In summary, it was unclear how reliable the IPA method is for predicting miRNA-regulated protein changes. The fact that the same proportions of protein changes could be explained by miRNA changes in the same and opposite directions was surprising, as in any cell type miRNAs would be predicted to impact gene expression, to some extent. Therefore, to investigate connections directly between miRNA expression and Nrf2, instead a more manual candidate analysis was completed. The list of 163 miRNAs predicted to target Nrf2 by TargetScan was manually compared against the whole-cell sncRNAseq data. Only 2/163 Nrf2-targeting miRNAs were upregulated upon astrocyte reactivity, therefore it is unlikely that the dampened Nrf2 pathway is due to elevated miRNA-induced suppression of Nrf2.

Counter intuitively, 47/163 Nrf2-targeting miRNAs were *downregulated* upon astrocyte reactivity (data not shown). This included 7 conserved miRNAs out of the total 15 conserved miRNAs predicted to target Nrf2 (therefore with high confidence of their targeting activity). While only a hypothesis at this stage, perhaps this represents a compensatory mechanism whereby reactive astrocytes attempt to rescue Nrf2 levels by downregulating transcription of Nrf2-targeting miRNAs. Regardless, in the future more experimental work should be done to investigate the connection between miRNAs and Nrf2 in astrocytes. This would complement these bioinformatic studies and provide further insight as to whether this is an important pathway for the regulation of oxidative stress in astrocytes, with possible downstream consequences regarding astrocyte reactivity or ALS pathogenesis.

4.6 Selection of candidate Nrf2-targeting miRNAs for subsequent inhibition in astrocytes

A major aim of this thesis is to investigate whether inhibiting specific miRNAs in astrocytes may be a viable therapeutic approach in ALS. Chapter 3 presented the reduced expression of the neuroprotective transcription factor Nrf2 and many of its downstream antioxidant genes in FBS-induced reactive astrocytes compared to quiescent astrocytes, mirroring a cellular phenotype often observed in ALS. Meanwhile Chapter 4 presented the miRNA profile of our human astrocyte models, including how expression changes may be connected to astrocyte reactivity and loss of homeostatic mechanisms in ALS. Leading on from these findings, I then aimed to inhibit specific miRNAs with a goal of increasing Nrf2 expression in astrocytes, to work towards a possible neuroprotective strategy. Selection of appropriate candidate Nrf2-targeting miRNAs for these aims was therefore the first step. Two candidates were selected, the rationale for which will now be discussed.

4.6.1 Selection of miR-27b-3p as an Nrf2-targeting candidate miRNA

To identify the first suitable candidate miRNA, the list of 163 miRNAs predicted to target Nrf2 according to TargetScan was compared against the top 100 most abundant miRNAs in each of the four sample types profiled by sncRNAseq – reactive and quiescent astrocytes and their ADEVs. This was because for a miRNA inhibitor to have significant biological effect, I reasoned the targeted miRNA must be abundant in astrocytes, to be an important Nrf2 regulator. Whilst miRNA abundance in ADEVs is not necessary for the inhibitor to have significant effects, it may still be beneficial. Perhaps inhibiting a miRNA in astrocytes may result in reduced functional miRNA in the EVs, including when taken up by other astrocytes to propagate the effect of increased Nrf2 signalling. The Venn diagram in Figure 4.12 shows the miRNAs that are predicted to target Nrf2 and are among the top 100 most abundant miRNAs in the indicated astrocyte or ADEV condition, in terms of average raw readcount from the sncRNAseq analysis.

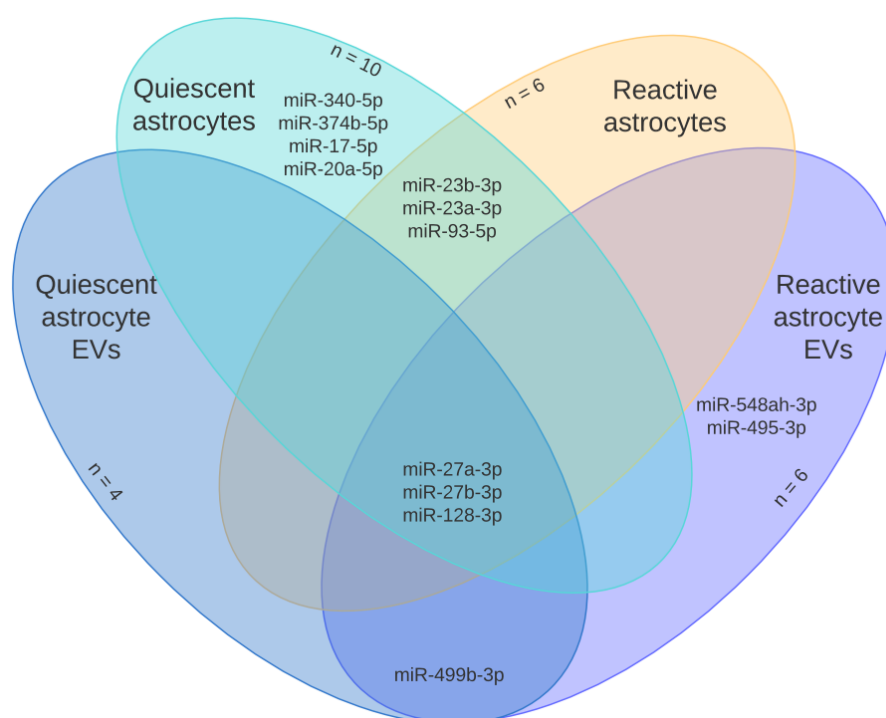


Figure 4.12. MiRNAs predicted to target Nrf2 that are abundant in human reactive or quiescent astrocytes or ADEVs. The list of miRNAs predicted to target Nrf2 was taken from TargetScan and compared against the top 100 most abundant miRNAs for each of the 4 sncRNAseq sample types – reactive and quiescent astrocytes and their ADEVs. N = total number of Nrf2-targeting miRNAs among the top 100 most abundant miRNAs for the indicated condition. Figure produced using Lucidchart.

From Figure 4.12, three candidate miRNAs for inhibition were identified as miR-27a-3p, miR-27b-3p and miR-128-3p, as they were among the top 100 most abundant miRNAs for all four sample types (centre of the Venn diagram of

Figure 4.12), and were predicted to target Nrf2. Notably, miR-27b-3p and miR-128-3p were already downregulated in reactive compared to quiescent astrocytes, which could be interpreted as a (failing) cellular attempt to restore Nrf2 activity. There was also a slight but non-significant downregulation of miR-27a-3p. None of the miRNAs were differentially expressed between reactive and quiescent ADEVs. The fact that these miRNAs are expressed more in quiescent astrocytes could be a limitation of these candidates, as perhaps they are associated with a more neuroprotective phenotype. However, since the aim here is to enhance Nrf2 antioxidant signalling, which has been shown previously to be neuroprotective (Vargas *et al.*, 2008), and they were still relatively abundant in reactive astrocytes, they were still all suitable candidates.

Notably all three miRNAs have also been reported as downregulated in ALS patient samples in a limited number of studies. Both miR-27b-3p and miR-128-3p were reported to be downregulated in peripheral blood samples from sALS patients compared to healthy controls, while higher blood levels of miR-27b-3p have been associated with a reduced risk of ALS (Liguori *et al.*, 2018; Zhu *et al.*, 2023). MiR-27a-3p was reported to be downregulated in serum exosomes from ALS patients compared to healthy controls (Xu *et al.*, 2018). Logically, inhibiting a miRNA which is already downregulated in ALS may be undesirable, as if this downregulation has pathogenic effects, these could be worsened. However, the downregulation of these miRNAs is only reported in the small number of studies described above, and they have not been suggested as major pathogenic miRNAs.

Despite these considerations, overall the three miRNAs were still determined as suitable candidates to inhibit in astrocytes in an attempt to induce the Nrf2 pathway. Whilst any one of the miRNAs would have been suitable, the decision to select miR-27b-3p as the final candidate was made as it has the highest average readcount in reactive astrocytes (221,877). This makes miR-27b-3p the 11th most abundant miRNA in reactive astrocytes, the cell type it will ultimately be inhibited in. In addition to bioinformatic target prediction, several studies experimentally support that miR-27b-3p targets Nrf2, in cell types including hepatocytes, esophageal squamous cell carcinoma cell lines and ARPE-19 retinal pigment epithelial cells. This included confirmation of a direct miR-27b-3p-Nrf2 interaction by luciferase reporter assay, as well as detecting the expected effects of miR-27b-3p mimics and inhibitors on Nrf2 and downstream gene expression (Han *et al.*, 2020; Lai *et al.*, 2023; Li *et al.*, 2022a).

Expression of miR-27b-3p in our models of reactive and quiescent astrocytes was confirmed by RT-qPCR, as presented in Figure 4.13. Although non-significant, there was a trend towards the downregulation of miR-27b-3p in reactive astrocytes, supporting the sncRNAseq data.

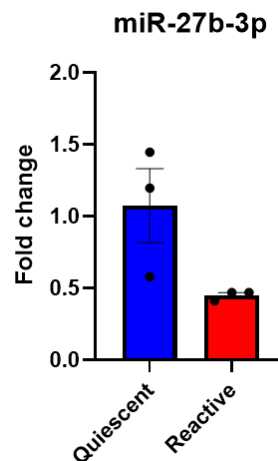


Figure 4.13. RT-qPCR of miR-27b-3p expression in quiescent and reactive astrocytes. No suitable housekeepers were found for normalisation. All Ct values were normalised to the average of the three quiescent astrocyte replicates. Welch's t-test, $n=3$.

4.6.2 Selection of miR-340-5p as a candidate Nrf2-targeting miRNA

As noted earlier, inhibition of two Nrf2-targeting miRNAs will be subsequently attempted in FBS-induced reactive astrocytes. Rather than selecting a further miRNA with the same criteria as miR-27b-3p (i.e. miR-27a-3p and miR-128-3p), a miRNA was selected based on previous experimental data produced in our research group.

This miRNA is miR-340-5p, which was shown by Dr Sophie Foggin to regulate autophagy, an ALS-related pathway, by directly targeting the autophagy regulator protein (and ALS gene product) TBK1. MiR-340-5p was previously inhibited by Dr Foggin using a synthetic miRNA 'power inhibitor' in the FBS-induced reactive human astrocyte model, a method which will be discussed more in Chapter 5. This was associated with an increase in Nrf2 protein, as measured by western blot, although TBK1 expression was variable. I sought to replicate this finding, as well as investigating the effect of miR-340-5p inhibition on downstream Nrf2 genes and ROS levels (Foggin, 2022).

In addition to the experimental data produced by our research group, TargetScan prediction and several other studies support that Nrf2 is a direct target of miR-340-5p. A luciferase reporter assay confirmed that miR-340-5p directly targets Nrf2 in the HepG2 hepatocellular carcinoma cell line and HEK 293T cells (Mei *et al.*, 2019; Shi *et al.*, 2015). In the C2CL12 mouse myoblast cell line, miR-340-5p mimics reduced Nrf2 protein, while miR-340-5p inhibitors increased Nrf2. Nrf2 mRNA levels were unchanged after both treatments, suggesting miR-340-5p regulates Nrf2 post-transcriptionally (Mei *et al.*, 2019).

MiR-340-5p dysregulation has been reported in several ALS patient studies, with downregulation reported in ALS patient spinal cord and serum, but upregulation in ALS neuromuscular junction compared to healthy control blood

(Campos-Melo *et al*, 2013; De Felice *et al*, 2018; Raheja *et al.*, 2018). MiR-340-5p was also detected in plasma-derived EVs from *SOD1*-ALS patients, but not *C9orf72*-ALS patients, perhaps indicating biomarker potential for certain ALS subtypes. However more work is required to validate this, as only 3 patients were in each group, including healthy control (Cheng *et al*, 2023). The variation in direction of dysregulation means that miR-340-5p is not clearly neurotoxic or neuroprotective in the ALS context, so it should not be a barrier to investigations.

Figure 4.12 shows that miR-340-5p is one of the top 100 most abundant miRNAs only in quiescent astrocytes, but not reactive astrocytes or either ADEV condition. In reactive astrocytes, the target cell type for miR-340-5p inhibition, its average raw readcount was 290, making it the 255th most abundant miRNA out of 1378 miRNAs detected at least once in reactive astrocytes. While this is not nearly as abundant as miR-27b-3p, it is still in the top 20%. As with miR-27b-3p, miR-340-5p is significantly enriched in quiescent astrocytes compared to reactive according to the sncRNAseq data. While not statistically significant, this trend was confirmed by RT-qPCR, as shown in Figure 4.14.

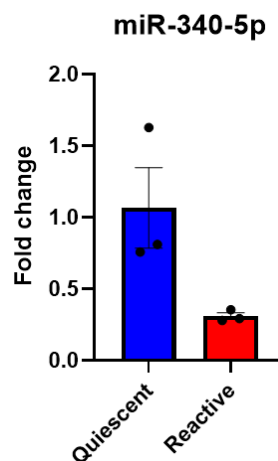


Figure 4.14. RT-qPCR of miR-340-5p expression in quiescent and reactive astrocytes. No suitable housekeepers were found for normalisation. All Ct values were normalised to the average of the three quiescent astrocyte replicates. Welch's t-test, $n=3$.

4.7 Conclusions

To conclude, Chapter 4 presented a sncRNAseq analysis of our published model of FBS-induced reactive and quiescent human primary astrocytes and their ADEVs. Differential expression analysis allowed the identification of miRNAs which show altered expression at the whole-cell level either as a cause or consequence of FBS-induced astrocyte reactivity. This included the upregulation of miR-206 and miR-155-5p and downregulation of miR-153-3p and miR-136-5p, which may be predicted to have a functional role in promoting astrocyte reactivity.

MiR-206 was identified as a miRNA which may be upregulated in ALS patient biofluids in part due to upregulation in reactive astrocytes, providing a possible

example of ‘source-mapping’. While RT-qPCR was ultimately unsuccessful for confirming miR-206 expression in astrocytes, the investigation of a hypothetical Nrf2/miR-206/NURR1 axis led to the discovery of downregulated *NURR1* mRNA upon astrocyte reactivity, which encodes another neuroprotective transcription factor. NURR1 downregulation in astrocytes may therefore be another pathogenic effect of astrocyte reactivity in ALS patients which should be investigated further, for example by immunohistochemistry of postmortem tissue. NURR1 upregulation has been reported in ALS patient blood and mSOD1 mouse spinal cord (Valsecchi *et al.*, 2020), however astrocyte-specific expression should be determined. Investigations were limited by the difficulty in detecting endogenous NURR1 protein by western blot and SWATH-MS, therefore optimising detection techniques may be required for further investigation, for example using a streptavidin-biotin method to amplify western blot signal.

Motif analysis of ADEV-enriched and whole-cell-enriched miRNAs revealed the possible involvement of nine ALS-associated RBPs including FUS, TDP-43 and HNRNPA1 in miRNA sorting in astrocytes, consistent with previous observations for FUS (Garcia-Martin *et al.*, 2022; Gordillo-Sampedro *et al.*, 2024). Overall, the data suggested that these RBPs were more important for the loading of miRNAs into EVs than cellular retention. Strikingly, there were no miRNAs containing motifs for ALS-associated RBPs that were enriched in reactive astrocytes compared to ADEVs. This may suggest a potential failure or intentional block of ALS-associated RBP-mediated retention of miRNAs in astrocytes upon reactivity. Dysregulated miRNA sorting by ALS-associated RBPs may therefore be a pathomechanism in ALS and other disorders associated with astrocyte reactivity, impacting astrocyte gene expression and cell-cell communication via EVs. TDP-43 or FUS pathology and/or a mutation in an RBP would be predicted to accentuate this dysregulation. FUS has previously been shown to have a role in loading miRNAs containing CGGGAG motif, termed an ‘EXOmotif’, into EVs from a range of mouse cell lines (Garcia-Martin *et al.*, 2022). I therefore predict that loss of this ADEV-miRNA sorting activity upon astrocyte reactivity or ALS-associated mutation may be an ALS pathomechanism, which our research group is now investigating.

Integration of sncRNAseq and proteomic data revealed that the Nrf2 pathway deficit upon FBS-induced astrocyte reactivity is unlikely to be due to widespread increased direct targeting of Nrf2 by miRNAs. In fact, significant downregulation of Nrf2-targeting miRNAs upon astrocyte reactivity may point towards a compensatory mechanism aiming to increase neuroprotective Nrf2 signalling.

Finally, along with previous experimental data, the sncRNAseq analysis guided the selection of two Nrf2-targeting miRNAs which I aim to inhibit in Chapter 5, to investigate their potential as astrocyte-targeted ALS therapeutics. By experimentally inhibiting miR-340-5p and miR-27b-3p, I aimed to drive an increase in expression of Nrf2 and its downstream target genes, which may result in reduced ROS and oxidative stress in reactive astrocytes.

Chapter 5. Targeting miRNAs in human astrocytes, towards a new ALS therapeutic strategy

5.1 Introduction

Nrf2 activation as a therapeutic approach for ALS has received considerable attention in preclinical research and in clinical trials, as discussed in Chapter 1. However, there is not yet an approved Nrf2-activating compound for ALS treatment. Whilst edaravone, currently used to treat ALS, may activate the Nrf2 pathway by an unknown mechanism (Arslanbaeva & Bisaglia, 2022), this has not been investigated thoroughly in an ALS context, which I begin to approach in Chapter 6. In this chapter, an alternative approach to chemical Nrf2 activation was investigated, via miRNA targeting, specifically in human astrocytes. This aimed to build upon the work by Vargas *et al.*, which showed the efficacy of genetic Nrf2 activation specifically in astrocytes for protecting against neurodegeneration (investigated by crossing mSOD1 mice with transgenic mice overexpressing astrocytic Nrf2 via the GFAP promoter) (Vargas *et al.*, 2008).

All work in this chapter involved cultured human FBS-induced reactive astrocytes, as opposed to quiescent, since ultimately reactive astrocytes would likely be the target cell of an ALS astrocyte-directed therapeutic, to combat astrocyte-mediated neurotoxicity. Furthermore, in Chapter 3 reduced expression of Nrf2 pathway genes was identified in reactive astrocytes (relative to quiescent), a molecular phenotype which I now aimed to rescue. The method by which I aimed to upregulate the Nrf2 pathway in reactive astrocytes was by inhibiting an Nrf2-targeting miRNA, to alleviate miRNA-mediated repression of Nrf2. Two Nrf2-targeting miRNAs identified in Chapter 4 as candidates for inhibition were miR-340-5p and miR-27b-3p. In addition to measuring the effects of inhibiting these miRNAs on the Nrf2 pathway (primarily by RT-qPCR), it was also important to consider off-target effects of a miRNA inhibitor, since miRNAs target many genes. To achieve this, a proteomic analysis of miRNA inhibitor-treated astrocytes was completed, which also allowed indirect measurement of expression of additional Nrf2 target genes.

The field of RNA therapeutics is rapidly developing, especially in recent years following the COVID-19 mRNA vaccine, with many ASO drugs now FDA-approved, including Tofersen for SOD1-ALS treatment (Miller *et al.*, 2022; Parvin *et al.*, 2024). Whilst no miRNA therapeutics are currently FDA-approved, although many are in clinical trials, lessons from the broader RNA therapeutics field are likely to advance miRNA therapeutic technology (Diener *et al.*, 2022). The rapid advancements in RNA therapeutics include for delivery methods, for example involving nanoparticles and viral vector-based systems (Parvin *et al.*, 2024). Methods for delivering miRNA therapeutics to cultured cells (although some are also applicable to *in vivo* studies) will now be reviewed, since

optimisation of miRNA inhibitor delivery to primary astrocytes was a major aspect of the work presented in this chapter.

5.1.1 Methods of delivering miRNA therapeutics to cells

The main classes of current tools to experimentally manipulate miRNAs (referred to here as therapeutics, for simplicity) are miRNA inhibitors (antagomiRs or anti-miRs) and mimics. Most miRNA inhibitors bind to the guide strand of target miRNA in the miRISC complex, preventing it from binding to its mRNA target. MiRNA sponges can also be used to reduce miRNA activity as they contain competitive binding sites for a miRNA, therefore preventing it from binding to its natural mRNA targets (Beavers *et al*, 2015). MiRNA mimics are synthetic versions of endogenous miRNAs which have the same mRNA-targeting functionality, therefore effectively upregulating expression of a miRNA (Garreau *et al*, 2024).

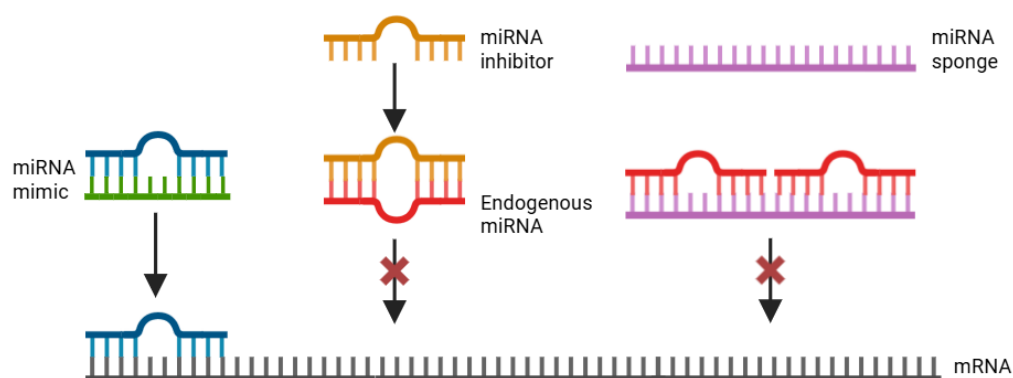


Figure 5.1. Schematic illustrating the mechanism of action of common miRNA therapeutics. MiRNA mimics replace endogenous miRNAs to upregulate their function, while miRNA inhibitors and sponges block endogenous miRNAs to inhibit their function. Created in BioRender.

The systems which will be discussed below all facilitate the delivery of miRNA therapeutics to cells. These systems aim to increase delivery efficiency, while protecting therapeutics from extracellular nucleases, preventing damage to recipient cells and genetic material, and preventing toxicity and an immune response where possible. MiRNA therapeutic delivery systems can be categorised as viral or non-viral vectors. Overall, viral vectors facilitate more efficient and long-lasting delivery of miRNA therapeutics, but can be more toxic and immunogenic, while non-viral vectors are generally less efficient but safer (Liu *et al*, 2022; Yang, 2015). The balance of these factors therefore must be considered for specific research aims and may result in different techniques being used for *in vitro* basic research to *in vivo* research or clinical trials. For example, for *in vivo* studies of CNS-targeted miRNA therapeutics, factors such as BBB penetration and tissue tropism must also be considered (Yeoh *et al*, 2024).

5.1.1.1 Viral-based RNA delivery methods

The viral-based miRNA therapeutic delivery methods most useful for neurodegenerative disease research are adeno-associated viruses (AAVs) and lentiviruses, as they can infect both dividing and non-dividing cells, including differentiated neurones. Non-lentiviral retroviruses are less useful as they can only infect dividing cells. Adenoviruses are also less widely used for miRNA therapeutics, probably because the delivery is more transient and can elicit a strong immune response (Sung *et al*, 2001; Yang, 2015).

AAVs are perhaps the most common and successful viral-based method of miRNA therapeutic delivery in ALS rodent models. AAVs are single-stranded DNA viruses which are favourable for delivery of miRNA therapeutics over some other gene therapies due to their relatively low size limit for encapsulated sequences (<4.8 kilobase) (Yang, 2015). AAVs have different serotypes, each with different tissue tropisms, meaning the cell or tissue types they preferentially infect (Srivastava, 2016). Serotypes with efficient CNS-targeting that have been used for ALS miRNA preclinical studies include AAV9, AAV5 and AAV recombinant human (rh)10. Specifically, these studies include delivery of artificial SOD1-targeting miRNAs AAVrh10-miR^{SOD1} and AAV9-amiR^{SOD1} to mSOD1 mice, and the artificial C9Orf72-targeting miRNA AAV5-miC to iPSC-derived neurones (Lind *et al*, 2020; Martier *et al*, 2019; Stoica *et al*, 2016a).

Lentiviruses are a subgroup of retroviruses - viruses which reverse transcribe their RNA to produce DNA that integrates into the host cell genome. This integration ensures long-term stable expression of miRNA therapeutics, whereas many AAVs do not integrate genetic material. Lentiviruses can deliver slightly longer nucleic acids than AAVs, with a sequence limit of approximately 8 kilobases (Yang, 2015). Lentiviruses have also been used to deliver miRNA therapeutics in ALS experimental research, for example to deliver hsa-miR-17~92, a miRNA cluster, to SOD1^{L144F} iPSC-derived motor neurones (Tung *et al*, 2019).

5.1.1.2 Non-viral RNA delivery methods

Non-viral methods of miRNA therapeutic delivery can be either physical or chemical. Physical methods exert forces on cells to induce permeability and facilitate therapeutic uptake, for example by electroporation, ultrasound, lasers, hydrodynamic force (exerted by rapid injection of a large volume) or 'gene gun' delivery of nucleic acid-coated microprojectiles (O'Brien & Lummis, 2007; Suda & Liu, 2007; Yang, 2015). Nucleofection is an example of a modified electroporation method, which can be tailored to specific cell types using certain electrical parameters and transfection reagents (Kane *et al*, 2008).

Chemical methods include lipid, polymer and inorganic-based approaches. Liposomes or lipoplexes are lipid/nucleic acid complexes whereby lipids form vesicles and encapsulate nucleic acids in their core. The charges of liposomes can vary, with cationic liposomes used most often since they are easy to produce, have a high affinity for cells membranes and typically do not induce

an immune response (Yang, 2015). Commercial liposome-based transfection reagents that can be used for miRNA therapeutic delivery include Lipofectamine® (e.g. Lipofectamine 2000, 3000 or RNAiMAX) [Invitrogen] and DharmaFECT® [Dharmacon]. ALS patient-derived iAstrocytes have previously been transfected with a miR-29b-3p mimic using HiPerFect® Transfection Reagent [Qiagen] to upregulate the Nrf2 pathway (Provenzano *et al.*, 2022). While a newer concept, a possible ‘natural’ alternative to synthetic liposomes may be to use EVs for miRNA therapeutic delivery, since they are already a ‘natural’ carrier of miRNAs between cells. Surface modifications may be employed to improve selectivity of delivery to target cells, for example by conjugating antibodies that bind specifically to neuronal or astrocytic surface proteins (Lim *et al.*, 2025).

Polymer nanoparticles used for delivery include polyethylenimine (PEI), poly(amidoamine) (PAMAM) dendrimers and poly(lactide-co-glycolide) (PLGA) (Yang, 2015). Like liposomes, cationic groups in polymers typically enhance both encapsulation of nucleic acids and delivery to cells due to opposing charge interactions (Piotrowski-Daspiet *et al.*, 2020). Inorganic materials have been used for miRNA therapeutic delivery in a few studies, including gold-based, Fe₃O₄-based and silica-based nanoparticles (Bitar *et al.*, 2012; Crew *et al.*, 2012; Schade *et al.*, 2013; Yang, 2015).

5.1.2 Experimental aims

The broad experimental aims of Chapter 5 are as follows:

- Determine the most efficient method for miRNA inhibitor delivery to human reactive astrocytes.
- Using this method, deliver Nrf2-targeting miR-27b-3p and miR-340-5p inhibitors to reactive astrocytes and determine whether the Nrf2 pathway is upregulated.
- Conduct a proteomic analysis of reactive astrocytes treated with the most promising miRNA inhibitor to determine the wider effects on gene expression, including other Nrf2 and other miRNA target genes.

5.2 Lipofectamine-based transfection of human reactive astrocytes

In Chapter 4, miR-340-5p and miR-27b-3p were selected as Nrf2-targeting miRNAs which will be inhibited in human primary FBS-induced reactive astrocytes, with the aim of potentiating Nrf2 signalling. First, an efficient method of miRNA inhibitor delivery to reactive astrocytes had to be determined. Transfection using Lipofectamine 2000 was selected as the initial method, due to its relative simplicity and widespread reported use. Before attempting delivery of commercial miRNA inhibitors, first reactive astrocytes were transfected with pmaxGFP plasmid to determine general transfection efficiency. Astrocytes were imaged 24 and 48 hours after transfection. At both

timepoints, less than 1% of cells fluoresced green from subjective visual microscopy assessment, illustrating an extremely low transfection efficiency. A representative image is shown in Figure 5.2, however in many fields of view there were no fluorescing cells.

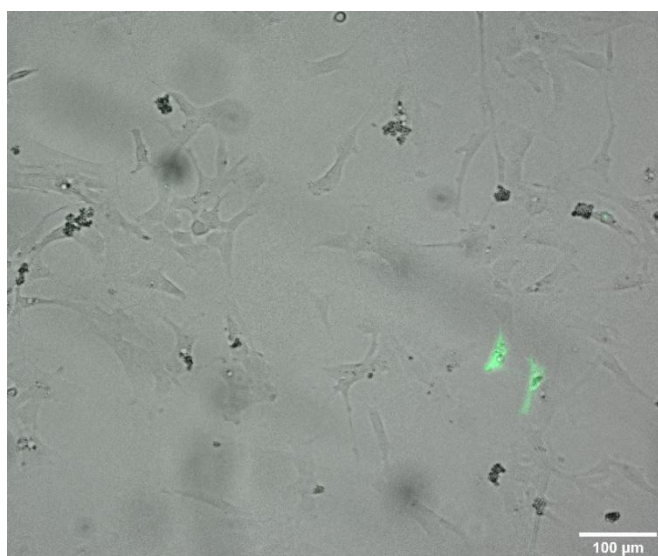


Figure 5.2. Reactive human astrocytes transfected with pmaxGFP plasmid. Reactive astrocytes were seeded at a density of 70,000 cells/well of a 6-well plate. After 24 hours, cells were transfected with pmaxGFP plasmid using Lipofectamine 2000. Specifically, 5 μ l Lipofectamine 2000 was incubated for 20 minutes with 125 μ l Opti-MEM, while 1 μ g pmaxGFP plasmid was incubated for 5 minutes with 125 μ l Opti-MEM. The two solutions were mixed and incubated for 30 minutes, before dropwise addition to cells in 2ml astrocyte medium. Cells were imaged using a widefield microscope after 24 and 48 hours. This representative image (24 hours) shows an overlay of the brightfield and GFP channels with two transfected/fluorescent cells. From subjective visual assessment, <1% of all cells fluoresced. Scale bar = 100 μ m.

Lipofectamine RNAiMAX reagent was also tested, as this transfection reagent is reportedly optimised for delivery of siRNAs and miRNAs. For the first experiment, pmaxGFP plasmid was not included in the transfection mix as RNAiMAX is not designed for plasmid delivery, therefore the aim of this first experiment was only to monitor cells for toxicity, rather than determining transfection efficiency. A mix of 25 μ l Opti-MEM and 1 μ l RNAiMAX reagent were briefly incubated, then added to a suspension of 10,000 reactive human astrocytes and seeded in an ibidi dish. After 24 hours, RNAiMAX was found to be highly toxic to reactive astrocytes by subjective visual assessment using a brightfield microscope, with very few surviving cells (data not shown). Therefore, this method was not taken further for delivery of miRNA inhibitors.

Transfection has previously been shown to be difficult in mouse primary astrocytes (Gutiérrez-Vázquez & Quintana, 2022). Primary cells are typically not susceptible to transfection due to their limited expansion capacity and lifespan, reflecting the findings in Figure 5.2 using Lipofectamine 2000 (Chong *et al*, 2021). Furthermore, a transfection reagent optimised for short RNA

therapeutic delivery (Lipofectamine RNAiMAX) was highly toxic to reactive astrocytes. Therefore, a method of introducing miRNA inhibitors to reactive astrocytes that does not require classical transfection was required.

5.3 Synthetic miRNA power inhibitor treatment of human reactive astrocytes

‘Standard’ miRNA inhibitors such as miRCURY LNA miRNA Inhibitors™ [Qiagen] typically require an additional system to facilitate their delivery to cells, such as Lipofectamine-based transfection. However, miRCURY LNA miRNA Power Inhibitors™ [Qiagen], from now on referred to as power inhibitors (PIs), do not require transfection, so were selected for further investigation. PIs are ASOs that bind to target miRNA sequences, preventing the miRNA from forming interactions with its own target mRNAs. As opposed to standard inhibitors with a phosphodiester backbone, PIs have a fully phosphorothioate (PS) modified backbone. This renders them highly resistant to enzymatic degradation, meaning they have improved stability and potency (Dowdy, 2017). They do not require transfection, instead addition directly to culture media reportedly allows them to be taken up by cells by a process known as gymnosis (Stein *et al*, 2010).

Since PIs do not require a delivery system (that could also be used to co-deliver pmaxGFP reporter plasmid) and they are not fluorescently tagged, it was not possible to first measure delivery efficiency via GFP. Therefore, I began with directly measuring the effect of miRNA PIs on Nrf2 pathway components as a readout. Reactive astrocytes were treated initially with miR-340-5p PI (100nM) for 48 hours, with 100nM selected as it is the lowest concentration recommended by the supplier, therefore avoiding toxicity issues and sparing enough PI for multiple experiments. RNA extraction and RT-qPCR was performed to measure Nrf2 pathway genes, as shown in Figure 5.3A-D. However, there were no significant changes in expression of any measured genes by RT-qPCR. It is perhaps unsurprising that *NRF2* expression was unaffected by miR-340-5p inhibition, since miRNAs sometimes prevent translation rather than inducing mRNA degradation. However, since expression of the downstream Nrf2 target genes *HMOX1*, *NQO1* and *IDH1* was unaffected by the treatment it suggests that miR-340-5p PI does not increase Nrf2 expression or activity in this system.

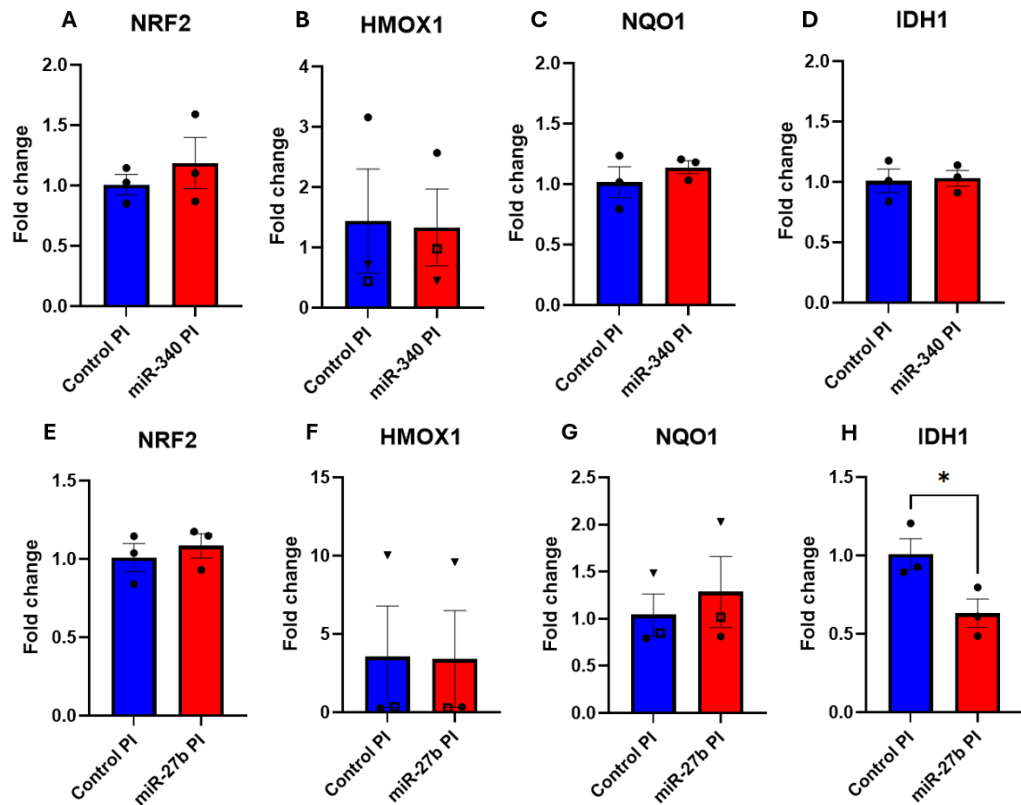


Figure 5.3. Treatment of human reactive astrocytes with miR-340-5p or miR-27b-3p power inhibitor does not upregulate Nrf2 pathway components, as measured by RT-qPCR. 24 hours after seeding, human reactive astrocytes were treated for 48 hours with miR-340-5p (miR-340, 100nM) or miR-27b-3p (miR-27b, 200nM) PI or equivalent scrambled control PI before RT-qPCR (n=3). GAPDH and B2M were used as housekeepers, and all data was normalised to the average control PI value for each gene. Different datapoint symbols (circle, triangle and square) represent biological replicates for highly variable genes (B, F and G). Welch's t-test used. The miR-340-5p and miR-27b-3p experiments were performed separately, so normalisation was made to different controls. A-D) Expression of NRF2, HMOX1, NQO1 and IDH1 after miR-340-5p PI treatment in serum-free astrocyte medium, to avoid possible interference of serum. Astrocytes had been seeded at a density of 150,000 (replicate 1) or 100,000 (replicates 2 and 3), with densities varied due to the different growth speeds of different astrocyte batches. E-H) Expression of NRF2, HMOX1, NQO1 and IDH1 after miR-27b-3p PI treatment in 2% FBS-containing astrocyte medium. Astrocytes were seeded at a density of 70,000 cells/well of a 6-well plate.

In a subsequent experiment, reactive astrocytes were treated for 48 hours with miR-27b-3p PI, this time at the higher concentration of 200nM due to the non-significant results of Figure 5.3A-D. As shown in Figure 5.3E-H, miR-27b-3p PI did not significantly affect *NRF2*, *HMOX1*, or *NQO1* expression. MiR-27b-3p inhibition significantly reduced expression of *IDH1*, which alone is unlikely to suggest major changes to Nrf2 activity, and regardless is the opposite direction as would predicted after inhibition of an Nrf2-targeting miRNA. *HMOX1* expression was highly variable between replicates after both miR-340-5p and

miR-27b-3p inhibition, as represented with different symbols (circle, triangle and square) in Figure 5.3B and F. However, even when datapoints were normalised to their own biological replicate control, rather than the average control value (the standard method used in this thesis), there was no clear *HMOX1* trend upon miR-340-5p or miR-27b-3p inhibition.

It must be noted that the miR-340-5p and miR-27b-3p inhibition experiments cannot be directly compared, due to differences in inhibitor concentration and nucleotide sequences, astrocyte seeding density and the FBS content of the astrocyte medium used for inhibitor treatment (FBS-free for miR-340-5p PI, 2% FBS for miR-27b-3p). Serum-free astrocyte medium was only used briefly in this chapter, for miR-340-5p PI treatments in this sub-section, in case FBS-derived miRNA carriers such as Argonaute and lipoproteins (Mannerström *et al*, 2019) are also able to sequester miRNA inhibitors. In retrospect, additional optimisation experiments could have been performed to investigate the effect of serum on miRNA inhibitor delivery, and serum-free medium should have also been used for miR-27b-3p PI treatment, however the lack of significant results led to the presumption that serum was not a critical factor. Despite these differences between miR-340-5p and miR-27b-3p PI treatments, overall there was no significant Nrf2 pathway upregulation in reactive astrocytes in Figure 5.3. In case this was a cell type-specific effect, HEK293T cells were also treated for 48 hours with miR-27b-3p PI (200nM, the same concentration used in astrocytes). However, this did not significantly alter Nrf2 pathway gene expression (data not shown), perhaps suggesting ineffective delivery in both cell types, or low miR-27b-3p expression in HEK293T cells.

Next, to ensure that effects of miRNA power inhibitors in astrocytes were not 'missed' at a 48-hour timepoint, reactive astrocytes were treated with control or miR-340-5p PI (100nM, the same miR-340-5p PI concentration used in other experiments) for 24 or 72 hours before RT-qPCR. Only miR-340-5p was inhibited in this instance, for simplicity. A single exploratory analysis was conducted as there was not a large change in expression in any gene, at any timepoint. As shown in Figure 5.4B-D, while *HMOX1*, *NQO1* and *IDH1* expression increased ~1.2x after 24 hours compared to control PI, this is a very modest increase that even if replicated would not suggest a major biological impact. After 72 hours there was a slight decrease in *HMOX1* and *IDH1* expression, as shown in Figure 5.4F and H, with fold changes of 0.7 and 0.8 compared to control PI, respectively. If replicated, a decrease in Nrf2 pathway gene expression would be undesirable.

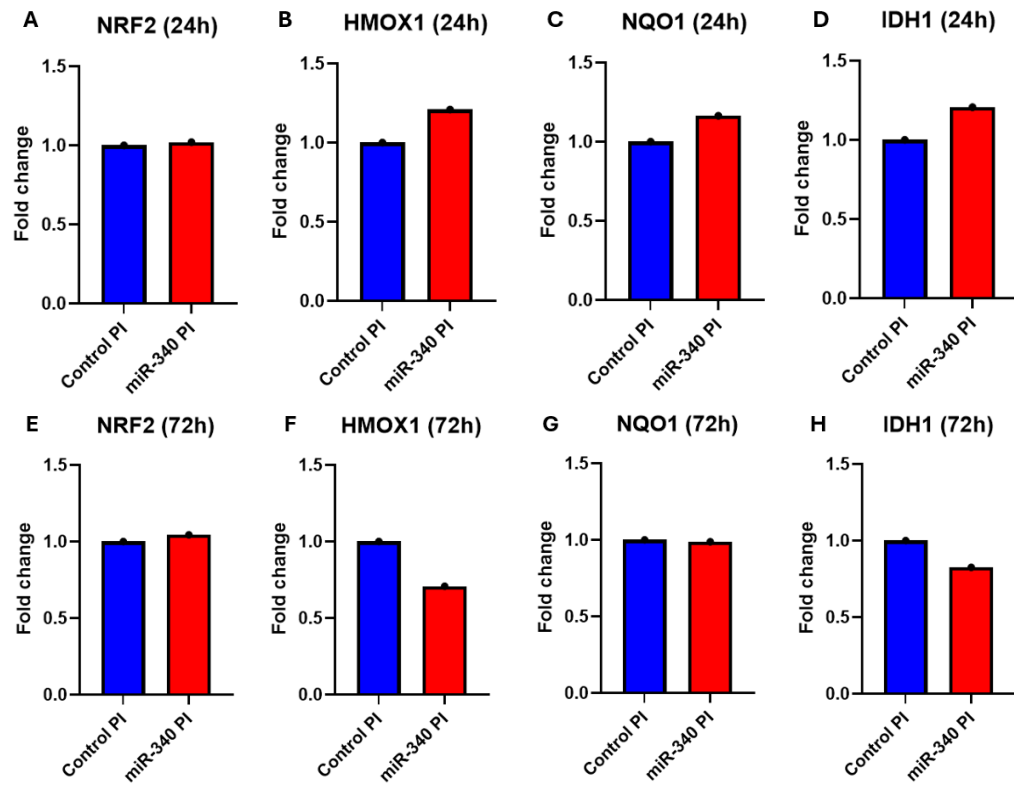


Figure 5.4. Treatment of human reactive astrocytes with miR-340-5p power inhibitor for 24 or 72 hours does not upregulate the Nrf2 pathway, as measured by RT-qPCR (n=1). Human reactive astrocytes were seeded at densities of 70,000 cells/well (for 72-hour inhibitor treatment) and 150,000 cells/well (for 24-hour inhibitor treatment) in a 6-well plate. After 24 hours, astrocytes were treated with control or miR-340-5p (miR-340) PI (100nM) for 24 or 72 hours in serum-free astrocyte medium, before RT-qPCR of Nrf2 pathway genes. A-D) Expression of NRF2, HMOX1, NQO1 and IDH1 after 24 hours. E-H) Expression of NRF2, HMOX1, NQO1 and IDH1 after 72 hours. GAPDH, B2M and 18S were used as housekeepers, and each miR-340-5p PI value was normalised to the respective control. No statistical test performed (n=1).

As noted earlier, the transfection efficiency of the PIs could not easily be determined as they were not visibly detectable in cells (e.g. via GFP fluorescence). However, I hypothesised that there was at best low-level uptake, since the Nrf2 pathway was not affected by miR-340-5p or miR-27b-3p PI treatment, including when different incubation times were tested for miR-340-5p inhibition. I therefore aimed to enhance miRNA PI delivery, which was first attempted using EV-mediated delivery. As discussed, EVs are natural carriers of miRNAs that are readily taken up by cells, therefore they were predicted to passively take up miRNA PI (as demonstrated in a pre-print study by our research group (Mesquita-Ribeiro *et al.*, 2025)) and enhance their uptake by astrocytes.

Reactive ADEVs were selected for this method, as they were predicted to be readily accepted by other reactive astrocytes and exert fewer functional changes in the recipient cells than EVs derived from other cell types. Reactive

ADEVs were isolated from conditioned medium and characterised by the method described in section 2.1.3. The ADEVs (10^9 per condition) were then incubated for 4 hours with control or miR-340-5p PI with rocking at room temperature to facilitate passive inhibitor uptake into ADEVs, a method previously described (Mesquita-Ribeiro *et al.*, 2025). The total volume (300 μ l of miRNA-loaded ADEVs in PBS) was then applied to reactive astrocytes via addition to culture media, giving a final inhibitor concentration of 100nM, the same as previous miR-340-5p experiments shown in Figure 5.3 and Figure 5.4. These treated astrocytes had been seeded 24 hours previously, and were not the same culture that had been used for ADEV isolation. After 48 hours of miR-340-5p PI and ADEV treatment, RT-qPCR was performed to measure Nrf2 pathway gene expression, as shown in Figure 5.5.

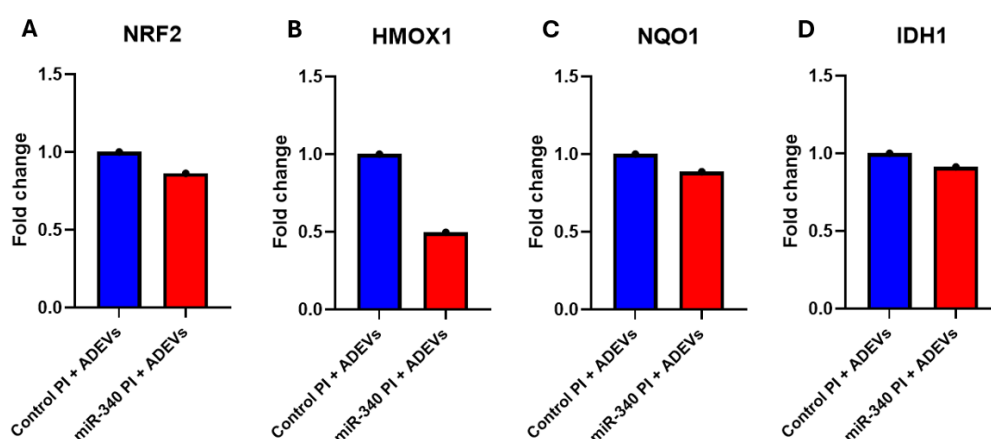


Figure 5.5. Treatment of human reactive astrocytes with miR-340-5p power inhibitor and ADEVs does not upregulate the Nrf2 pathway, as measured by RT-qPCR (n=1). Human reactive astrocytes were seeded at a density of 150,000 cells/well in a 6-well plate. 10^9 reactive ADEVs (isolated from other reactive astrocytes) were incubated for 4 hours with miR-340-5p (miR-340) PI in a total volume of 300 μ l PBS, then applied to reactive astrocytes at a final PI concentration of 100nM in serum-free astrocyte medium. After 48 hours, RT-qPCR was performed to measure Nrf2 pathway gene expression. A-D) Expression of NRF2, HMOX1, NQO1 and IDH1. GAPDH, B2M and 18S were used as housekeepers, and each miR-340-5p PI +ADEVs value was normalised to the respective control. No statistical test performed (n=1).

The results of this exploratory analysis in Figure 5.5 suggested that the Nrf2 pathway was not upregulated in reactive astrocytes by treatment with miR-340-5p PI and reactive ADEVs that had been pre-incubated, relative to ADEVs pre-incubated with control PI. There was no significant change in expression of *NRF2*, *NQO1* or *IDH1*, while *HMOX1* expression decreased, therefore only one replicate was performed. These results may suggest that ADEVs do not enhance astrocytic uptake of miRNA PI, since the results broadly reflect those of Figure 5.3A-D, where miR-340-5p was added directly to cells in culture media. Alternatively, the method may require further optimisation, including the optimal incubation time of ADEVs with miRNA PI, and ensuring that the EVs were not damaged by 4-hour agitation, using NTA.

However, this method was not pursued further, since the lack of fluorescent tag on miRNA PIs would make optimisation difficult. Ideally, all initial optimisations would be performed based on the percentage of cells that take up a fluorescent (or other) tag, as with Lipofectamine 2000 in the previous section, to determine conditions for the most efficient delivery. Then, the Nrf2 pathway could be measured by RT-qPCR following a fully optimised miRNA inhibition protocol.

To summarise the results of this section, there was no evidence of successful Nrf2 pathway upregulation induced by treatment of reactive human astrocytes with miR-340-5p or miR-27b-3p PI, including when ADEVs were used to enhance uptake of miR-340-5p PI. While the reasons for this are unclear, it was hypothesised to be due to low-level inhibitor uptake, since the low transfectability of these primary astrocytes had already been demonstrated. Alternate methods for delivering miRNA inhibitors to reactive astrocytes were therefore explored, focussing on those known to be efficient in hard-to-transfect cell types, and that allow visual monitoring and identification of transfected cells.

5.4 Lentivirus-mediated delivery of miRNA inhibitors to human reactive astrocytes

All work up to this point relied on non-viral methods of miRNA inhibitor delivery, which are known to generally be less efficient than viral methods (Yang, 2015). Therefore, a viral method was explored as the next miRNA inhibitor delivery system. As discussed in section 5.1.1.1, AAVs and lentiviruses are the most appropriate and perhaps most widely used for delivery of miRNA modulators in neurodegenerative disease preclinical research, in part due to their ability to transduce terminally-differentiated cells.

Ultimately, lentiviruses were selected as the viral delivery method, since they integrate genetic material into the host cell genome so should facilitate long-term expression of a miRNA inhibitor (Yang, 2015). Furthermore, lentiviruses are used more broadly for *in vitro* research, while AAVs are often preferred for *in vivo* research and clinical trials (Gutierrez-Guerrero *et al*, 2020). Lentiviruses have previously been used to deliver miRNA therapeutics to astrocytes, such as a miR-136-5p inhibitor delivery to cultured mouse primary astrocytes, and pre-miR-145 delivery to astrocytes in mouse spinal cord using a GFAP promoter (Peng *et al.*, 2017; Wang *et al.*, 2015).

Lentiviruses encoding a miR-27b-3p, miR-340-5p or scramble (control) miRNA inhibitor sequence were purchased from Applied Biological Materials Inc. (Abm), with further details provided in Chapter 2. Briefly, vectors encoding the inhibitor sequences were purchased pre-packaged into third generation VSV-G pseudotyped lentivirus particles, with a titre of 10^7 IU/ml. The vectors also encoded GFP, allowing direct monitoring of transduction efficiency.

5.4.1 Optimisation of lentivirus treatment of human reactive astrocytes

First, several steps of the lentivirus delivery required optimisation, which are summarised in Table 5.1 and described afterwards. Optimisation was performed to ensure the maximum possible transduction efficiency, as determined by subjective visual assessment of the proportion of GFP-fluorescing cells. Optimisation experiments were carried out in 96-well plates, with human reactive astrocytes typically seeded at 4,000 cells per well and treated 24 hours later (note that this was increased to 5,000 for immunocytochemistry experiments described in the subsequent section). A mix of control lentivirus in a small volume of astrocyte medium (typically 60µl total volume) was added to cells, along with polybrene to enhance lentivirus uptake. Polybrene is a positively charged cation, therefore it reduces the electrostatic repulsion between the typically negatively charged cell membrane and virus envelope (Denning *et al*, 2013). A minimum of 18 hours after lentivirus transduction, the virus-containing medium was removed followed by two washes and replacement with fresh astrocyte medium, to ensure safety before imaging or other analysis.

Table 5.1. A summary of the optimised experimental procedures of lentivirus treatment of human reactive astrocytes.

Feature to be optimised	Conditions tested	Condition selected
MOI (approximate)	2.5, 5, 7.5, 12.5, 30	12.5
Polybrene concentration	0.5, 1, 2, 3, 4 and 8µg/ml	8µg/ml
Transduction time	18, 24, 48 and 72 hours	24 hours
Treatment method	Direct treatment and spinoculation	Direct treatment
Reagent for washes	DPBS, Advanced DMEM-F12	Advanced DMEM-F12
Time after virus removal before subsequent analysis	0, 24, 48, 72, 96 and 120 hours	24 hours

Control (scramble) miRNA inhibitor lentivirus was used for optimisation. Optimal conditions were selected based on efficiency of transduction, as measured by subjective visual assessment of the proportion of GFP-fluorescing cells using a widefield microscope. MOI = multiplicity of infection.

One factor to be optimised was the multiplicity of infection (MOI), which is the ratio of the number of viral particles to the number of target cells at the time of transduction, which was generally assumed to be double the number of cells that had been seeded 24 hours previously (Zhang *et al*, 2004). All MOIs tested (2.5-30) resulted in low transduction efficiency of astrocytes despite the use of 8µg/ml polybrene, with less than ~5% of cells expressing GFP. Unexpectedly, an MOI of 30 did not improve efficiency compared to 2.5-12.5, likely because

more medium was required to dilute the larger volume of virus, as a virus:medium ratio of over 1:5 was likely to be toxic. Therefore, the MOI selected was 12.5 as transduction efficiency was slightly higher than 2.5-7.5, but allowed preservation of reagents compared to higher MOIs. This equated to 10 μ l virus in a total volume of 60 μ l in a 96-well plate, assuming the 4,000 cells had doubled to 8,000.

As mentioned, optimal polybrene concentration was determined to be 8 μ g/ml, a commonly used polybrene concentration which has previously been used for astrocyte-like (glioma) cells (Liu *et al*, 2004). Unexpectedly, in some experiments polybrene at any concentration was completely toxic, killing all cells, while in other experiments it did not kill any. In case this was due to differences between polybrene aliquots, the stock was remixed and re-aliquotted. A polybrene-only control was also always included in lentivirus experiments.

Initial experiments used DPBS for washes to remove lentiviral particles after transduction, however this caused some cell death, or lifting of the cells. Therefore, Advanced DMEM-F12 was used instead, as astrocytes were known to survive in this medium during the preparation of quiescent astrocytes, as described in section 2.1.1.3. It was not necessary to use a supplement-containing medium for the short washes.

The length of transduction time was also investigated, after astrocytes were treated with 10 μ l virus and 8 μ g/ml polybrene in a final volume of 60 μ l. For this experiment, miR-27b-3p inhibitor virus was tested as well as the control, to check that the low efficiency seen so far was not specific to the control virus. While 18-hour transductions had so far been tested, here 24, 48 and 72-hour transductions were tested. When the virus was removed from the first plate after 24 hours, 10 μ l of medium was added to the 48-hour plate, and 20 μ l was added to the 72-hour plate to prevent drying out. All three plates were monitored for several days after removing the virus using a widefield microscope. Immediately after the 24-hour virus treatment was washed off there were no visible GFP-positive cells, but some were visible from a minimum of 24 hours later. For the 48 and 72-hour conditions, GFP-positive cells were visible as soon as the virus was removed. The number of GFP-positive cells was very similar for all conditions after the initial 48 hours, although slightly higher in miR-27b-3p than control virus, and this continued up to 5 days after initial transduction. Therefore, increasing transduction time did not increase efficiency, if there was at least 48 hours including both transduction and the subsequent recovery time. A transduction time of 24 hours was therefore selected, with a further 24 hours after virus removal.

All experiments so far involved direct treatment of human astrocytes with lentivirus in culture medium. Another method which can increase transduction efficiency in hard-to-transduce cell types is spinoculation. Here cells and lentivirus are centrifuged together to increase the local concentration of the virus, thus improving viral binding to cells (Remley *et al*, 2021). A suspension of astrocytes, control lentivirus (MOI 12.5) and polybrene (8 μ g/ml) was gently

mixed and incubated at room temperature for 20 minutes, followed by centrifugation at 800g for 30 minutes. The pelleted cells were then resuspended in the same suspension and transferred to a plate. However, spinoculation did not appear to increase the transduction efficiency, so experiments continued to use the simpler direct lentivirus treatment method.

Figure 5.6A shows an example of human astrocytes which have undergone the optimised lentivirus treatment protocol. Despite the optimisation performed so far, the transduction efficiency was still low (~5-10%), prompting the investigation of whether this was an astrocyte-specific effect. Therefore, HEK293T cells were treated with the control lentivirus as a comparison, since they are a highly transducible cell line (Timmins *et al*, 2024). Due to their smaller size, HEK293T cells were seeded at 25,000 per well of a 96-well plate, and after 24 hours the cells did not appear firmly settled. They were therefore treated 48 hours after plating with 25 μ l control virus in a total volume of 100 μ l, which assuming the cells have doubled to 50,000 was an MOI of 5. It was not possible to use a higher MOI due to limits of total volume and virus:medium ratio. While the transduction efficiency therefore could not be directly compared to that of astrocytes in Figure 5.6A with an MOI of 12.5 in a 60 μ l total volume, it still gave an indication of cell-specific effects.

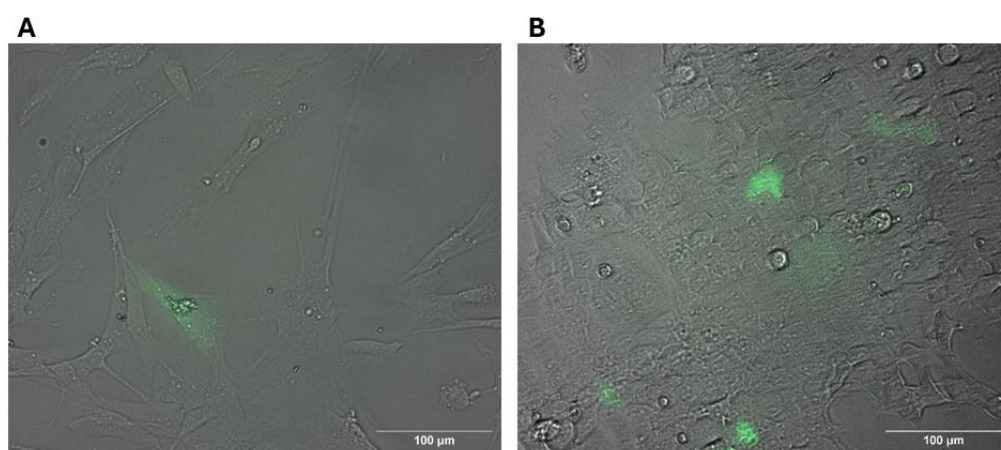


Figure 5.6. Reactive human astrocytes and HEK293T cells transduced with a GFP-expressing lentivirus. A) Human reactive astrocytes transduced with control miRNA inhibitor lentivirus (MOI ~12.5). B) HEK293T cells transduced with control miRNA inhibitor lentivirus (MOI ~5). For both conditions, cells were incubated with lentivirus and 8 μ g/ml polybrene for 24 hours, then the virus was removed and cells were replenished with fresh medium. Cells were imaged 24 hours later at 10x magnification using the brightfield and GFP channel of a widefield microscope. Scale bar = 100 μ m.

As shown in Figure 5.6B, transduction efficiency in HEK293T cells was lower than expected (~5%) for a highly transducible cell line. This may suggest the commercial virus itself was relatively inefficient. Unfortunately, very high MOIs could not be used as the titre is 10⁷ IU/ml, meaning very high volumes would be required to achieve a high MOI, which is likely to be toxic and would not be

possible to treat in a small volume of medium. Therefore, in the future, a virus with a higher titre would ideally be purchased or produced.

To summarise, while more efficient than Lipofectamine-based transfection, treatment of human reactive astrocytes with these commercially supplied lentiviruses still resulted in a very low transduction efficiency. Therefore, bulk approaches to measure gene expression changes, such as RT-qPCR or western blotting, were not practical.

5.4.2 Single cell analysis of lentivirus-mediated inhibition of miR-340-5p and miR-27b-3p in astrocytes

As an alternative to bulk gene expression measurement approaches, immunocytochemistry-directed single cell analysis was explored, as it allows proteins of interest to be measured only in transduced cells, by co-staining with an anti-GFP antibody. Since the aim of this refinement was to measure the Nrf2 pathway by immunocytochemistry (as opposed to RT-qPCR), this first had to be optimised before lentivirus treatments. In Chapter 3 immunocytochemistry was found not to be the most accurate method for comparing the Nrf2 pathway between reactive and quiescent astrocytes, due to the very different cell morphologies. However, it may be appropriate when comparing different treatment groups in reactive astrocytes, which I sought to confirm.

Two commercial Nrf2 antibodies, ProteinTech [16396-1-AP] and Santa Cruz [sc-13032], were used in Chapter 3. The Santa Cruz antibody was used for immunocytochemistry, however it was important to confirm that this was an appropriate choice. As shown in Figure 5.7, the ProteinTech antibody stains relatively evenly throughout reactive astrocytes, contrasting the assumption that transcription factors primarily reside in the nucleus. The Santa Cruz antibody displays a more typical transcription factor staining, with most Nrf2 expression in the nucleus, therefore this antibody was used for future analyses, with concentration reduced slightly from 1:75 to 1:100.

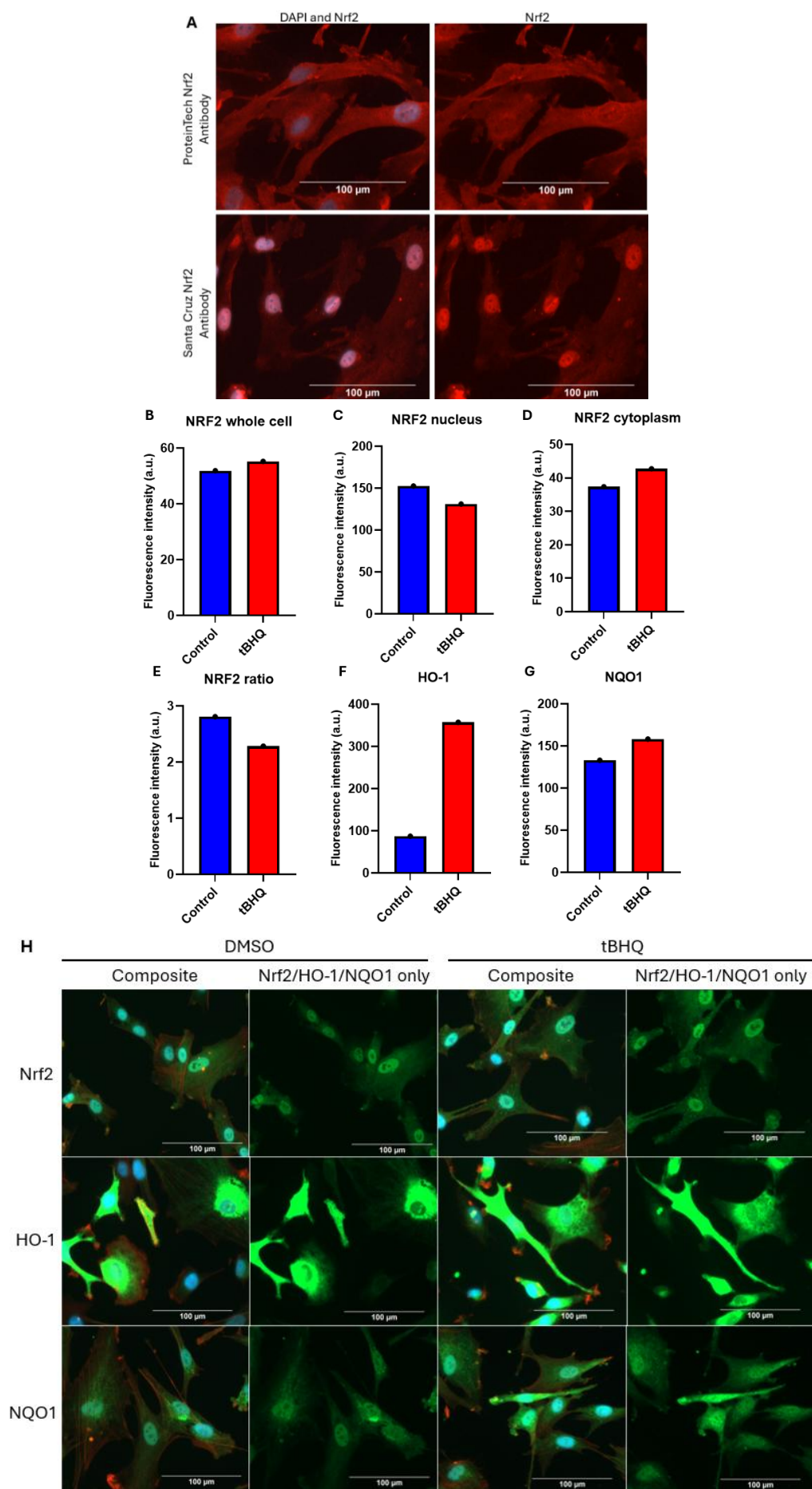


Figure 5.7. Optimisation of immunocytochemistry for single-cell Nrf2 pathway measurement in human reactive astrocytes. A) Human reactive astrocytes were stained for Nrf2 using the ProteinTech [16396-1-AP] antibody (1:300) or Santa Cruz [sc-13032] antibody (1:75, red), while nuclei were stained with DAPI (100nM, blue) (n=1). Cells were imaged at 20x magnification using a widefield microscope (green and blue channels 20% laser/150ms exposure, red channel 40%/150ms). B-G) Reactive astrocytes were treated with tBHQ (10 μ M) or DMSO (control) for 24 hours before immunocytochemistry (n=1). Cells were stained for Nrf2 (1:100, Santa Cruz), HO-1 or NQO1 (1:200, green), in combination with DAPI (100nM) and phalloidin (1:500, red), then imaged (red and blue channels 20%/150ms, green channel 30%/300ms). Fluorescence intensity (a.u.) was measured for each cell using FIJI, then the average background intensity of each image was subtracted. B-E) Fluorescence of Nrf2 was measured in the B) whole cell, C) nucleus only and D) cytoplasm only. E) A nucleus:cytoplasm ratio was calculated for each cell. F) Whole-cell HO-1 fluorescence. G) Whole-cell NQO1 fluorescence. >20 cells were measured from 4 images for each condition. No statistical tests performed (n=1). H) Representative images of DMSO and tBHQ treated reactive astrocytes. Composite images show DAPI (blue), phalloidin (red) and either Nrf2, HO-1, or NQO1 as indicated (green).

To ensure that changes in the expression of Nrf2 and target genes could be quantified by immunocytochemistry, astrocytes were treated with the commercial Nrf2 activator tBHQ (10 μ M, as used for Figure 3.8 in Chapter 3) 24 hours before fixation and staining, for a single exploratory analysis. Representative images are shown in Figure 5.7H. The whole-cell fluorescence intensities of Nrf2, HO-1 and NQO1 protein staining after tBHQ treatment were measured relative to the DMSO control, as shown in Figure 5.7B, F and G. Since Nrf2 translocates from the cytoplasm to the nucleus upon activation, Nrf2 was also measured separately in the nucleus and cytoplasm (Figure 5.7C and D), which was also presented as a nucleus:cytoplasm ratio for each cell (Figure 5.7E).

From the single biological replicate used to optimise methods, it appeared that tBHQ treatment caused a large increase in HO-1 protein expression (~4-fold), while no major change was seen in Nrf2 or NQO1 expression. While tBHQ may not be predicted to increase Nrf2 overall expression, it was surprising that there was not an increase in the nucleus:cytoplasm ratio, since tBHQ activates Nrf2 by blocking its repression by KEAP1 (Abiko *et al.*, 2011). This may suggest that with this method, antibody and astrocyte model, Nrf2 and NQO1 changes cannot be accurately measured by immunocytochemistry, therefore they were not measured in following lentivirus experiments. However, Figure 5.7C indicates that increased HO-1 can be confidently measured by this method. Therefore, to determine whether lentivirus-mediated miRNA inhibition upregulated the Nrf2 pathway, HO-1 expression was used as a readout, measured by immunocytochemistry of transduced cells.

So far, experiments had been performed to determine the optimal conditions for efficient lentivirus treatment and immunocytochemistry measurement of

Nrf2 pathway components. To determine the effect of lentivirus-mediated miRNA inhibition on the Nrf2 pathway, reactive human astrocytes were first seeded in 96-well plates at a density of 5,000 per well. Cells were transduced for 24 hours with control, miR-340-5p or miR-27b-3p inhibitor viruses (12.5µl, MOI 12.5) with polybrene (8µg/ml). The virus was then removed and cells were washed, then after 24 hours the cells were fixed and co-stained for both GFP and HO-1. Cells were imaged using a widefield microscope, with representative images shown in Figure 5.8C. FIJI was used to measure the intensity (mean grey value) of HO-1 in transduced (GFP expressing) cells. These transduced cells will co-express the miRNA inhibitor as it is in the same lentivirus construct as GFP. The threshold for a cell to be considered transduced was a minimum GFP fluorescence value of 5 a.u. (after background intensity removal).

Figure 5.8 shows that neither miR-340-5p or miR-27b-3p inhibition increased HO-1 expression in transduced cells (expressing GFP and miRNA inhibitor), as measured by immunocytochemistry. A limitation of using immunocytochemistry for this study is that expression of the target miRNAs may vary between cells. Since a relatively small number of cells were analysed due to the low transduction efficiency (>20 per condition), this cell-cell variability may have a significant impact. Furthermore, due to the GFP expression induced by lentiviruses, HO-1 had to be measured using a red secondary antibody (Alexa Fluor™ 568 goat anti-rabbit), which from experience may not be as sensitive as a green secondary antibody in this system. Some transduced cells may also not have been 'visually' green (GFP fluorescence <5a.u.), but still had low-level miRNA inhibitor expression.

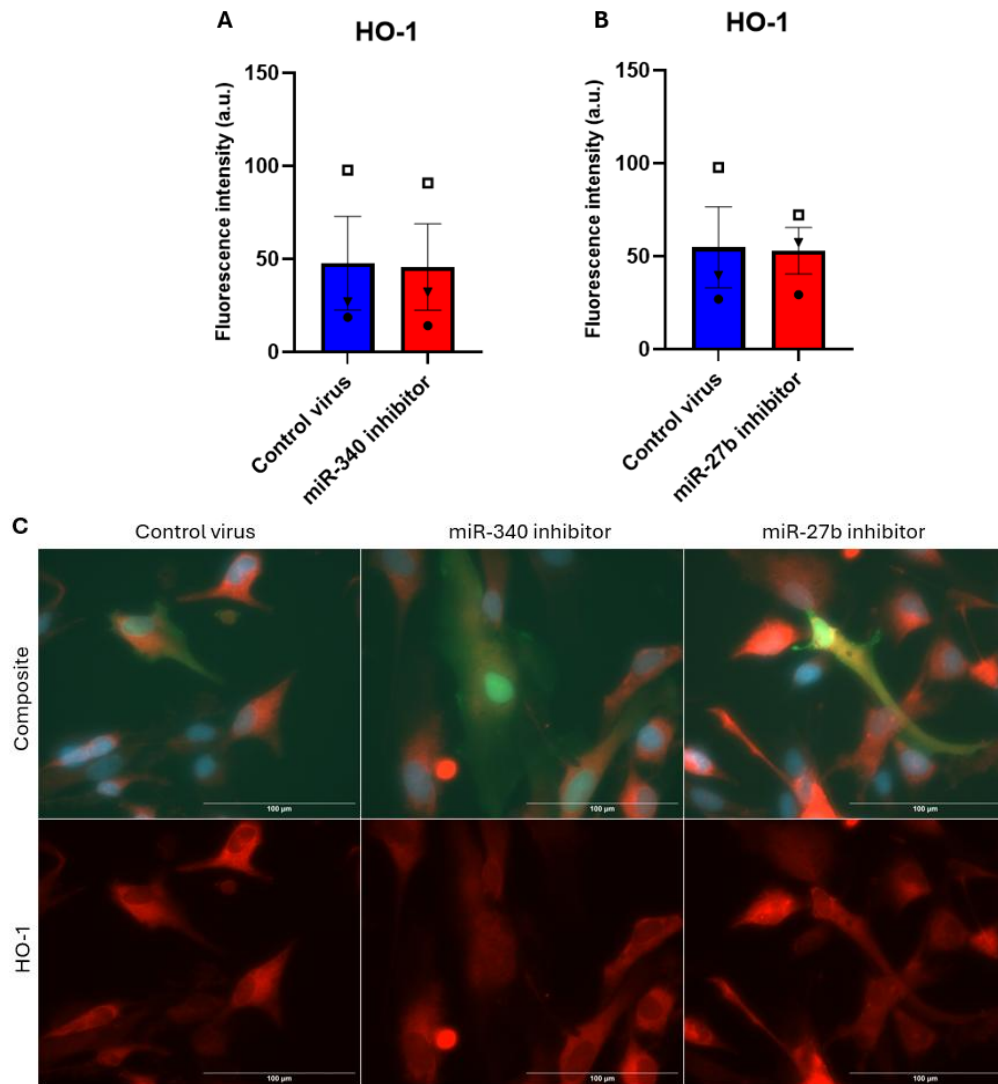


Figure 5.8. Lentivirus-mediated inhibition of miR-340-5p or miR-27b-3p does not upregulate HO-1 expression in reactive human astrocytes, as measured by single-cell immunocytochemistry. Human reactive astrocytes were seeded at a density of 5,000 cells/well of a 96-well plate. After 24 hours they were transduced for 24 hours with control, A) miR-340-5p (miR-340) or B) miR-27b-3p (miR-27b) inhibitor lentivirus constructs (MOI ~12.5) and polybrene (8 μ g/ml). The virus was then removed and medium replaced, then cells were fixed 24 hours later. Immunocytochemistry was used to stain for transduced GFP (1:500) and endogenous HO-1 protein (1:150, red). Nuclei were stained with DAPI (100nM). Cells were imaged at 40x magnification using a widefield microscope in the green and blue channels (20% laser, 150ms exposure) and red channel (50%, 700ms). FIJI was used to measure whole-cell intensity of each protein, then background (average intensity of 3 areas per image) was removed. >20 cells were measured from a minimum of 10 images. HO-1 expression data was only included for cells with a minimum GFP intensity of 5 a.u. Datapoint symbols illustrate the variation between biological replicates. Welch's t-test, n=3. C) Representative images of astrocytes after transduction with control, miR-340 or miR-27b inhibitor lentivirus. Composite = DAPI (blue), HO-1 (red) and GFP (green).

Therefore, RT-qPCR was used for a bulk approach to measure the effects of

miRNA inhibition on the Nrf2 pathway, as RT-qPCR may be a more sensitive method. Due to the low transduction efficiency, significant alteration to the Nrf2 pathway was not anticipated. However, it was important to confirm this by RT-qPCR, before moving on to alternate miRNA inhibition methods. Figure 5.9 shows that as anticipated, lentiviral transduction with neither miR-340-5p or miR-27b-3p inhibitors successfully upregulated the expression of *NRF2* or its target genes *HMOX1*, *NQO1* and *IDH1*. Therefore, the search for a more efficient miRNA inhibitor delivery system for human astrocytes continued, as this would allow sensitive RT-qPCR measurement of the Nrf2 pathway, with confidence that a significant fraction of cells had taken up the inhibitor.

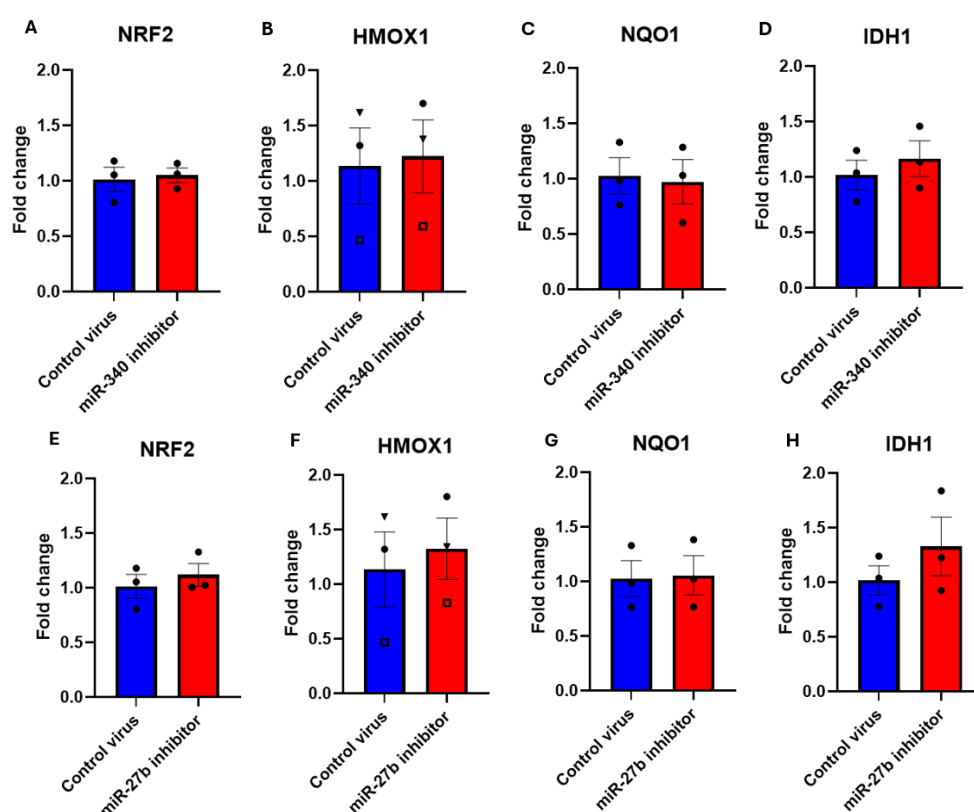


Figure 5.9. Lentivirus-mediated inhibition of miR-340-5p or miR-27b-3p does not upregulate the Nrf2 pathway in human reactive astrocytes, as measured by RT-qPCR. Human reactive astrocytes were seeded in 12-well plates then transduced for 24 hours with control (A-D) miR-340-5p (miR-340) or (E-H) miR-27b-3p (miR-27b) inhibitor lentivirus constructs (MOI ~9.4, reduced from 12.5 in immunocytochemistry experiments due to limited reagents and budget constraints) and polybrene (8 μ g/ml). The virus was then removed and medium was replaced, then RT-qPCR was performed 24 hours later. GAPDH, B2M and 18S were used as housekeepers, and all data was normalised to the average control virus value for each gene. Datapoint symbols illustrate biological replicates for the highly variable HMOX1 gene. A-D) Expression of NRF2, HMOX1, NQO1 and IDH1, respectively, in astrocytes transduced with control or miR-340-5p inhibitor lentivirus. E-H) Expression of NRF2, HMOX1, NQO1 and IDH1, respectively, in astrocytes transduced with control or miR-27b-3p inhibitor lentivirus. Welch's t-test, $n=3$.

5.5 Nucleofection-mediated delivery of miRNA inhibitors to human reactive astrocytes

The final tested method of delivering miRNA inhibitors to primary astrocytes was nucleofectionTM. Nucleofection is a non-viral transfection method that uses electroporation and transfection reagents to open pores in the cell membrane, with reagents and electrical parameters optimised for different cell types (Kane *et al.*, 2008). Nucleofection can be used to deliver many types of therapeutic into the nucleus and cytoplasm, including siRNA, miRNAs, proteins, DNA and mRNA. Compared to traditional electroporation methods, nucleofection facilitates efficient transfection of hard-to-transfect cell types such as primary cells, neurones and iPSCs [Lonza]. However, a possible limitation of electroporation (and nucleofection) is cell injury and toxicity (Batista Napotnik *et al*, 2021).

First, reactive human astrocytes were nucleofected with pmaxGFP plasmid to determine transfection efficiency. Briefly, 3 million cells were incubated in P3 (primary cell) nucleofection reagents (including 4µg pmaxGFP) for 15 minutes, transferred to a 100µl Nucleocuvette® and nucleofected in the 4D-NucleofectorTM Core Unit using the CA 137 pulse code for primary cell types. The suspension was immediately added to fresh astrocyte medium and seeded, including 15,000 cells per well of an ibidi dish. Such a high number of cells were nucleofected, and seeded at ~3x the normal density, as it was predicted that nucleofection may be highly toxic. Cells were then imaged 24 hours later. As the representative image in Figure 5.10 shows, nucleofection did not appear to be toxic, although I aimed to confirm this by resazurin assay, which will be presented in the next section. Therefore, for all future nucleofection studies, cell numbers were scaled down considerably while the ratio of pmaxGFP plasmid to cells was kept constant, for example 0.7µg plasmid with 500,000 cells. Anecdotally, nucleofection appeared to be less toxic in primary astrocytes than in iPSC-derived motor neurones used by other members of our research group (personal communications). Conversely, Lipofectamine RNAiMAX reagent was highly toxic to astrocytes but not iPSC-motor neurones, reinforcing that the most effective method for miRNA inhibitor delivery must be optimised for individual cell types.

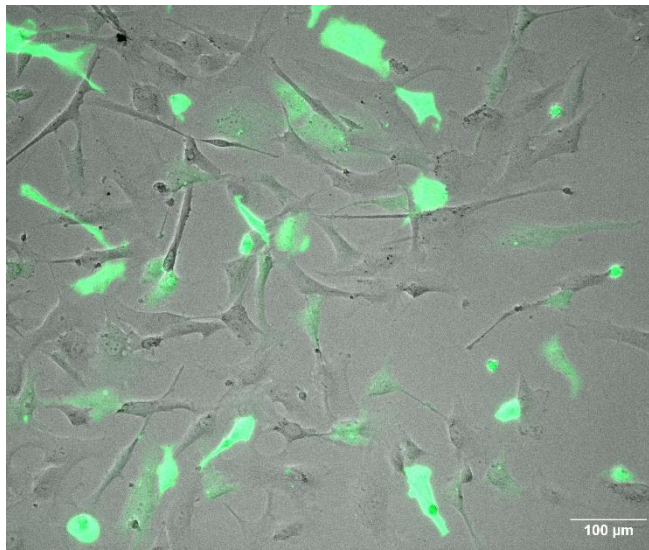


Figure 5.10. Nucleofection is an efficient method for delivering pmaxGFP plasmid to human reactive astrocytes. ~3 million human reactive astrocytes were nucleofected with 4μg pmaxGFP plasmid and seeded at a density of 15,000 cells/well in ibidi dishes. A representative image from 24 hours later is shown with overlaid brightfield and GFP channels, taken using a widefield microscope at 10x magnification. Scale bar = 100μm.

Most importantly, Figure 5.10 shows that ~50% of reactive astrocytes were successfully transfected with pmaxGFP plasmid. Comparing the efficiencies of different delivery methods in a quantitative manner was not possible, since power inhibitors do not fluoresce, and lentiviruses use a different GFP expression system to the pmaxGFP plasmid. Despite this, it was clear from Figure 5.10 that nucleofection was by far the most efficient delivery system tested, at least for those which can be monitored using a GFP reporter. Therefore, I moved on to deliver miRNA inhibitors via nucleofection to human primary astrocytes.

5.5.1 Nucleofection-mediated delivery of miR-340-5p and miR-27b-3p power inhibitors to human reactive astrocytes

For initial nucleofection experiments, miR-340-5p and miR-27b-3p PIs (power inhibitors) were used, as opposed to 'standard' non-PIs. While the modifications of PIs were not required here, it allowed pilot studies to be performed using existing reagents. The concentration of PI selected was a ratio of 8μl (50μM stock) to 400,000 cells, counted before nucleofection, which was the highest concentration possible with the existing reagents. When all cells were seeded in 1 well of a 6-well plate, this approximately equated to a final PI concentration of 200nM.

Reactive astrocytes were nucleofected with control, miR-340-5p and miR-27b-3p PI, cultured for 24 hours, then RT-qPCR was performed to measure Nrf2

pathway gene expression. Figure 5.11 shows that from two replicates, there were no obvious Nrf2 pathway changes following PI treatment (although statistical tests were not performed) other than a possible increase in *HMOX1* expression after miR-340-5p inhibition, and a decrease in *NQO1* expression after miR-27b-3p inhibition. MiR-340-5p inhibition was also tested in a third experiment (n=1), where cells were left for 48 hours to allow more time to recover from the stress of nucleofection (data not shown). However, the HO-1 increase was not observed, perhaps suggesting the miRNA inhibitor effect is short lived.

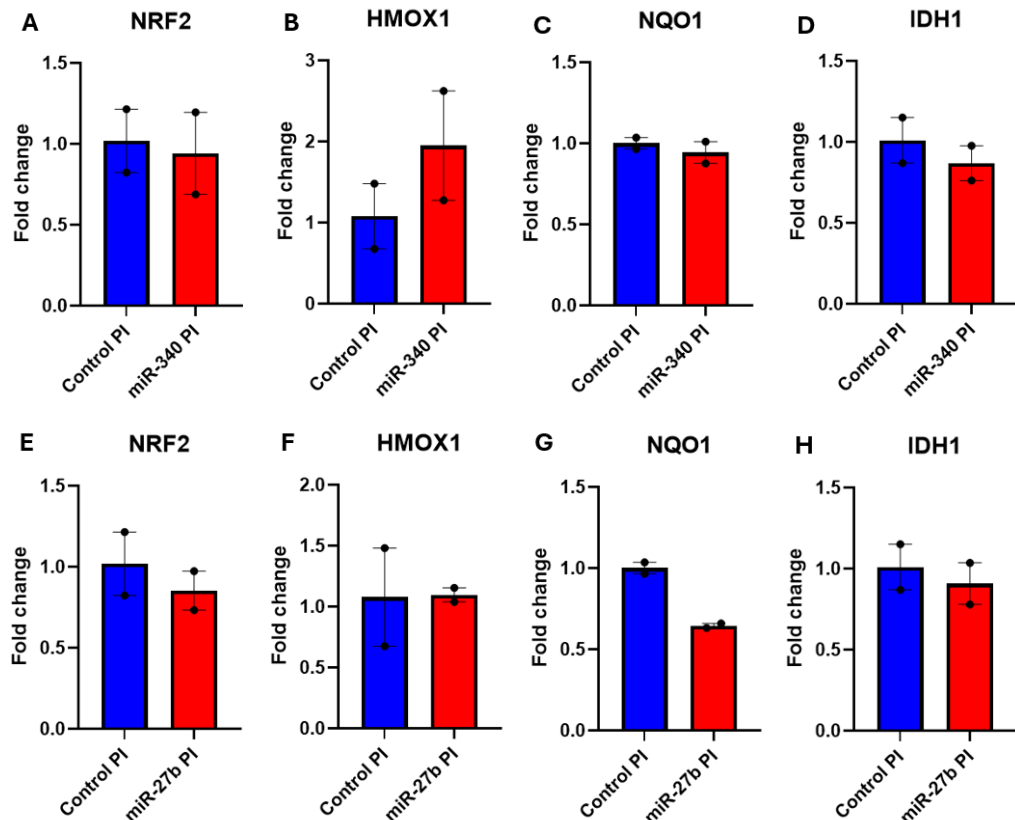


Figure 5.11. Nucleofection of reactive human astrocytes with miR-340-5p power inhibitor may increase HMOX1 expression. Human reactive astrocytes were nucleofected with control, miR-340-5p (miR-340) or miR-27b-3p (miR-27b) PI at a final concentration of 200nM, then plated in a 6-well plate. After 24 hours, RT-qPCR was performed to measure expression of Nrf2 pathway genes. A-D) Expression of NRF2, HMOX1, NQO1 and IDH1 in astrocytes treated with control or miR-340-5p PI. E-H) Expression of NRF2, HMOX1, NQO1 and IDH1 in astrocytes treated with control or miR-27b-3p PI. GAPDH and B2M used as housekeepers, and all values were normalised to the average control PI value for each gene. No statistical test performed (n=2).

5.5.2 Nucleofection-mediated delivery of standard miR-340-5p inhibitor to human reactive astrocytes

In case the overall lack of Nrf2 pathway changes was due to the PI phosphorothioate modifications interfering with the nucleofection process, standard non-power inhibitors were evaluated for future investigations. These inhibitors were also miRCURY LNA miRNA Inhibitors [Qiagen], therefore were otherwise identical to the PIs, except from possessing normal phosphodiester nucleotide bonds. Standard inhibitors require transfection, with nucleofection used as the transfection method here. Only miR-340-5p inhibition was continued with, since there was only a trend towards *HMOX1* upregulation when miR-340-5p was inhibited in Figure 5.11, not miR-27b-3p. Furthermore, the trend towards decreased *NQO1* expression after miR-27b-3p inhibition in Figure 5.11G may indicate an undesired decrease in Nrf2 activity.

While subjective visual assessment had so far suggested that nucleofection was relatively non-toxic to reactive astrocytes, a resazurin cell viability assay which measures metabolically active cells was performed to confirm this assumption. This was performed at this time, not earlier with a pmaxGFP plasmid, as it was important to account for any toxicity of the selected miRNA inhibitors. 600,000 reactive astrocytes were nucleofected with 12µl control or miR-340-5p standard inhibitor (the same inhibitor:cell ratio as for the PIs) and seeded in a black 96-well plate in triplicate at a density of 5,000 cells/well, in addition to non-nucleofected controls. The spare nucleofected cells were used for the experiments described in the next section. PmaxGFP plasmid was not included, to avoid fluorescence interference during the resazurin assay and the other experiments (next section). 24 hours after seeding, cells were washed with PBS and incubated for 2 hours with resazurin in PBS (1mg/ml), before fluorescence was measured at 540nm excitation/590-10nm emission. Figure 5.12 confirmed that nucleofection with either control or miR-340 inhibitor is not toxic to reactive astrocytes, compared to a non-nucleofected control.

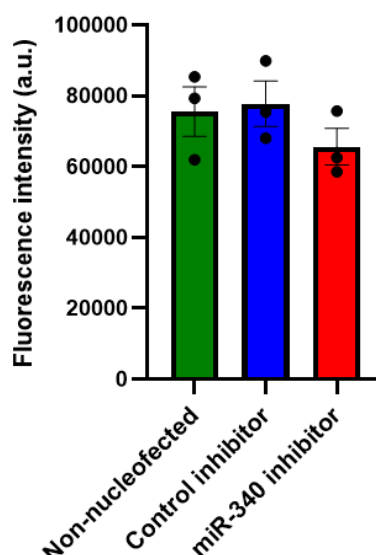


Figure 5.12. Nucleofection with control or miR-340-5p inhibitor is not toxic to reactive human astrocytes. Human reactive astrocytes were nucleofected with control or miR-340-5p (miR-340) standard inhibitor and seeded in black 96-well plates at a density of 5,000 cells/well along with non-nucleofected astrocytes. After 24 hours, cells were incubated with resazurin (1mg/ml) for 2 hours, before measuring relative fluorescence (540ex/590-10em) which corresponds to cell viability. Cell-free buffer-only (resazurin solution) fluorescence was subtracted from each value. Three biological replicates (n=3) were each the average of 3 technical replicates (wells). One-way ANOVA used.

To conclude, nucleofection had so far been shown to be an efficient and non-toxic method of delivering material to reactive astrocytes. The RT-qPCR data in Figure 5.11 suggested that nucleofection with miR-340-5p PI may cause a slight increase in *HMOX1* expression, perhaps indicating subtle activation of the Nrf2 pathway. To explore this further using a standard miR-340-5p inhibitor, another proteomic analysis was performed.

5.5.2.1 Proteomic analysis of human reactive astrocytes nucleofected with a miR-340-5p standard inhibitor

Instead of continuing with a candidate RT-qPCR approach, and given that the challenge of efficient nucleic acid delivery to astrocytes appeared to have been overcome using nucleofection, a proteomic analysis was conducted to more broadly determine the impact of miR-340-5p inhibition (via nucleofection) at the molecular level. This allowed the measurement of a large panel of Nrf2 target gene products in astrocytes, as previously presented in the proteomic analysis of Chapter 3 (section 3.2.2.1), in addition to other gene targets of miR-340-5p. An additional benefit of proteomics over RT-qPCR is the measurement of protein rather than mRNA, since mRNA changes are not always reflected at the protein level. Importantly, the SWATH-MS proteomic analysis method used here was the same that identified the Nrf2 pathway deficit in reactive astrocytes in Chapter 3, meaning it was an appropriate method to determine whether the Nrf2 pathway had been rescued by miR-340-5p inhibition.

To generate samples for proteomic analysis, 600,000 reactive human astrocytes were nucleofected with 12µl miR-340-5p or control standard inhibitor (50µM stock). A GFP plasmid was not added since the efficiency of nucleofection had already been confirmed to be ~50%. Furthermore, this avoided additional alteration to the proteome and allowed some of the nucleofected cells to be used for calcium imaging, which will be discussed shortly, and the resazurin assay, all in parallel. For the proteomic samples, 350,000 nucleofected astrocytes were seeded in one well of a 6-well plate, therefore the final concentration of inhibitor was approximately 175nM (~7µl in ~2ml medium). After 24 hours, cells were pelleted and frozen at -20°C. All three biological replicates were lysed simultaneously in RIPA buffer with protease and phosphatase inhibitors (1:1000) then quantified by BCA assay. While 5µg of cell lysate was used for SWATH-MS in Chapter 3, here 20µg was used since more protein was available, which was reasoned to improve proteome-wide detection.

The same methods for SWATH-MS and data analysis were used as described in Chapter 3. Briefly, control and miR-340-5p inhibited astrocyte samples were analysed in triplicate, with trypsin digestion of proteins followed by SWATH-MS in DIA mode. For analysis, after the removal of non-human contaminant proteins, 3954 protein groups were detected across the six samples. This was lower than the number of protein groups in batch 1 in Chapter 3 (4858 groups), however it should be noted that the proteins detected in batch 1 were used as a reference library for this proteomic analysis, so no additional proteins could be detected. StatsPro analysis was conducted to remove protein groups (unique proteins or groups of similar (ambiguous) proteins which cannot be distinguished) with more than 30% variance or detection in less than 4/6 samples, resulting in a total of 2009 protein groups. Numbers of unique and ambiguous protein groups before and after StatsPro analysis are presented in Table 5.2.

Table 5.2. Number of protein groups detected by SWATH-MS proteomic analysis of reactive human astrocytes nucleofected with control or miR-340-5p standard inhibitor.

Protein groups detected in at least 1 astrocyte sample (before StatsPro)			Protein groups detected after StatsPro filtering		
Total protein groups	Unique proteins	Ambiguous protein groups	Total protein groups	Unique proteins	Ambiguous protein groups
3954	3603	351	2009	1832	177

Control and miR-340-5p standard inhibitor nucleofected human reactive astrocytes were analysed (n=3). Protein groups consist of unique proteins, where peptide sequences can be assigned to one protein with certainty, and ambiguous protein groups, where peptide sequences may belong to multiple proteins. StatsPro filtering removed proteins with variance of >30% or detection in less than 4/6 samples.

As in Chapter 3, ambiguous protein groups were removed for further analysis, since these similar proteins (such as isoforms) could not be distinguished. Using the same conditions for differential expression analysis as in Chapter 3 (limma test $p_{adj} < 0.05$, $\text{Log}_2\text{FC} > \pm 0.5$), there were no differentially expressed proteins between control and miR-340-5p inhibitor nucleofected astrocytes, as shown in Figure 5.13A. This indicates that the miRNA inhibitor treatment may not have been successful, at least to the extent required to cause significant changes in protein expression.

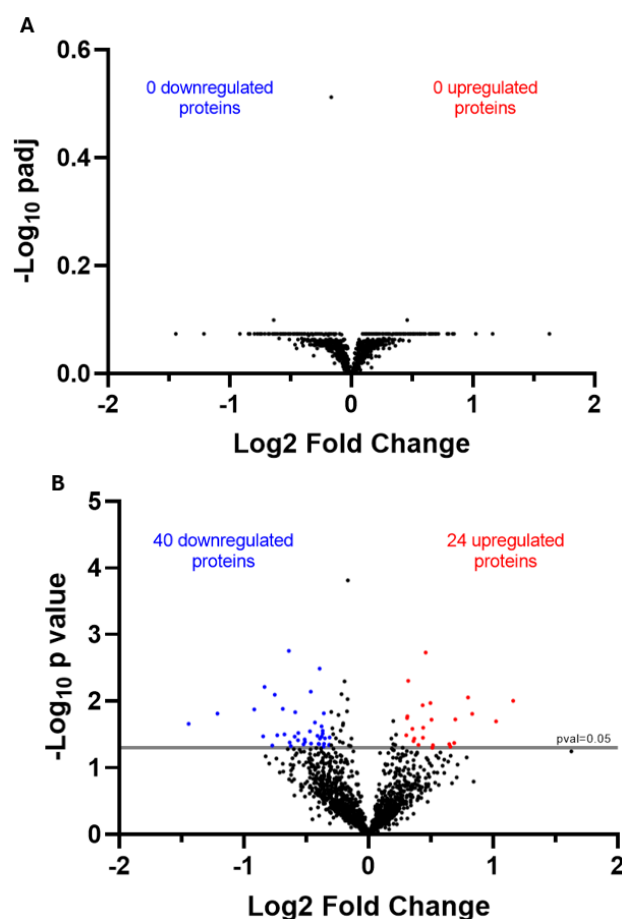


Figure 5.13. Volcano plots showing differential expression of proteins upon miR-340-5p inhibition in reactive human astrocytes, determined by SWATH-MS. Human reactive astrocytes were nucleofected with control or miR-340-5p standard inhibitor and lysed 24 hours later for SWATH-MS proteomic analysis ($n=3$). Data was analysed using StatsPro and the limma statistical test. A) Log_2 fold change (FC) plotted against $-\text{Log}_{10}p_{adj}$. There were no significantly differentially expressed proteins using the criteria $\text{Log}_2\text{FC} > \pm 0.5$, $p_{adj} < 0.05$ (note that $p_{adj}=0.05$ is above the scale of the y axis). B) Log_2FC plotted against $-\text{Log}_{10}p$ value (p_{val}). Grey line is at $p_{val}=0.05$. Criteria for differential expression was reduced to $\text{Log}_2\text{FC} > \pm 0.3$, $p_{val} < 0.05$. Colour change is at $\text{Log}_2\text{FC} > \pm 0.3$ (for statistically significant proteins).

To identify any possible protein expression changes with lower FCs than above, the stringency for differential expression was reduced to p value < 0.05 and $\text{Log}_2\text{FC} > \pm 0.3$ (equating to a fold change of only ± 1.23). This identified 64 proteins differentially expressed between control and miR-340-5p inhibitor

nucleofected human astrocytes, as represented in Figure 5.13B. None of these proteins were one of the 47 Nrf2 target genes listed in section 1.4.1, of which 12 were differentially expressed between quiescent and reactive astrocytes in the proteomic analysis presented in Chapter 3. This illustrated that Nrf2 pathway upregulation had not been successful by nucleofection with miR-340-5p inhibitor.

Expression of other miR-340-5p target gene products was then examined, to evaluate whether miR-340-5p inhibition altered expression of other miR-340-5p target genes. All 1393 predicted gene targets of miR-340-5p (according to TargetScan, with no additional stringency for target prediction) were compared against the 64 differentially expressed proteins. Of these proteins, 6 were predicted miR-340-5p targets, namely DPP9, PDS5B, PRKCA, LLPH, EIF2S1 and GNA13. All 6 proteins were downregulated upon miR-340-5p inhibition, the *opposite* direction to what would be predicted of a miR-340-5p target gene. Considering this general failure to upregulate expression of miR-340-5p target genes, and the lack of effect on the Nrf2 pathway, it therefore appears highly unlikely that miR-340-5p inhibition was successful by this method.

While this lack of efficacy may be due to the use of a standard miR-340-5p inhibitor, rather than PI, the high transfection efficiency of nucleofection should facilitate efficient delivery of both inhibitor types. Both miRNA inhibitors should be protected from nucleases as they are LNA-modified. The RT-qPCR results using miR-340-5p PI in Figure 5.11 did not provide strong evidence for Nrf2 pathway upregulation, causing only a very slight *HMOX1* increase (n=2). This result therefore may be due to chance since *HMOX1* expression is generally highly variable, instead suggesting that miRNA inhibitors had not been successfully delivered by nucleofection.

As mentioned earlier, calcium imaging was also performed on reactive astrocytes that had been nucleofected with control or miR-340-5p standard inhibitor 24 hours previously. Calcium imaging was selected as a functional readout of miR-340-5p inhibitor treatment (in the event that molecular measurements were problematic), as Nrf2 had previously been shown to regulate intracellular calcium, including in human astrocytes. Specifically, oxidative protein folding in the ER produces H₂O₂, which activates Nrf2. Nrf2 then activates expression of glutathione peroxidase 8 (GPx8), a calcium-regulating ER protein (Granatiero *et al*, 2019; Yoboue *et al*, 2017). This pathway was shown to be disrupted in human mSOD1 iPSC-derived astrocytes, resulting in Nrf2-mediated calcium dysregulation, which induced motor neurone death (Granatiero *et al.*, 2019).

However as discussed, the proteomic analysis confirmed that miR-340-5p inhibition was not successful in these reactive astrocytes, since there were no rational or statistically significant changes in protein expression. As a result, miR-340-5p inhibition via nucleofection did not affect Ca²⁺ signal in reactive astrocytes after stimulation with glutamate or ATP, as shown in Appendix Figure 9.4. Therefore, Chapter 6 pivots to the commercial Nrf2 activator tBHQ to

further investigate possible effects of chemical Nrf2 activation on reactive human astrocyte calcium signalling.

5.6 Conclusions

In this chapter, I aimed to upregulate the Nrf2 pathway in human primary FBS-induced reactive astrocytes by inhibiting the Nrf2-targeting miRNAs miR-340-5p and miR-27b-3p. One of the major technical challenges of this chapter was achieving efficient delivery of miRNA inhibitors, which in most systems could be monitored via GFP expression. The delivery systems tested in reactive astrocytes were Lipofectamine 2000 and Lipofectamine RNAiMAX transfection reagents, direct and ADEV-mediated delivery of miRNA PIs, lentiviruses and finally nucleofection of both power and standard miRNA inhibitors. The main findings from these methods are summarised in Table 5.3.

Table 5.3. Summary of methods tested for miRNA inhibitor delivery to reactive human astrocytes.

Astrocyte treatment/delivery method	Delivery efficiency	Main findings
Lipofectamine 2000	Very low (<1%)	Poor delivery of pmaxGFP plasmid.
Lipofectamine RNAiMAX	Unknown	Highly toxic.
miRNA PIs	Unknown	Nrf2 pathway unaffected by miR-27b-3p or miR-340-5p PIs, when delivered directly or via ADEVs.
Lentivirus miRNA inhibitors	Low (~5-10%)	Nrf2 pathway unaffected at single cell or bulk level by miR-27b-3p or miR-340-5p lentivirus-mediated inhibition.
Nucleofection of miRNA PIs	Relatively high (~50%)	Efficient delivery of pmaxGFP plasmid, but Nrf2 pathway unaffected by nucleofection with miR-340-5p or miR-27b-3p PI.
Nucleofection of standard miRNA inhibitors	Relatively high (~50%)	Nucleofection with miR-340-5p standard inhibitor caused no rational protein expression changes of Nrf2 pathway genes or miR-340-5p target genes, determined by proteomic analysis.

Some methods were not used for miRNA inhibitor delivery due to toxicity or low transfection efficiency. Delivery efficiencies are estimated from subjective visual microscopic assessment of GFP expression.

Neither Lipofectamine transfection reagent was taken forward for delivery of miRNA inhibitors, since RNAiMAX was highly toxic and Lipofectamine 2000 delivered a pmaxGFP plasmid to <1% of cells. While it was not possible to

determine the efficiency of miRNA PIs as there was no fluorescent tag to monitor delivery, neither miR-340-5p or miR-27b-3p PI upregulated the Nrf2 pathway, as measured by RT-qPCR, including when miR-340-5p was incubated with reactive ADEVs to enhance transfection efficiency (n=1). Lentiviruses were more efficient than Lipofectamine 2000 at inducing GFP expression in astrocytes, however lentivirus-encoded miR-340-5p and miR-27b-3p inhibitors also did not upregulate the Nrf2 pathway, as measured by immunocytochemistry of transduced cells, or by bulk RT-qPCR. If lentiviruses were used again for miRNA inhibitor studies, fluorescence-activated cell sorting (FACS) could be used to isolate the population of only GFP-expressing cells before RT-qPCR. However, due to the low transduction efficiency considerable reagents and cells would be required, therefore this approach could not be pursued. Similarly, a stable cell line of transduced cells could be selected for as the lentiviruses encoded a puromycin resistance gene, however this was not possible in this astrocyte model due to the low passage limit (p7).

Finally, nucleofection was found to be by far the most efficient method for delivering pmaxGFP plasmid to reactive astrocytes (~50% transfection efficiency), which was assumed to also extend to miRNA inhibitor delivery. Despite involving electroporation, nucleofection was also non-toxic, as quantified by a resazurin cell viability assay. RT-qPCR (n=2) of reactive astrocytes nucleofected with miR-340-5p PI suggested a slight increase in *HMOX1* expression compared to control, while miR-27b-3p PI may have reduced *NQO1* expression, therefore miR-340-5p was taken forward as the more promising candidate. However, a SWATH-MS proteomic analysis of reactive astrocytes nucleofected with a standard miR-340-5p inhibitor (since phosphorothioate linkages were no longer required) found no evidence of successful miR-340-5p inhibition. Specifically, no miR-340-5p target gene products or Nrf2 pathway gene products were significantly upregulated in miR-340-5p inhibited cells, even when the significance threshold for expression changes was reduced from $\text{Log2FC} \geq \pm 0.5$, $\text{padj} < 0.05$ to $\text{Log2FC} \geq \pm 0.3$, $\text{pval} < 0.05$. Therefore, I did not identify an effective miRNA-targeted treatment for astrocytic Nrf2 pathway upregulation, as a possible future therapeutic strategy for ALS.

It is unfortunately unclear why miR-27b-3p and miR-340-5p inhibition did not appear to be 'functional' in these human astrocytes. MiRNA candidates were selected based on multiple avenues of evidence, and delivery of inhibitors to astrocytes was optimised thoroughly. It is also unlikely to be due to inaccuracies in measuring the Nrf2 pathway as multiple methods were used, including a thorough proteomic analysis to analyse additional miRNA target genes. In the future, perhaps further investigation into the best concentration and timepoints for inhibitor treatment should be carried out. While these factors were considered in this chapter, decisions were generally made based on the best conditions for efficient inhibitor delivery, or Nrf2 pathway RT-qPCR results. However, expression changes in other miRNA target genes were not considered, which perhaps would aid decision making for optimisation. A possible factor contributing to the poor miRNA targeting in reactive astrocytes

may be RBP dysregulation (as indicated by the motif analysis in Chapter 4) impacting the RNA interference pathway, for example RBPs may aberrantly sequester miRNA inhibitors. Over 50 RBPs have previously been shown to bind to ASOs (Liang *et al*, 2015), including the ALS-associated hnRNPA1 (Abdul-Manan & Williams, 1996), therefore similar may be predicted for miRNA inhibitors.

Despite this failure to establish a miRNA-targeted approach, Nrf2 upregulation in astrocytes remains a possible ALS therapeutic strategy which should be explored further. In Chapter 6, the commercial Nrf2 activator tBHQ was used to explore the broader impact of Nrf2 activation in reactive astrocytes, and to characterise these reactive astrocytes as a simple disease-relevant model for screening Nrf2-activating compounds. Furthermore, the effects of riluzole and edaravone on astrocyte function will also be investigated, including to establish the extent to which Nrf2 activation in astrocytes may already be achieved by current ALS therapeutics.

Chapter 6. Investigating the effects of Nrf2 pathway manipulation and ALS therapeutics on human primary astrocyte phenotypes

6.1 Introduction

In Chapter 5, I was unable to demonstrate upregulation of the Nrf2 pathway in human reactive astrocytes through attempts to inhibit specific miRNAs. One possible rationale for this observation is that this reactive astrocyte model is somehow 'resistant' to therapeutic upregulation of Nrf2. Preliminary data (Figure 5.7, n=1) in Chapter 5 suggested that tBHQ treatment increased expression of Nrf2 target gene product HO-1 judged by immunocytochemistry, however Nrf2 and NQO1 expression and Nrf2 subcellular localisation was not affected. Therefore, further validation of this exploratory analysis was required to confirm that a chemical Nrf2 activator is functional in this model. If chemical rescue of the Nrf2 pathway deficit phenotype could be demonstrated, these reactive astrocytes could still represent a platform to screen for modulators of this ALS-relevant phenotype.

This chapter therefore broadened the investigation of tBHQ-induced Nrf2 activation in reactive astrocytes, including measurement of several Nrf2 pathway gene products by RT-qPCR and the resultant impact on cellular ROS levels. Cell viability was also measured to investigate the effects of tBHQ-mediated Nrf2 activation on general astrocyte health. Calcium imaging was used to measure astrocyte activity and facilitate predictions as to the effects of Nrf2 activation on excitotoxicity, since Ca^{2+} influx can lead to gliotransmitter release (Goenaga *et al.*, 2023). As discussed in Chapter 5, Nrf2 may have a role in regulating Ca^{2+} homeostasis (Granatiero *et al.*, 2019), therefore the effects of inhibiting an Nrf2-targeting miRNA (miR-340-5p) on Ca^{2+} signalling was investigated. However, because the Nrf2 pathway was not upregulated by the miRNA inhibitor, unsurprisingly there were no significant effects on Ca^{2+} signalling, therefore here a commercial Nrf2 activator was substituted. Although these studies could in the future be expanded to further Nrf2 activators, as a simple proof-of-concept study they were limited to tBHQ (Arslanbaeva & Bisaglia, 2022).

By thoroughly investigating the effects of Nrf2 activation in astrocytes using several functional assays, I hope to demonstrate the utility of this reactive astrocyte model for screening for future Nrf2 activators. This convenient disease-relevant cell model is a valuable tool for the ALS field, since many similar studies rely on animal models and expensive, time-consuming iPSC-derived cell models which only model certain genetic forms of the disease (Keerie *et al.*, 2025; Mead *et al.*, 2013; Vargas *et al.*, 2008). The absence of a genetic lesion in this model means that it may also be of relevance to sporadic

ALS, where astrocyte reactivity is also a driving mechanism. Initial drug screening could be performed in our simple model, before validation of promising candidates in ALS-specific models. Furthermore, astrocyte reactivity is not specific to ALS, so this model could also be useful for researching other neurological or neurodegenerative diseases.

In this thesis, rather than screening further Nrf2 activators, the described assays were extended to investigate the effects of ALS therapeutics riluzole and edaravone. Despite the importance of astrocytes in ALS pathogenesis, the mechanistic effects of riluzole and edaravone on astrocyte function are poorly understood. When pursuing novel astrocyte-targeting Nrf2 activating therapeutics, it is important to consider whether they improve upon the current standard of care ALS therapeutics. If edaravone or riluzole adequately activate and rescue the Nrf2 pathway and reduce ROS in reactive astrocytes, then this may suggest that an additional astrocyte Nrf2-targeted therapy is not warranted. Conversely, if these compounds do not affect or in fact repress the Nrf2 pathway, or have any undesirable effects on cell viability or Ca^{2+} signalling, this may partially explain their limited efficacy for ALS treatment and solidify the need for a novel astrocyte-targeted Nrf2-activating therapeutic.

To conclude, this chapter investigated the effects of direct Nrf2 activation (via tBHQ), edaravone and riluzole on reactive astrocyte health, function and antioxidant response. This may help direct the best small molecule ALS therapeutic strategy for targeting astrocyte-mediated neurodegeneration. In the following sections, brief summaries of tBHQ, edaravone and riluzole will be presented, focussing on any evidence for Nrf2 pathway modulation and astrocytic effects. Astrocyte excitability and Ca^{2+} signalling will then be discussed, since calcium imaging is an important functional readout of astrocyte activity in this chapter.

6.1.1 tBHQ

Tert-butylhydroquinone (tBHQ) is an FDA-approved food additive which is a well-characterised antioxidant and Nrf2 activator (Khezerlou *et al.*, 2022). tBHQ auto-oxidises to tert-butylbenzoquinone (tBQ), which binds covalently to thiol groups of critical KEAP1 (an E3 ubiquitin ligase for Nrf2) cysteine residues, therefore releasing Nrf2 from Keap1-mediated repression (Abiko *et al.*, 2011). In a mixed mouse cortical culture, tBHQ selectively activated Nrf2 in astrocytes over neurones, which was sufficient to protect neurones from H_2O_2 -induced oxidative stress. Importantly, the neuroprotective effects of tBHQ were dependent on Nrf2 activation, confirming the suitability of tBHQ for studying Nrf2 activation in astrocytes as a neuroprotective strategy (Kraft *et al.*, 2004).

The neuroprotective effects of tBHQ extend beyond its antioxidant function, for example tBHQ treatment of glia was shown to protect neurones from glutamate-induced excitotoxicity in rat mixed cortical cultures (Shih *et al.*, 2003). Nrf2 activation via tBHQ has also been shown to reduce astrocyte reactivity in a rat model of traumatic brain injury, illustrated by reduced hypertrophy and GFAP expression (Zhang *et al.*, 2020). This may be

hypothesised to be due to tBHQ-mediated promotion of the anti-inflammatory functions of Nrf2, for example blocking NF- κ B signalling (Liu *et al.*, 2023b). To my knowledge, tBHQ has not been suggested as an ALS treatment, although it has been shown to improve the neuroprotective effects of astrocytes (Vargas *et al.*, 2005). A tBHQ-containing diet was shown to reduce amyloid β load in the brain of AD model mice (Akhter *et al.*, 2011).

6.1.2 Edaravone

Edaravone (3-Methyl-1-phenyl-2-pyrazolin-5-one/ Radicava®) was approved for ALS treatment by the FDA in 2017, however it is not currently approved by the European Medical Agency (EMA) (Rothstein, 2017). Edaravone is a free radical scavenger and antioxidant. It scavenges lipid peroxy radicals to prevent lipid peroxidation and scavenges peroxynitrite (ONOO⁻) to prevent its reaction with protein tyrosine residues to produce 3-nitrotyrosine residues (Fujisawa & Yamamoto, 2016). Consequently, edaravone was shown to reduce 3-nitrotyrosine levels in ALS patient CSF, indicating reduced oxidative stress (Yoshino & Kimura, 2006).

In addition to scavenging free radicals, edaravone may combat oxidative stress by upregulating the Nrf2 pathway. Edaravone was shown to activate the Nrf2 pathway in neurone-like PC12 cells, an interaction which was necessary for edaravone-mediated neuroprotection after chlorpyrifos (pesticide)-induced oxidative stress injury (Shou *et al.*, 2019; Wu *et al.*, 2017). Edaravone also activated the Nrf2 pathway in human corneal epithelial cells and a mouse model of asthma (Li *et al.*, 2017; Pan *et al.*, 2020). Edaravone counteracts the downregulation of the Nrf2 pathway induced by amyloid β_{25-35} , kainate and depression, in SH-SY5Y cells, rats and mice respectively (Dang *et al.*, 2022; Liu *et al.*, 2018; Zhang *et al.*, 2019c).

Regarding the effects of edaravone on astrocytes, oxidative stress induced by LPS or H₂O₂ in rat astrocytes was attenuated by edaravone, via reactivation of Akt signalling, an important pathway for regulating the antioxidant response (Goetting *et al.*, 2022; Guo *et al.*, 2020). The antioxidant activity of edaravone was also shown to reduce carbon monoxide-induced apoptosis of rat astrocytes, as the reduction in ROS inhibited the mitochondrial apoptosis pathway (Xu *et al.*, 2017).

Several studies also suggest that edaravone can attenuate astrocyte inflammation and reactivity, for example it reduced traumatic brain injury-induced astrocyte activation in rats (Wang *et al.*, 2011a). Edaravone reduced inflammation in LPS-treated rat astrocytes, indicated by reduced expression of TNF- α , IL-1 β , IL-6 and NOS2 (also indicating reduced oxidative stress), and reduced LPS-induced morphological changes such as smaller nuclei and dense branching (Guo *et al.*, 2020). Similarly, a preprint study by Liu *et al.* found that edaravone repressed the JAK2/STAT3 pathway after cerebral ischemia, a pathway which has been shown to regulate astrocyte reactivity (Herrmann *et al.*, 2008; Liu *et al.*, 2023a). It also reduced expression of A1 (neurotoxic) astrocyte markers C3 and Serpin G1 and increased A2 (neuroprotective)

markers pentraxin 3 (PTX3) and sphingosine kinase 1 (SPHK1) (Liu *et al.*, 2023a). Edaravone in a 4:1 combination with (+)-borneol (an anti-inflammatory, neuroprotective natural product), known as edaravone dexborneol, was shown to prevent activation of neurotoxic A1 astrocytes in response to cerebral ischemia via inhibition of NF- κ B signalling, resulting in reduced neuronal death (Chen *et al.*, 2024; Gao *et al.*, 2023). Edaravone also stimulates GDNF release from human foetal neural stem cell-derived astrocytes, indicating another neuroprotective mechanism (Li *et al.*, 2022b).

6.1.3 Riluzole

Riluzole (6-(Trifluoromethoxy)-2-aminobenzothiazole) was the first licenced ALS treatment, which was approved by the FDA in 1995 and the EMA in 1996 (Tzeplaeff *et al.*, 2023). Riluzole modestly improves survival and time before tracheostomy, with different studies suggesting survival benefits of 2-3 months up to 19 months (Andrews *et al.*, 2020; Lacomblez *et al.*, 1996; Tzeplaeff *et al.*, 2023). Riluzole is an anti-excitotoxic therapeutic, which appears to function via multiple mechanisms (Bellingham, 2011). For example, it reduces glutamatergic neurotransmission by inhibiting glutamate release and blocking NMDA glutamate receptors (Fumagalli *et al.*, 2008; Hubert *et al.*, 1994; Jehle *et al.*, 2000; Martin *et al.*, 1993). Riluzole was shown to enhance expression and activity of the GLT-1 (EAAT2) glutamate transporter in mouse astrocytes, and increased glutamate re-uptake by rat astrocytes (Carbone *et al.*, 2012a; Frizzo *et al.*, 2004). Other contributing factors to the anti-excitatory effect of riluzole may include inhibition of voltage-gated Na⁺ channels and potentiation of calcium-dependent K⁺ currents (Bellingham, 2011).

Only a few studies have focused on potential links between riluzole and the Nrf2 pathway or explored the effects of riluzole on astrocytes. Riluzole was found to block the activation of Nrf2 induced by methylmercury in the rat cortex (Deng *et al.*, 2014). In contrast, riluzole increased Nrf2 and HO-1 expression in rat white matter exposed to hypoxia. However, riluzole did not enhance the antioxidant effect of the Nrf2 activator curcumin in human primary spinal cord astrocytes treated with H₂O₂ (Daverey & Agrawal, 2020). Overall, this may suggest the effects of riluzole on the Nrf2 pathway vary, including in different cell types.

Most studies regarding the effects of riluzole in astrocytes investigate its effect on glutamate transporters and glutamate reuptake. Riluzole may also reduce astrocyte reactivity, as it was shown to reduce GFAP levels in a PD rat model (Carbone *et al.*, 2012b). Other studies have shown that riluzole increases neurotrophic factor synthesis or release, which may improve astrocyte-mediated neuroprotection (Dennys *et al.*, 2015; Mizuta *et al.*, 2001). Dennys *et al.* found that this was only the case after acute riluzole treatment of mouse astrocytes, not chronic treatment, therefore the authors argued for an acute treatment regimen in patients (Dennys *et al.*, 2015).

6.1.4 Astrocyte excitability and Ca²⁺ signalling

Astrocytes are not electrically excitable, meaning they do not generate action potentials like neurones. However, they are chemically excitable, since synaptic activity can induce calcium signalling in astrocytes, which allows them to modulate synaptic transmission by releasing gliotransmitters. Specifically, neurotransmitters bind to astrocyte receptors, such as glutamate binding to AMPA and NMDA ionotropic receptors, or ATP binding to purinergic P2X receptors. This induces Ca²⁺ influx, both from the extracellular space via ion channels and internal Ca²⁺ stores such as the ER, which may induce the release of gliotransmitters such as glutamate, GABA, ATP and D-serine (Goenaga *et al.*, 2023).

Glutamate and ATP were used to stimulate human astrocytes in this chapter; therefore, they will now briefly be discussed. In addition to being stimulated by glutamate via NMDA or AMPA receptors, astrocytes also uptake glutamate from the synapse. As described in Chapter 1, this function is crucial for preventing overstimulation of post-synaptic neurones and excitotoxicity (neuronal death induced by excessive Ca²⁺ influx). In ALS patients, excitotoxicity is induced by several mechanisms, including the reduced uptake of glutamate by astrocytes via the EAAT2 transporter (Provenzano *et al.*, 2023). Excitotoxicity may affect astrocytes as well as motor neurones in ALS, since glutamate was shown to induce degeneration of spinal astrocytes in the mSOD1 mouse model, which contributed to disease progression (Rossi *et al.*, 2008).

There is generally an increase in expression and activation of purinergic receptors in different ALS models and cell types, often promoting disease progression, as reviewed in (Cieślak *et al.*, 2019). ATP is released from damaged and dying neurones, which acts as a neuroinflammatory signal, for example to promote their removal by microglia (Cieślak *et al.*, 2019; Skaper *et al.*, 2010). Therefore overall, a detrimental upregulation of purinergic signalling may be predicted in ALS, in addition to the well characterised excitotoxicity induced by increased glutamatergic signalling. Excessive ATP stimulation of astrocytes may contribute to pathogenic mechanisms such as astrocyte reactivity, oxidative stress, and potentiating excitotoxicity by stimulating glutamate release, which will be discussed further in section 6.4 (Franke *et al.*, 2012).

6.1.5 Experimental aims

The broad experimental aims of Chapter 6 are as follows:

- Determine whether tBHQ upregulates (rescues) the Nrf2 pathway in our human reactive astrocyte model and results in a functional decrease in ROS.
- Explore the broader effects of tBHQ-mediated Nrf2 activation on reactive astrocyte health and function, by considering cell viability and calcium signalling.
- Determine whether the ALS therapeutics edaravone and riluzole affect the Nrf2 pathway and ROS in reactive astrocytes.
- As with tBHQ, explore the broader effects of edaravone and riluzole on reactive astrocyte health and function via cell viability assays and calcium imaging.

These experimental aims will allow evaluation of this human reactive astrocyte model as a potential platform for screening future Nrf2-activating therapies. They will also improve understanding of the mechanism of action of current ALS therapeutics, while comparing their efficacy to a specific Nrf2-activating treatment (tBHQ), regarding astrocyte function. Unlike previous chapters where results were discussed as they were presented, here most discussion and interpretation will be presented in the final section (6.4), to allow consideration of all astrocyte assays and consider the effects of tBHQ, edaravone and riluzole in parallel.

6.2 Investigating the effects of tBHQ-mediated Nrf2 activation on human reactive astrocyte phenotypes

6.2.1 tBHQ improves the antioxidant response and viability of human reactive astrocytes

Firstly, RT-qPCR was performed to confirm whether tBHQ induced an Nrf2 pathway increase in human reactive astrocytes (building on the exploratory immunofluorescence analysis in Chapter 5). Astrocytes were treated with tBHQ (10 μ M, as used previously) for 24 hours, before RT-qPCR of *NRF2* and its target genes *HMOX1*, *NQO1* and *IDH1*. Figure 6.1 shows that tBHQ significantly upregulates expression of *HMOX1* and *NQO1*, overall evidencing upregulation of the Nrf2 pathway, therefore confirming that this model is not 'resistant' to therapeutic upregulation of Nrf2.

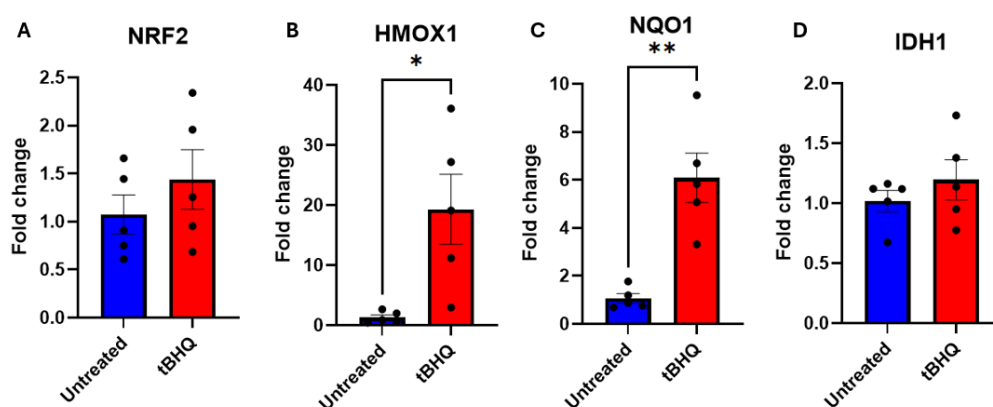


Figure 6.1. The commercial Nrf2 activator tBHQ upregulates the Nrf2 pathway in human reactive astrocytes. Human reactive astrocytes were seeded at a density of 70,000 cells/well in a 6-well plate. After 24 hours, they were treated for 24 hours with tBHQ (10 μM) or left untreated (if repeated DMSO vehicle should be included, however effect of DMSO is expected to be minimal). RT-qPCR then measured expression of A) NRF2, B) HMOX1, C) NQO1 and D) IDH1 expression from total RNA. GAPDH, B2M and 18S were used as housekeepers, and all data was normalised to the average control value for a given gene. Welch's t-test, n=5.

To establish whether the increase in Nrf2 pathway gene expression leads to an improved cellular antioxidant response, and thus a reduction in oxidative stress, the DCFDA/H2DCFDA – Cellular ROS Assay Kit [Abcam] was used to measure ROS. 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) is a non-fluorescent compound, which when internalised by cells is hydrolysed by esterases. This removes the two acetate groups, producing 2',7'-dichlorodihydrofluorescein (DCFH). ROS present in the cell then oxidises DCFH to the highly fluorescent green compound 2',7'-dichlorofluorescein (DCF). Quantification of fluorescence therefore measures relative cellular ROS levels (Kato, 2025).

Initial experiments were required to determine optimal DCFDA concentration, incubation time and buffer used for both DCFDA and test compound incubation (data not shown). This resulted in the following method, with the commonly used oxidative stress inducer H₂O₂ included as a positive control in the first three replicates, to ensure the assay was functional (Ransy *et al.*, 2020). Briefly, human reactive astrocytes were seeded in triplicate in a black 96-well plate, then 44 hours later cells were washed in 1x Dilution Buffer (provided in the Abcam kit) and incubated for 30 minutes with DCFDA (10 μM, the lowest supplier-recommended concentration) in 1x Dilution Buffer with 10% FBS (termed supplemented buffer). Astrocytes were washed again then were incubated for 4 hours with DMSO (vehicle control), tBHQ (1 μM, 10 μM), H₂O₂ (200 μM, commonly used to induce ROS (Ransy *et al.*, 2020)) or left untreated. A no-DCFDA negative control was also included, in addition to supplemented buffer without cells (for background measurement). Fluorescence was measured at 485nm excitation/520nm emission, within the range for GFP measurement. An average value was calculated across the three technical replicates, and the average background value was subtracted.

The ROS assay is designed for short incubations with compounds of interest, since cells are pre-incubated with DCFDA, which may only be stable for up to 6 hours according to Abcam. Optimisation of the ROS assay to allow 24-hour drug incubation via multiple methods was attempted with H₂O₂ as a positive control, although all were ultimately unsuccessful, either due to toxicity of longer H₂O₂ treatment or a failure of H₂O₂ to increase ROS levels, suggesting the assay was not reliable. Therefore, it was not possible to incubate cells with tBHQ for 24 hours as for the RT-qPCR analysis in Figure 6.1, instead cells were treated for 4 hours. A schematic depiction of the DCFDA ROS assay is presented in Figure 6.2.

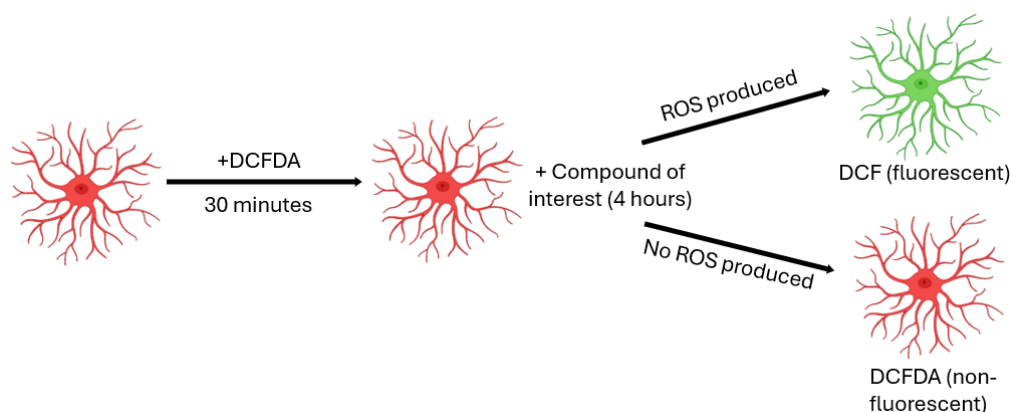


Figure 6.2. Schematic representation of the DCFDA assay to measure ROS levels in human reactive astrocytes. Compounds of interest included H₂O₂ (positive control), tBHQ, DMSO (vehicle control) or untreated. Created using BioRender.

Figure 6.3A shows that H₂O₂ caused a drastic increase in ROS levels after 4 hours, as expected, with an average increase in fluorescence of 11,401% compared to the untreated control. However, this was not significant, likely due to variation. Note that raw fluorescence values (a.u.) are presented for Figure 6.3A unlike B and C, as there was no additional vehicle control for normalisation. However, Figure 6.3A still demonstrates clearly that the assay is suitable for measuring changes in ROS.

The ROS assay was also used to measure the effects of 4-hour tBHQ treatment on ROS levels in human reactive astrocytes. Both 1µM and 10µM were tested to establish if there were any dose-dependent effects. Higher concentrations were not used as preliminary investigations revealed some cell toxicity, based on subjective visual assessment. Figure 6.3B shows that both concentrations reduced the average ROS levels by approximately 50% compared to both the DMSO and untreated controls. Although this was not statistically significant, the trend may suggest that Nrf2 pathway upregulation ameliorated the antioxidant response and reduced oxidative stress. The lack of significance may be due to the short incubation time, while a greater impact on Nrf2 and ROS levels may be predicted after 24 hours.

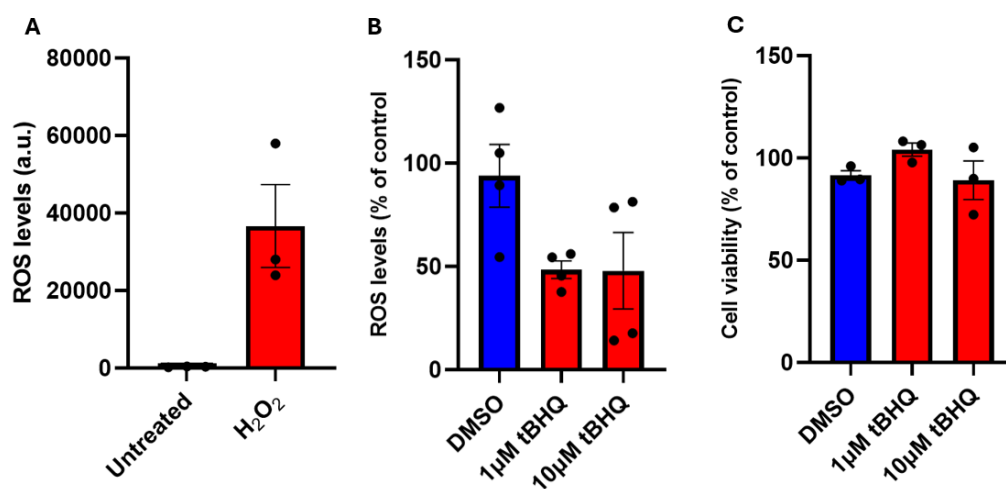


Figure 6.3. tBHQ induced a non-significant reduction in ROS levels in human reactive astrocytes. Human reactive astrocytes were seeded at a density of 5,000 cells/well in black 96-well plates, for A and B) ROS assays or C) resazurin assays. All incubations were at 37°C. For ROS assays, 44 hours after seeding, astrocytes were incubated with DCFDA (10µM) for 30 minutes then A) H₂O₂ (200µM) or B) tBHQ (1µM and 10µM) for 4 hours, along with untreated and DMSO controls. DCFDA fluorescence was quantified (485ex/520em) to measure relative ROS levels. C) For the resazurin assay, 44 hours after seeding, astrocytes were treated with tBHQ (1µM and 10µM) along with untreated and DMSO controls. After 4 hours, cells were treated with resazurin (1mg/ml) for 2 hours. Cell viability was then measured by fluorescence (540ex/590-10em). Cell-free buffer-only fluorescence was subtracted from each value (10% FBS-supplemented dilution buffer [Abcam] for A and B, resazurin solution for C). A) Raw fluorescence values presented (a.u.). B and C) All values are presented as a percentage of the untreated control. Each biological replicate was an average of 3 technical replicates (wells). A) Welch's t-test, $n=3$. B) One-way ANOVA, $n=4$. C) One-way ANOVA, $n=3$.

To confirm that changes in ROS produced by each cell was being measured, and not a change in overall cell number, it was important to normalise the results of Figure 6.3B to cell number. This could theoretically be achieved by counting cells or measuring protein content by BCA assay after treatment, however these methods were predicted to be inaccurate due to the low cell number. Instead, a resazurin assay was performed to measure cell viability. If a significant change in cell viability was induced by a treatment, then ROS levels could be normalised to the percentage change in cell viability. Resazurin is a blue dye, which when internalised by cells is metabolically reduced to produce the fluorescent pink compound resorufin. Resorufin is released by metabolically active cells, therefore fluorescence intensity of the culture medium can be used to measure relative cell viability (Petiti *et al*, 2024).

Reactive astrocytes were plated in a black 96-well plate at a density of 5,000 per well. After 44 hours, cells were treated in triplicate with tBHQ (1µM, 10µM), DMSO or left untreated in fresh astrocyte medium. After 4 hours, cells were washed with PBS and incubated with 100µl resazurin stock solution (0.1mg/ml in PBS) for 2 hours, as well as resazurin-only wells (no cells).

Fluorescence was measured at 540nm excitation/590-10nm emission. Figure 6.3C confirms that 4-hour treatment with tBHQ (1 μ M and 10 μ M) does not cause a significant change in cell viability compared to DMSO-treated cells. Therefore, no normalisation of ROS levels to cell number was performed to produce Figure 6.3B.

While the main function of the resazurin assay in Figure 6.3C was for normalising the results of the ROS assay, I also sought to determine whether longer 24-hour treatment with tBHQ had any effect on cell viability. Since this was the same timepoint as the Nrf2 pathway increase shown in Figure 6.1 (measured by RT-qPCR), any viability changes may be predicted to be due to Nrf2 pathway upregulation. Only 10 μ M tBHQ was tested, not 1 μ M, as this was the concentration used for RT-qPCR of Nrf2 pathway genes in Figure 6.1. Figure 6.4 shows that after 24 hours, tBHQ (10 μ M) treatment improves the viability of reactive astrocytes, in contrast to a 4-hour treatment (Figure 6.3C).

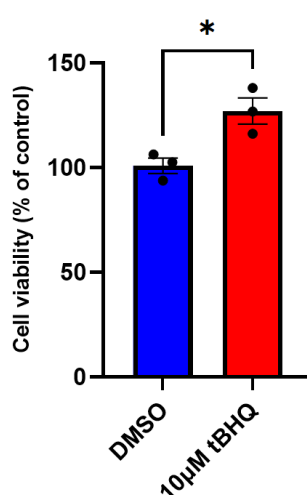


Figure 6.4. tBHQ (10 μ M) increased the viability of human reactive astrocytes after 24 hours. Human reactive astrocytes were seeded at a density of 5,000 cells/well in black 96-well plates. 24 hours after seeding, astrocytes were treated with tBHQ (10 μ M) or DMSO for 24 hours then resazurin (1mg/ml) for 2 hours. Cell viability was then measured by fluorescence (540ex/590-10em). Cell-free resazurin solution-only fluorescence was subtracted from each value. All values are presented as a percentage of the untreated control. Welch's t-test used. N=3, where each biological replicate was an average of 3 technical replicates (wells).

6.2.2 tBHQ treatment increases ATP-induced Ca²⁺ signal in human reactive astrocytes

After investigating the effects of tBHQ on the antioxidant response and survival of human reactive astrocytes, I next sought to determine whether tBHQ may impact glutamate or ATP-induced Ca²⁺ signalling. Calcium imaging was therefore conducted after 24-hour treatment of reactive astrocytes with tBHQ (10 μ M) or DMSO vehicle control. Only one tBHQ concentration was tested due to the complexity of calcium imaging, with 10 μ M selected as it had been shown

to activate the Nrf2 pathway (Figure 6.1). Cells were incubated with Fluor-4 calcium dye before de-esterification, then they were imaged using the Olympus IX70 microscope. Images were taken every 0.5 seconds for 8 minutes, resulting in 960 frames. After 1 minute of no stimulation, cells were stimulated with glutamate (200 μ M) for 2 minutes then ATP (300 μ M) for 3 minutes, and finally ionomycin (1 μ M) for 2 minutes. While glutamate and ATP are physiological neurotransmitters, ionomycin is a calcium ionophore (ion carrier) antibiotic which induces rapid Ca²⁺ influx to the cytoplasm from internal calcium stores (Morgan & Jacob, 1994). In addition to serving as a positive control for the assay, this also facilitates data analysis, since all cells fluoresce brightly after ionomycin stimulation so are clearly visible to trace around. Fluorescence intensities were measured for each cell at each timepoint using FIJI. Ca²⁺ signal upon each stimulation was calculated by quantifying the change in fluorescence intensity relative to resting (baseline) intensity, which is referred to as " $\Delta F/F$ ", as described in detail in section 2.5.4 (Macleod, 2012). To produce an overall trace for cells that respond to glutamate and/or ATP (Figure 6.5A), the baseline was defined as the first 15 seconds of the experiment, while for all other analyses baseline was 15 seconds before addition of the required neurotransmitter. The definition of a 'responding cell' was defined as a maximum peak $\Delta F/F$ value of >0.01 during the glutamate or ATP stimulation.

Figure 6.5A presents the Ca²⁺ traces (average for each biological replicate) of DMSO and tBHQ treated astrocytes, to show how the Ca²⁺ signal (intracellular Ca²⁺ concentration) changed throughout the experiment. The ionomycin stimulation was removed from the trace, as the strong Ca²⁺ increase would hide the more subtle responses to glutamate and ATP. In Figure 6.5B-G, separate data for glutamate and ATP shows the percentage of responding cells (cells with a maximum peak of >0.1 $\Delta F/F$ during the indicated neurotransmitter stimulation), average maximum peak response (highest $\Delta F/F$ value during the stimulation) and total calcium response (area under the curve (AUC) of the Ca²⁺ trace). In summary, these methods aim to identify any differences in Ca²⁺ signal between tBHQ and DMSO treated astrocytes.

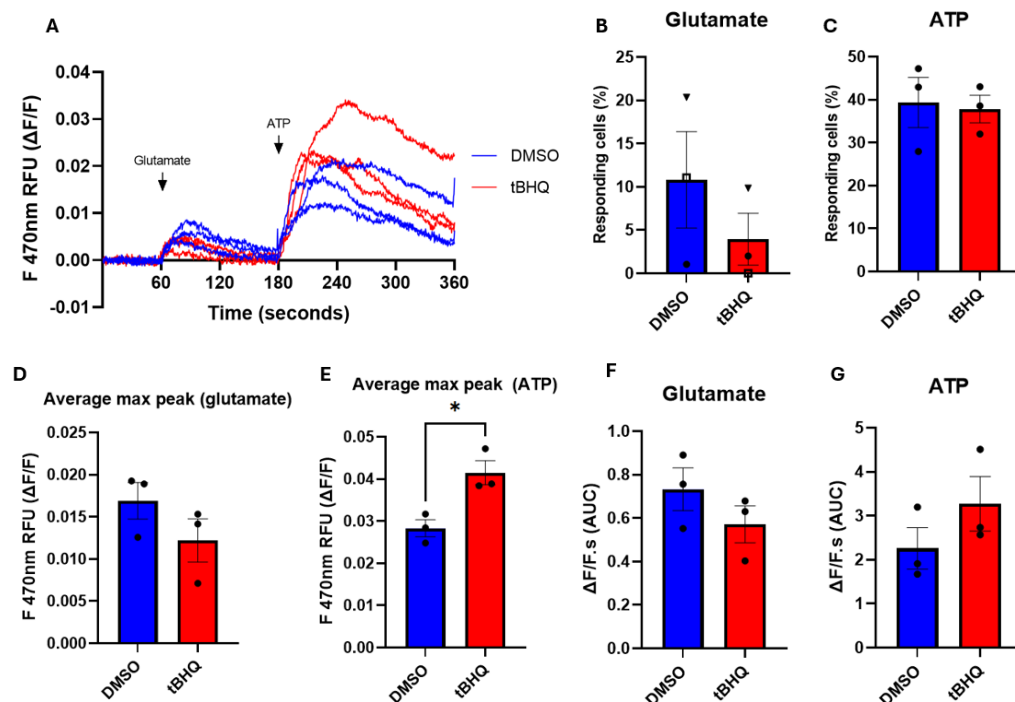


Figure 6.5. tBHQ increased Ca^{2+} signal of human reactive astrocytes in response to ATP stimulation. Human reactive astrocytes were seeded at a density of 60,000 cells/well in Nunc dishes. After 24 hours, cells were treated with tBHQ (10μM) or DMSO (control) for 24 hours. Calcium imaging was then performed using the Fluor-4 calcium indicator (1μM). After 1 minute without stimulation, cells were stimulated for 2 minutes with glutamate (200μM) then 3 minutes with ATP (300μM), followed by 2 minutes with ionomycin (1μM) as a positive control. A maximum $\Delta\text{F}/\text{F}$ value of >0.01 was required to be considered a response and included for analysis. RFU = relative fluorescent units. AUC = area under curve. A) Trace showing Ca^{2+} response to glutamate and ATP stimulation for cells that respond to either neurotransmitter. Ionomycin not included. B+C) Percentage of cells that respond to B) glutamate and C) ATP. The different symbols in Figure B illustrate the variability between biological replicates. D+E) Average maximum (max) Ca^{2+} signal (peak height) of cells responding to D) glutamate and E) ATP. F+G) AUC indicating the total Ca^{2+} signal during the F) glutamate stimulation and G) ATP stimulation. Welch's t-tests used for B-G, no statistical test for A (representative image only). $N=3$, where each biological replicate was an average of 2 technical replicates (dishes).

The trace in Figure 6.5A and the percentages of responding cells in Figure 6.5B and C show that overall, there was a greater Ca^{2+} response to ATP than glutamate, regardless of tBHQ treatment. While tBHQ did not significantly affect the Ca^{2+} response to glutamate by any parameter, there was a trend towards a reduced response by all three measures, as shown in Figure 6.5B, D and F. In contrast, Figure 6.5E shows that tBHQ significantly increased ATP-induced Ca^{2+} signalling, in terms of the average maximum peak. There was also a trend towards increased AUC, representing the overall magnitude of the response, as shown in Figure 6.5G.

6.3 Investigating the effects of edaravone and riluzole on human reactive astrocyte phenotypes

6.3.1 Edaravone and riluzole may upregulate the Nrf2 pathway in reactive astrocytes

Since the previous section demonstrated that tBHQ successfully activated the Nrf2 pathway in human reactive astrocytes, I sought to determine whether a similar effect is induced by either riluzole or edaravone. As discussed previously, this was to consider whether a specific Nrf2 activating therapeutic may be more or less effective than current ALS therapeutics at promoting an astrocyte antioxidant response. Edaravone was predicted to upregulate the Nrf2 pathway in astrocytes, as this has been shown in several other cell types, whereas the effects of riluzole on the Nrf2 pathway are less clear and may be cell type specific (Daverey & Agrawal, 2020; Deng *et al.*, 2014; Pan *et al.*, 2020; Shou *et al.*, 2019).

Human reactive astrocytes were treated for 24 hours with several concentrations of each compound, with the resultant RT-qPCR measurement of the Nrf2 pathway shown in Figure 6.6. There were slight differences in the concentrations tested of each compound, specifically 1µM, 10µM and 100µM edaravone and 10µM, 50µM and 100µM riluzole. This was because the first replicate of riluzole treatment showed no effect of 1µM (data not shown), but possible effects of 10µM and 100µM, therefore 50µM was introduced as an intermediate concentration. In contrast, an increase in *HMOX1* expression was observed after the first replicate of 1µM, 10µM and 100µM edaravone treatment, therefore testing of all three concentrations was continued.

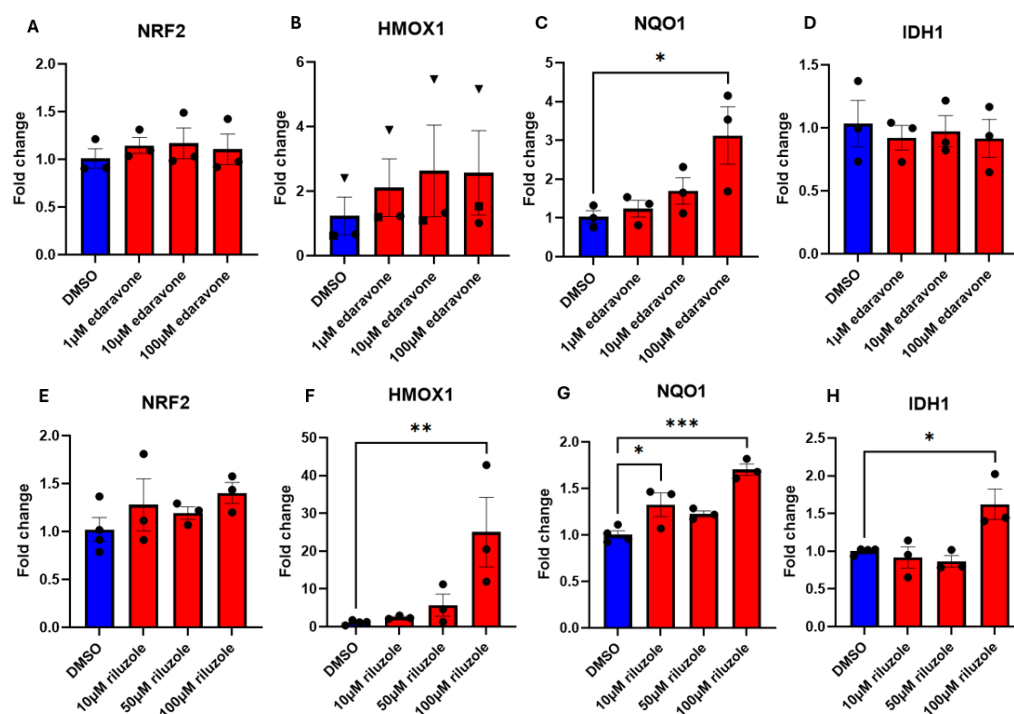


Figure 6.6. Edaravone and riluzole may activate the Nrf2 pathway in human reactive astrocytes. Human reactive astrocytes were seeded at a density of 125,000 cells/well in a 6-well plate. After 24 hours, astrocytes were treated with DMSO, edaravone (1µM, 10µM and 100µM) or riluzole (10µM, 50µM and 100µM) for 24 hours. RT-qPCR then was performed to measure the Nrf2 pathway. A-D) Expression of NRF2, HMOX1, NQO1 and IDH1 after edaravone treatment. Variance in HMOX1 expression between biological replicates illustrated with different datapoint symbols. E-H) Expression of NRF2, HMOX1, NQO1 and IDH1 after riluzole treatment. One-way ANOVA, $n=3$.

Only the highest concentration of edaravone tested (100µM) significantly affected expression of an Nrf2 pathway gene, with upregulation of *NQO1* shown in Figure 6.6C. However, considerable variation between replicates may be the reason for non-significance for other genes and doses. When each edaravone Ct value was normalised to its own biological replicate control, rather than the average of all control values for the gene (the standard method used in this thesis), this resulted in significant upregulation of the following genes: *NRF2* (after treatment with 10µM edaravone), *HMOX1* (1µM, 10µM and 100µM edaravone) and *NQO1* (100µM edaravone). Therefore overall, it appears that edaravone may upregulate the Nrf2 pathway in human reactive astrocytes, as has been reported in other cell types. This may even include at the low concentration of 1µM, illustrated by increased *HMOX1* expression.

Figure 6.6E-H shows that riluzole (100µM) may upregulate the Nrf2 pathway, with significant upregulation of all three target genes tested. *NQO1* expression was also increased by 10µM riluzole. If riluzole Ct values were normalised to their own controls, rather than the average control, the only significant expression changes were increased *NQO1* and *IDH1* (100µM riluzole).

6.3.2 Edaravone improves human reactive astrocyte antioxidant response and survival, while riluzole may induce cell stress

As presented previously for tBHQ, I sought to determine whether the possible Nrf2 pathway upregulation induced by edaravone and riluzole in astrocytes results in a functional reduction in ROS levels. Human reactive astrocytes were treated with DCFDA (10 μ M) for 30 minutes, then a range of edaravone and riluzole concentrations for 4 hours, before fluorescence (485ex/520em) was read to measure ROS. The same concentrations were used as for previous Nrf2 pathway measurement, in addition to 1 μ M riluzole. A resazurin assay was performed after 4-hour treatment with the same compound concentrations, to allow any necessary normalisation to cell number. As previously, limitations of the ROS assay meant that only 4-hour edaravone and riluzole treatments could be investigated, therefore the results do not directly correlate with the Nrf2 pathway RT-qPCR in Figure 6.6.

Figure 6.7A shows that the two higher concentrations of edaravone (10 μ M and 100 μ M) caused a non-significant decrease in ROS levels in reactive astrocytes of approximately 50% compared to DMSO control. The 100 μ M edaravone treatment caused a small but significant increase in cell viability, as shown in Figure 6.7C. Therefore, the ROS levels for this treatment were normalised to account for this increased cell number (ROS value divided by the percentage increase in cell number, x100). Although both the Nrf2 pathway RT-qPCR (Figure 6.6A-D) and ROS assay results (Figure 6.7A) were largely non-significant, overall there was a strong trend towards edaravone upregulating the Nrf2 pathway and downregulating ROS in human reactive astrocytes. This reduction in oxidative stress may be predicted to contribute to the improved cell viability observed after 4 hours.

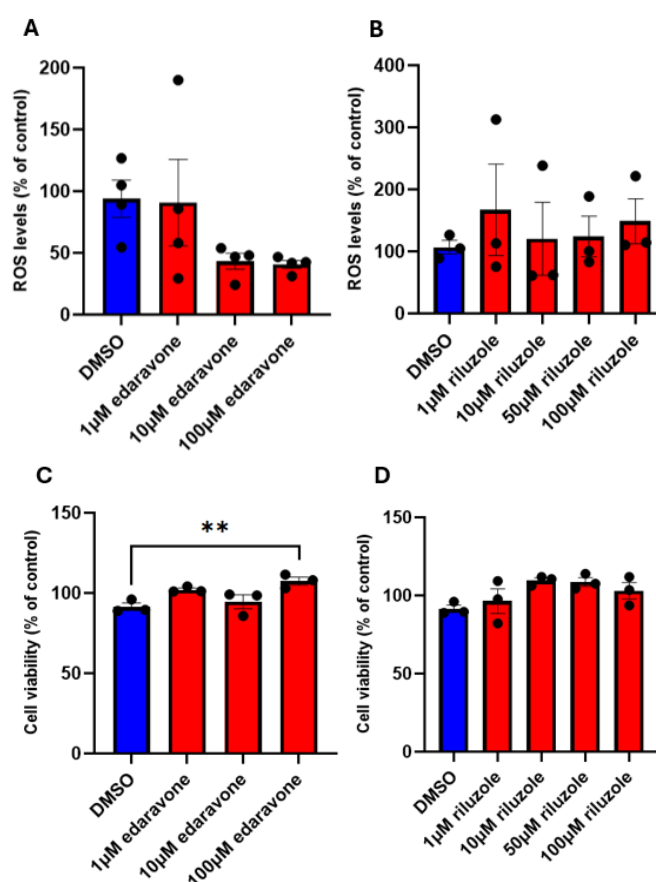


Figure 6.7. Edaravone may reduce ROS levels in human reactive astrocytes.

Human reactive astrocytes were seeded at a density of 5,000 cells/well in black 96-well plates, for A+B) ROS assays or C+D) resazurin assays. For ROS assays, 44 hours after seeding, astrocytes were incubated with DCFDA (10μM) for 30 minutes then A) Edaravone (1μM, 10μM, 100μM) or B) Riluzole (1μM, 10μM, 50μM, 100μM) for 4 hours, along with untreated and DMSO controls. DCFDA fluorescence was quantified (485ex/520em) to measure relative ROS levels. For resazurin assays, 44 hours after seeding, astrocytes were treated with C) Edaravone (1μM, 10μM, 100μM) or D) Riluzole (1μM, 10μM, 50μM, 100μM) along with untreated and DMSO controls. After 4 hours, cells were treated with resazurin (1mg/ml) for 2 hours. Cell viability was then measured by fluorescence intensity (540ex/590-10em). Cell-free buffer-only fluorescence was subtracted from each value (10% FBS-supplemented dilution buffer [Abcam] for A and B, resazurin solution for C and D). All values are presented as a percentage of the untreated control. A) n=4. B-D) n=3. Each biological replicate was an average of 3 technical replicates (wells). One-way ANOVA used.

Figure 6.7B shows that riluzole did not affect reactive astrocyte ROS levels at any concentration. Contrasting the effects of edaravone, there was a slight trend towards increased ROS after riluzole treatment. Figure 6.7D shows that after 4 hours, riluzole did not significantly affect the viability of reactive astrocytes, therefore ROS assay normalisation was not required. Overall, the ROS assay results suggest that the Nrf2 pathway upregulation induced by 100μM riluzole after 24 hours does not lead to a reduction in ROS, with the

acknowledgment that the different incubation times may affect results. I hypothesised that here the Nrf2 pathway upregulation (rescue) may in fact be due to riluzole-induced oxidative stress. Riluzole has been shown to reduce the antioxidant protective effects of the Nrf2 activator curcumin in human primary astrocytes [SC1800], suggesting it may induce some oxidative stress in this cell model (Daverey & Agrawal, 2020). It was therefore predicted that due to this oxidative stress, longer riluzole treatment, perhaps at lower concentration too, may lead to cell toxicity.

A further resazurin assay was performed after 24-hour treatment of human reactive astrocytes with edaravone and riluzole, with the same incubation time and concentrations that were used for Nrf2 pathway RT-qPCR in Figure 6.6. Figure 6.8A shows that edaravone (10 μ M and 100 μ M) increased the viability of reactive astrocytes after 24 hours, therefore the increased viability induced by 100 μ M edaravone after only 4-hour treatment in Figure 6.7C was sustained. While speculative, this could be associated with Nrf2 upregulation, since 24-hour tBHQ treatment also increased cell viability, as shown in Figure 6.4.

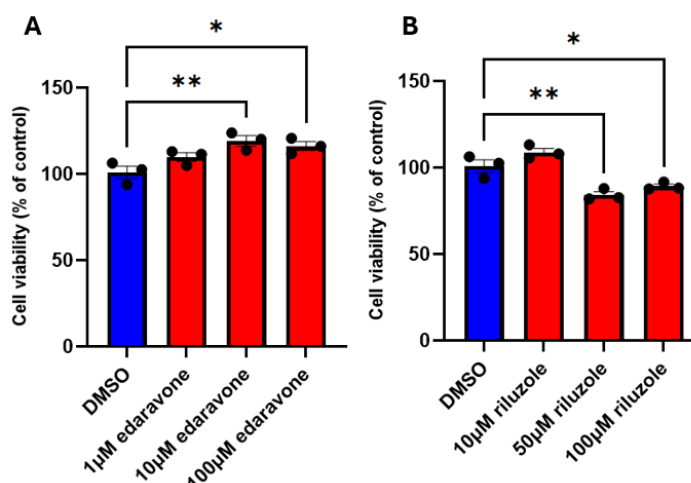


Figure 6.8. Edaravone improved the viability of human reactive astrocytes after 24 hours, while riluzole reduced viability. Human reactive astrocytes were seeded at a density of 5,000 cells/well in black 96-well plates. 24 hours after seeding, astrocytes were treated with A) Edaravone (1 μ M, 10 μ M, 100 μ M) or B) Riluzole (10 μ M, 50 μ M, 100 μ M) for 24 hours along with untreated and DMSO controls. Cells were then treated with resazurin (1mg/ml) for 2 hours. Cell viability was then measured by fluorescence intensity (540ex/590-10em). Cell-free resazurin solution-only fluorescence was subtracted from each value. All values are presented as a percentage of the untreated control. N=3, where each biological replicate was an average of 3 technical replicates (wells). One-way ANOVA used.

In addition, Figure 6.8B shows that riluzole (50 μ M and 100 μ M) reduced the viability of human reactive astrocytes after 24 hours. This further suggests that the Nrf2 pathway upregulation induced by 100 μ M riluzole shown in Figure 6.6E-H was likely due to cellular stress, rather than a beneficial antioxidant effect of the compound.

6.3.3 Edaravone increases ATP-induced calcium signalling in human reactive astrocytes

The effects of edaravone and riluzole on Ca^{2+} signal in reactive astrocytes were then explored, to further evaluate the impact of these ALS therapeutics on astrocyte function. These compounds were tested at the same time as tBHQ (10 μM , Figure 6.5), therefore all conditions were the same, including the stimulation regime with glutamate and ATP. For simplicity and consistency, 10 μM was also selected as the concentration for both edaravone and riluzole. However, the effects of each compound cannot be directly compared as they likely have different EC50 values and properties.

The effects of edaravone (10 μM) on astrocyte Ca^{2+} signalling after glutamate or ATP stimulation is presented in Figure 6.9, beginning with the overall Ca^{2+} trace for responding cells in Figure 6.9A. As discussed in section 6.2.2, Ca^{2+} signal was generally higher after stimulation with ATP than glutamate. Edaravone did not significantly alter astrocyte Ca^{2+} signal in response to glutamate stimulation, as there was no change in percentage of responding cells, average maximum peak or total calcium response (AUC), as presented in Figure 6.9B, D and F respectively. However, edaravone increases the Ca^{2+} response to ATP stimulation, as there was a significant increase in average maximum peak compared to control, as shown in Figure 6.9E. While not significant, there was also a trend towards increased AUC, shown in Figure 6.9G.

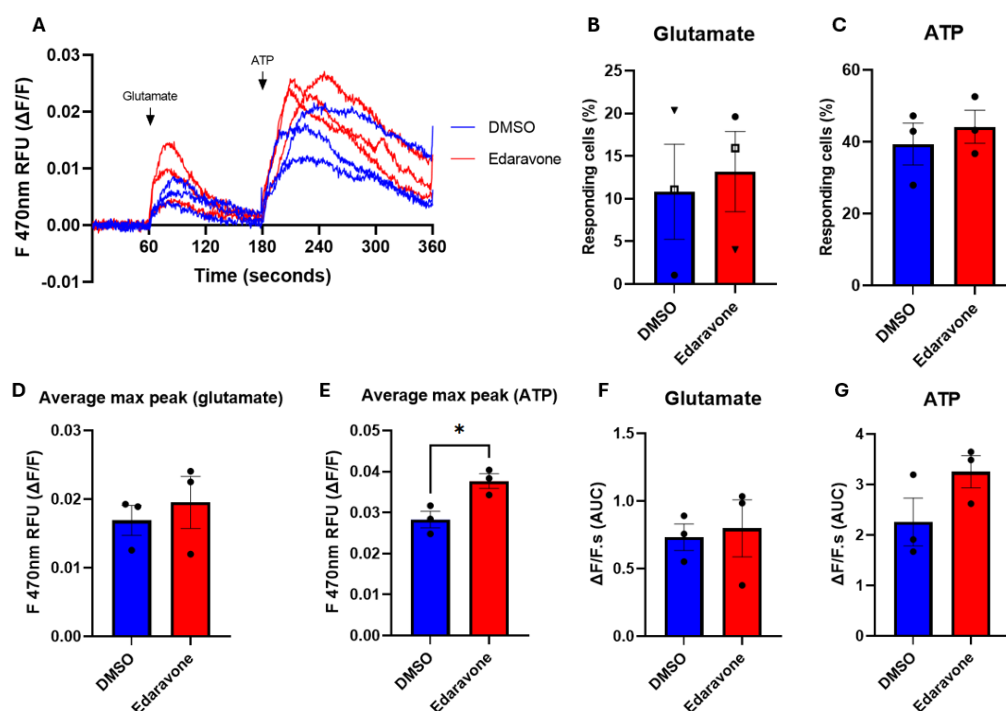


Figure 6.9. Edaravone increased ATP-induced Ca^{2+} signalling in human reactive astrocytes. Human reactive astrocytes were seeded at a density of 60,000 cells/well in Nunc dishes. After 24 hours, cells were treated with edaravone (10 μM) or DMSO (control) for 24 hours. Calcium imaging was then performed using the Fluor-4 calcium indicator (1 μM). After 1 minute without stimulation, cells were stimulated for 2 minutes with glutamate (200 μM) then 3 minutes with ATP (300 μM), followed by 2 minutes with ionomycin (1 μM) as a positive control. A maximum peak $\Delta\text{F}/\text{F}$ value of >0.01 was required to be considered a response and included for analysis. RFU = relative fluorescent units. AUC = area under curve. A) Trace showing Ca^{2+} response to glutamate and ATP stimulation for cells that respond to either neurotransmitter. Ionomycin not included. B+C) Percentage of cells that respond to B) glutamate and C) ATP. The different symbols in Figure B illustrate the variability between biological replicates. D+E) Average maximum (max) Ca^{2+} signal (peak height) of cells responding to D) glutamate and E) ATP. F+G) AUC indicating the total Ca^{2+} signal during the F) glutamate stimulation and G) ATP stimulation. Welch's *t*-tests used for B-G, no statistical test for A (representative image only). *N*=3, where each biological replicate was an average of 2 technical replicates (dishes).

The effects of riluzole on reactive astrocyte Ca^{2+} signalling are presented in Figure 6.10. Unlike tBHQ and edaravone, riluzole did not cause any significant changes in Ca^{2+} response to glutamate or ATP stimulation, in terms of percentage of responding cells (Figure 6.10B and C), average maximum peak (Figure 6.10D and E) or total calcium response (AUC) (Figure 6.10F and G).

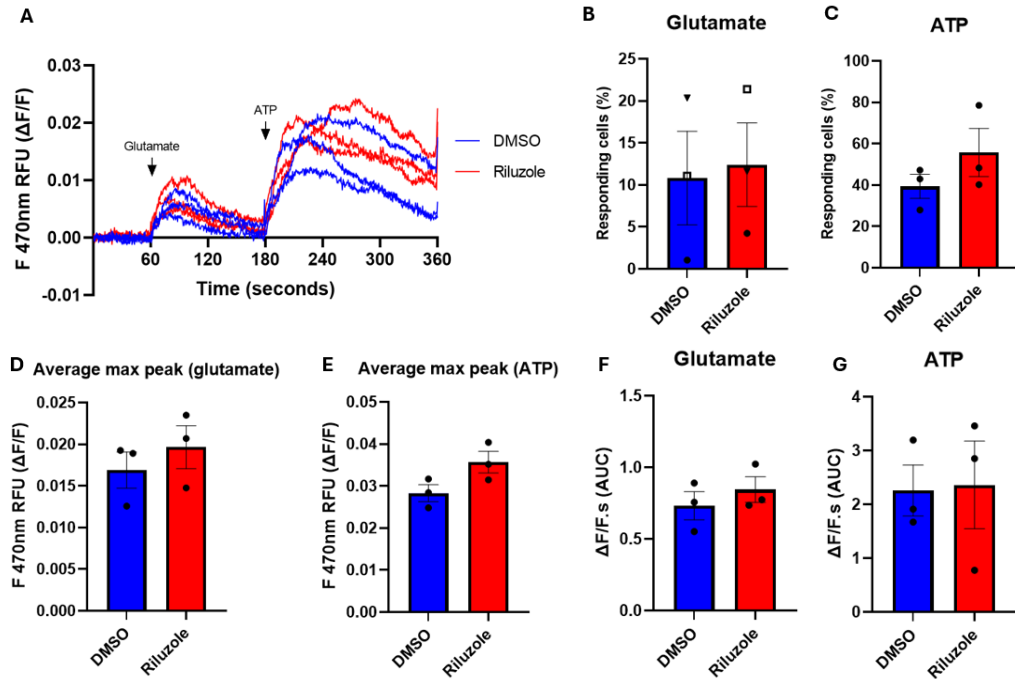


Figure 6.10. Riluzole did not affect glutamate or ATP-induced Ca^{2+} signalling in reactive astrocytes. Human reactive astrocytes were seeded at a density of 60,000 cells/well in Nunc dishes. After 24 hours, cells were treated with riluzole (10 μM) or DMSO (control) for 24 hours. Calcium imaging was then performed using the Fluor-4 calcium indicator (1 μM). After 1 minute without stimulation, cells were stimulated for 2 minutes with glutamate (200 μM) then 3 minutes with ATP (300 μM), followed by 2 minutes with ionomycin (1 μM) as a positive control. A maximum peak $\Delta\text{F}/\text{F}$ value of >0.01 was required to be considered a response and included for analysis. RFU = relative fluorescent units. AUC = area under curve. A) Trace showing Ca^{2+} response to glutamate and ATP stimulation for cells that respond to either neurotransmitter. Ionomycin not included. B+C) Percentage of cells that respond to B) glutamate and C) ATP. The different symbols in Figure B illustrate the variability between biological replicates. D+E) Average maximum (max) Ca^{2+} signal (peak height) of cells responding to D) glutamate and E) ATP. F+G) AUC indicating the total Ca^{2+} signal during the F) glutamate stimulation and G) ATP stimulation. Welch's t-tests used for B-G, no statistical test for A (representative image only). $N=3$, where each biological replicate was an average of 2 technical replicates (dishes).

6.4 Discussion and conclusions

In this chapter, our model of FBS-induced human reactive astrocytes was demonstrated as a convenient disease-relevant cell model for screening Nrf2 activators. With tBHQ used as an example Nrf2 activator, upregulation of the Nrf2 pathway was demonstrated, in addition to a non-significant reduction in ROS. Cell viability assays and calcium imaging were used to profile astrocyte function beyond the antioxidant response, with tBHQ shown to increase cell viability and Ca^{2+} response to ATP stimulation. This cell model may therefore be a useful tool for the ALS field, since many Nrf2 activation studies rely on

mouse models or time-consuming iPSC-derived models (Keerie *et al.*, 2025; Vargas *et al.*, 2008). In contrast, this primary human cell model is commercially available, very simple to use and cost effective, which in the future could be used for high-throughput screening of Nrf2 activators to identify initial candidates, before further validation in ALS-specific models. In this thesis, this cell model was used to investigate the effects of the ALS therapeutics riluzole and edaravone, as exemplars. A summary of the main experimental findings of this chapter is presented in Table 6.1.

Table 6.1. Summary of the main findings regarding the effects of tBHQ, edaravone and riluzole on human reactive astrocyte phenotypes.

Assay	tBHQ	Edaravone	Riluzole
Nrf2 pathway RT-qPCR	Upregulates <i>NRF2</i> , <i>HMOX1</i> and <i>NQO1</i> expression (10µM)	Upregulates <i>NQO1</i> expression (100µM). If non-standard analysis method used, upregulates <i>NRF2</i> (10µM), <i>HMOX1</i> (1µM, 10µM, 100µM) and <i>NQO1</i> (100µM)	Upregulates <i>HMOX1</i> , <i>NQO1</i> , <i>IDH1</i> expression (100µM) and <i>NQO1</i> expression (10µM)
ROS assay	Non-significant ~50% decrease in ROS (1µM, 10µM)	Non-significant ~50% decrease in ROS (10µM, 100µM)	No change (slight trend towards increased ROS - 1µM, 10µM, 50µM, 100µM)
Resazurin assay	Increased viability after 24 hours (10µM)	Increased viability after 4 hours (100µM) and 24 hours (10µM, 100µM)	Reduced viability after 24 hours (50µM, 100µM)
Calcium imaging	Increased peak response to ATP (10µM)	Increased peak response to ATP (10µM)	No change

Human reactive astrocytes were treated with the compounds for 24 hours for Nrf2 RT-qPCR, calcium imaging and a resazurin assay. For the ROS assay and another resazurin assay, astrocytes were treated for 4 hours. Concentrations in brackets correspond to the concentration of the treatment compound. Most non-significant changes are not detailed, unless there were notable trends.

6.4.1 Evaluating the effects of tBHQ in human reactive astrocytes

To my knowledge, there are no studies which investigate tBHQ as a potential ALS therapeutic, however it is an FDA-approved food additive (Khezerlou *et al.*, 2022). Here, tBHQ was used for a proof-of-concept of Nrf2 activation, rather than to investigate tBHQ for ALS treatment. The reactive astrocyte model exhibits downregulated Nrf2 pathway components compared to quiescent astrocytes, as identified by proteomic analysis in Chapter 3, a phenotype which I therefore aimed to rescue. RT-qPCR was used to identify that after 24 hours, tBHQ (10 μ M) effectively upregulates the Nrf2 pathway in reactive astrocytes, leading to the expected decrease (although non-significant) in ROS. Limitations of the ROS assay meant that the effects of tBHQ could only be measured after 4 hours, unlike the 24 hours used for RT-qPCR. Therefore, 24-hour tBHQ treatment may be predicted to significantly reduce ROS and oxidative stress in the reactive astrocyte model.

24-hour tBHQ treatment also increased cell viability, as it increased the number of metabolically active cells as measured by a resazurin assay (Petiti *et al.*, 2024). This may indicate reduced cytotoxicity, suggestive of a desirable, healthier astrocyte phenotype. Alternatively, increased viability may be due to increased proliferation, a hallmark of astrocyte reactivity, however this would contrast a previous study showing that tBHQ reduces astrocyte reactivity after traumatic brain injury in rats (Zhang *et al.*, 2020). Further assays would be required to distinguish between proliferation and improved survival/reduced toxicity, for example a lactate dehydrogenase (LDH) assay to measure dying cells or Ki-67 staining to measure proliferating cells (Kumar *et al.*, 2018; Uxa *et al.*, 2021). Increasing the number of neurotoxic reactive astrocytes would not be desirable of an ALS therapeutic as this may accelerate neuronal death, however these effects may be specific to tBHQ not Nrf2 activators in general.

Calcium imaging data showed that tBHQ induced a greater Ca²⁺ signal than DMSO in response to ATP stimulation, in terms of the maximum peak response (highest Ca²⁺ signal during the stimulation). It must be noted that tBHQ is a known inhibitor of the SERCA pump (sarcoplasmic/endoplasmic reticulum Ca²⁺-ATPase), a Ca²⁺ transporter which moves Ca²⁺ from the cytosol to the sarcoplasmic reticulum (SR) or ER (Periasamy & Kalyanasundaram, 2007; Uddin & Thomas, 2024). This effect of tBHQ has also been confirmed in mouse astrocytes, resulting in a depletion of Ca²⁺ stores from the ER (Rodríguez-Prados *et al.*, 2020). Therefore, this SERCA-inhibiting effect may underly the tBHQ-mediated increase in intracellular Ca²⁺ concentration in response to ATP. The results of Figure 6.5 therefore cannot be interpreted as a *direct* effect of Nrf2 activation, however Nrf2 may still have an impact.

Based on a study by Granatiero *et al.*, Nrf2 activation was predicted to reduce Ca²⁺ signal due to upregulated expression of the glutathione peroxidase 8 (GPx8) ER protein, which regulates both oxidative stress and Ca²⁺ homeostasis. Nrf2 impairment was previously shown to result in Ca²⁺ overload in both the

ER and cytosol, with Nrf2 activation by DMF restoring Ca^{2+} homeostasis and reducing Ca^{2+} levels in both compartments (Granatiero *et al.*, 2019). This conflicts my data showing that tBHQ increases Ca^{2+} signal, in this case after ATP stimulation. Therefore, additional methods would be needed to determine whether the tBHQ-mediated effect is due to Nrf2 activation or SERCA inhibition. GPx8 was detected in the proteomic analysis of Chapter 3, in which there was a statistically significant upregulation in reactive astrocytes compared to quiescent, which approached the threshold for differential expression (padj 0.04, Log2FC 0.4). This GPx8 upregulation is therefore unlikely to be due to Nrf2, since the Nrf2 pathway is dampened in reactive astrocytes. However, GPx8 may still be upregulated by chemical upregulation of Nrf2 via tBHQ.

In the future, additional Nrf2 activators could be tested to explore this hypothesis. However, many other widely used Nrf2 activators such as sulforaphane, curcumin, resveratrol and DMF are also known calcium regulators (Herrmann *et al.*, 2019; Jara *et al.*, 2025; McCalley *et al.*, 2014; Poletto Bonetto *et al.*, 2022). Therefore siRNA-mediated knockdown of Nrf2, or inhibition of an Nrf2-targeting miRNA if this is optimised further, may be more appropriate methods to explore the potential impact of an Nrf2-modulating therapeutic on astrocyte calcium signalling.

6.4.2 Evaluating the effects of edaravone and riluzole in human reactive astrocytes

As discussed, several assays have so far been presented to demonstrate the utility of our astrocyte model for screening Nrf2 activators. While other Nrf2 activators beyond tBHQ were not investigated here, these assays were used to investigate the effects of riluzole and edaravone on reactive astrocyte phenotypes. I aimed to improve understanding of the mechanisms of action of these current ALS therapeutics by considering their effects on astrocyte health and function, and establish whether either compound may in part exert beneficial effects via astrocyte Nrf2 signalling.

Overall, edaravone had many similar effects as tBHQ in reactive astrocytes. Both compounds appeared to activate the Nrf2 pathway after 24 hours, however using the standard data analysis method edaravone (100 μM) only upregulated *NQO1* expression. This may illustrate relatively weak Nrf2 activation relative to tBHQ (although the compounds cannot be directly compared), suggesting that research is still needed to find a more effective astrocyte-targeted Nrf2 activator for ALS treatment. For both compounds, Nrf2 activation was also associated with a non-significant ~50% reduction in ROS (after 4 hours) and improved cell survival (after 24 hours). Both compounds also increased the Ca^{2+} response to ATP stimulation, although for tBHQ this was likely in part due to inhibition of the SERCA Ca^{2+} transporter. However, some of these shared properties may be predicted to be due to activation of the Nrf2 pathway.

ATP stimulation of astrocytes likely modulates certain astrocyte functions via changes in Ca^{2+} concentration (Murakami *et al*, 2003). There is considerable evidence that several of the known effects of ATP stimulation on astrocytes may be detrimental in an ALS context, which will now be discussed, therefore edaravone (and tBHQ) may be predicted to worsen these effects due to ATP-induced hyperexcitability.

One of these detrimental effects of ATP stimulation is the promotion of astrocyte reactivity, indicated by phenotypes such as GFAP increase and morphological changes (Franke *et al.*, 2012; Neary *et al*, 1994). This may be in part due to activation of cyclooxygenase-2 (COX-2), an enzyme involved in production of pro-inflammatory cytokines, free radicals and ROS (Brambilla & Abbracchio, 2001; Drachman *et al*, 2002). COX-2 also catalyses synthesis of the pro-inflammatory mediator prostaglandin E2 (PGE_2) which stimulates glutamate release from astrocytes, therefore this pro-inflammatory mechanism may also promote excitotoxicity (Drachman *et al.*, 2002). Purinergic signalling may also cause astrocyte reactivity by inducing phosphorylation and activation of STAT3, a signalling protein known to be important for regulating astrogliosis (Herrmann *et al.*, 2008; Washburn & Neary, 2006).

ATP stimulation of P2X_7 purinergic receptors has also been shown to induce astrocytes to release glutamate and GABA (Duan *et al*, 2003; Wang *et al*, 2002). Increased intracellular Ca^{2+} concentration induces rapid fusion of glutamate-containing vesicles with the cell membrane (Bezzi *et al*, 2004). Therefore, edaravone may trigger excess release of glutamate from ATP-stimulated astrocytes, which in ALS patients may contribute to excitotoxicity.

There is conflicting evidence regarding whether ATP stimulation reduces or promotes oxidative stress in astrocytes. ATP stimulation of the P2X_7 receptor was shown to increase iNOS expression in both LPS-treated rat astrocytes (in addition to elevated NO) and IL-1 β -treated human foetal astrocytes, suggesting oxidative stress (Murakami *et al.*, 2003; Narcisse *et al*, 2005). This ATP-induced iNOS increase may be a direct result of Ca^{2+} elevation (Franke *et al.*, 2012). In contrast, ATP stimulation was shown to enhance the antioxidant defence in rat astrocytes by activating SOD1 and SOD2 enzymes and increasing glutathione (Adzic *et al*, 2017).

My data may partly explain the limited efficacy of edaravone for ALS treatment, since it increases the response of human astrocytes to ATP stimulation. Edaravone may therefore worsen astrocyte-mediated neurotoxicity by exacerbating astrocyte reactivity, excitotoxicity and possibly oxidative stress. Purinergic signalling is likely elevated in ALS patients, creating a vicious cycle to potentiate pathology (Cieřlak *et al.*, 2019). This may be compounded further by certain ALS-associated genetic variants, since mouse mSOD1 astrocytes were previously shown to release excessive Ca^{2+} from the ER when stimulated with ATP (Kawamata *et al.*, 2014). ATP stimulation of the P2X_7 receptor was also shown to underly the neurotoxic phenotype of mSOD1 rat astrocytes (Gandelman *et al.*, 2010).

Further work would be required to confirm why ATP-mediated Ca^{2+} signalling is augmented by edaravone. Edaravone does not have a well described role in Ca^{2+} regulation, with known roles limited to attenuation of Ca^{2+} overload induced by amyloid β in PC12 cells and prevention of Ca^{2+} -induced ROS production in rat brain (He *et al*, 2012; Takayasu *et al*, 2007). Further experiments to elucidate a mechanism could include patch clamping to determine whether edaravone affects Ca^{2+} flux through any ion channels during ATP stimulation (Fellin *et al*, 2006). Only one purinergic receptor (P2RX3) was detected in batch 1 of the proteomic analysis presented in Chapter 3, in only 1 quiescent astrocyte sample, however it is likely that many expressed receptors were not detected that may mediate this Ca^{2+} influx. Detection of membrane proteins is challenging by standard proteomic methods due to their low abundance and hydrophobic properties, with additional solubilisation procedures required for a membrane protein-focussed proteomic analysis (Helbig *et al*, 2010).

Riluzole and edaravone have different advantages and limitations, therefore their efficacy may vary depending on the pathophysiology of individual patients. While the effects of these compounds cannot be directly compared, overall edaravone appeared to be a more effective antioxidant therapeutic and improves cell viability of the reactive astrocyte model. As discussed for tBHQ, this may suggest an overall healthier astrocyte phenotype or it may be a sign of astrocyte reactivity, which would be a significant limitation. The edaravone-induced increased Ca^{2+} response to ATP stimulation may also promote astrocyte reactivity and excitotoxicity. In contrast, riluzole does not have an antioxidant phenotype and reduces cell viability. Activation of the Nrf2 pathway by 100 μM riluzole did not reduce ROS and was associated with reduced cell survival, therefore likely indicating the cells were under significant stress, leading to the hypothesis that astrocytic stress may contribute to the limited efficacy of riluzole as an ALS treatment. While the same level of stress may not be observed in ALS patients as the concentration of riluzole in the brain is likely only 3-8 μM (predicted to be three to four-fold higher than the plasma concentration of 1-2 μM) (Bellingham, 2011), it still illustrates that riluzole is not an antioxidant therapeutic. Therefore, research into an astrocyte-targeting antioxidant therapeutic should continue, to be given to patients along with riluzole. This is especially true in the UK, where edaravone is not licenced for ALS treatment.

A possible advantage of riluzole over edaravone is that it is unlikely to accentuate any of the detrimental effects of ATP stimulation, since it did not affect Ca^{2+} signalling after ATP stimulation in our astrocyte model. It is somewhat surprising that riluzole did not reduce the Ca^{2+} response to glutamate, since riluzole is known to block NMDA glutamate receptors and prevent the downstream Ca^{2+} response (Hubert *et al*, 1994). However, riluzole increases expression of GluR1 and GluR2 AMPA receptor subunits in mouse hippocampal neurones and increases the uptake of glutamate by rat astrocytes (Frizzo *et al*, 2004). Therefore, the balance of these opposing functions may result in no significant changes in the astrocyte Ca^{2+} response to glutamate.

The only glutamate receptor detected by proteomic analysis (batch 1, Chapter 3) was the GRIK1 subunit of the kainate receptor, although it did not pass StatsPro filtering. As suggested for purinergic receptors, this poor detection of glutamate receptors is likely due to the challenges of detecting membrane proteins by standard proteomic methods. However, another hypothesis may be that these astrocytes express low levels of the NMDA receptors targeted by riluzole, perhaps explaining the unchanged Ca^{2+} response.

In conclusion, edaravone likely ameliorates the antioxidant response of reactive human astrocytes and is a better antioxidant ALS therapeutic than riluzole. However, edaravone also increases ATP-induced Ca^{2+} signalling, unlike riluzole, which may potentiate astrocyte reactivity and excitotoxicity. This data illustrates the complexity of evaluating the efficacy of ALS therapeutics in astrocytes, since multiple pathogenic mechanisms can be affected in different ways. Further research should aim to find a treatment without the undesirable astrocytic side effects of edaravone and riluzole, and that elicits stronger Nrf2 activation than edaravone.

Chapter 7. Final discussion and conclusions

Astrocytes are key contributors to motor neurone degeneration in ALS. Astrocytes undergo reactive astrogliosis, wherein they lose homeostatic, neuroprotective functions and gain pro-inflammatory, neurotoxic functions. This may include impaired antioxidant responses, such as impaired Nrf2 signalling and reduced glutathione release (Birger *et al.*, 2019; Moujalled *et al.*, 2017). The Nrf2 pathway is the primary astrocyte antioxidant defence, and its genetic activation (via a GFAP promoter) was demonstrated to be neuroprotective in ALS mSOD1 mice (Vargas *et al.*, 2008). In this thesis, I aimed to take this study further in a human primary reactive astrocyte model lacking genetic variants, as *SOD1* variants are rare and Nrf2 is a regulator of *SOD1*, therefore findings from the mSOD1 mouse model may not be applicable to other genetic subtypes or sALS (Kraft *et al.*, 2007). I also aimed to upregulate the Nrf2 pathway using miRNA inhibitors or small molecules, which have more therapeutic relevance than genetic activation.

Chapter 3 presented the human primary astrocyte model used in this thesis, commercially available foetal cortical astrocytes [SC1800] which were cultured in serum-free conditions to promote a quiescent phenotype, and 2% FBS to promote chronic reactivity. This model was published in (White *et al.*, 2024), for which I was a contributing author. The reactive astrocyte model reflected the Nrf2 pathway impairment observed in ALS patients (measured by RT-qPCR), providing a clinically-relevant platform for screening Nrf2 activators. Proteomic profiling (SWATH-MS) confirmed reduced Nrf2 signalling in the reactive astrocyte model and identified 55 ALS-associated proteins expressed in human astrocytes, 18 of which were differentially expressed upon reactivity, implicating a potential neuroinflammatory role in ALS pathogenesis. Key examples included downregulation of PON2 and upregulation of CAV1 in reactive astrocytes, which were both validated by additional proteomic analyses. I reasoned that reduced astrocytic PON2 may promote astrocyte-mediated neurotoxicity by reducing the antioxidant response, while increased CAV1 may promote astrocyte reactivity, as this has previously been shown in a mouse model of stroke (Blochet *et al.*, 2020; Giordano *et al.*, 2011).

Chapter 4 expanded this model characterisation by sncRNAseq to explore miRNA changes associated with FBS-induced astrocyte reactivity. Numerous studies report dysregulation of miRNAs in biofluids or tissue samples of ALS patients which may be viable ALS biomarkers, the most promising of which is likely a plasma neural-EV 8-miRNA signature (Banack *et al.*, 2020; Banack *et al.*, 2024; Banack *et al.*, 2022). However, the contribution of astrocytic miRNAs to ALS pathogenesis is poorly understood, in part because not much is known about the role of miRNAs in human astrocyte reactivity. Using sncRNAseq data, top candidates for astrocyte-reactivity regulating miRNAs were identified, including the pro-inflammatory miR-206 and miR-155-5p and anti-inflammatory miR-153-3p and miR-136-5p. MiR-206 was taken forward for experimental evaluation, with its pro-inflammatory mechanism of action predicted to include targeting of the anti-inflammatory transcription factor

NURR1. While I was unable to confirm this direct miR-206-NURR1 relationship, because a failure to confirm astrocytic miR-206 expression by RT-qPCR prevented further functional manipulations, reduced *NURR1* expression was confirmed in the reactive astrocyte model compared to quiescent astrocytes. Loss of astrocytic NURR1 may therefore contribute to ALS pathogenesis, warranting further investigation in patient-derived astrocyte models and postmortem tissue.

In addition to astrocyte whole-cell extracts, reactive and quiescent ADEVs were profiled by sncRNAseq. Astrocyte reactivity had a far smaller impact on ADEV miRNA expression than on whole-cell miRNAs (1.2% vs. 34% differentially expressed). Motif analysis of ADEV-enriched (compared to their respective whole-cell) miRNAs identified sequences predicted to bind nine ALS-associated RBPs, including TDP-43, FUS, and HNRNPA1, suggesting these RBPs may be involved in miRNA selection for ADEV loading. This function has already been demonstrated for FUS in multiple mouse cell lines and predicted in human iPSC-astrocytes (Garcia-Martin *et al.*, 2022; Gordillo-Sampedro *et al.*, 2024). The same motifs were also enriched in miRNAs retained in quiescent astrocytes relative to ADEVs, indicating ALS-RBP involvement in selective cellular retention. Strikingly, this pattern was entirely absent in reactive astrocytes, where no miRNAs containing ALS-RBP motifs were preferentially retained, implying that astrocyte reactivity disrupts RBP-mediated miRNA sorting. Such disruption likely alters astrocyte gene regulation and could be exacerbated in ALS patients with RBP mutations or TDP-43/FUS pathology. Thus, impaired astrocyte miRNA sorting by ALS-associated RBPs may be a widespread pathogenic mechanism associated with neuroinflammation in ALS.

By integrating the proteomic analysis of Chapter 3 with the sncRNAseq analysis of Chapter 4, I aimed to identify miRNA-regulated pathways which may regulate astrocyte reactivity and therefore may have a role in ALS pathogenesis. However, IPA-based integration was inconclusive, since comparable numbers of proteins were predicted to be targeted by miRNAs changing in both the same and opposite directions, obscuring genuine interactions. Manual integration showed that miRNA regulation did not explain the Nrf2 pathway deficit in reactive astrocytes, as most Nrf2-targeting miRNAs were downregulated (43) rather than upregulated (2), indicating the deficit arises from other mechanisms.

Finally, sncRNAseq analysis and previous experimental data led to the selection of miR-27b-3p and miR-340-5p as Nrf2-targeting miRNAs to be inhibited in reactive astrocytes in Chapter 5, to test their therapeutic potential. However, miRNA manipulation in primary astrocytes proved technically challenging. Methods trialled included Lipofectamine-based transfection, direct and ADEV-mediated delivery of synthetic miRNA power inhibitors, and commercial lentivirus miRNA inhibitors. Only nucleofection achieved relatively high transfection efficiency (indicated by GFP plasmid expression), which was unexpectedly non-toxic. Unfortunately, delivery of both power and standard miRNA inhibitors by nucleofection did not successfully upregulate the Nrf2 pathway. Proteomic analysis confirmed minimal broader impact of a standard

miR-340-5p inhibitor, suggesting inefficacy of the inhibitors (at least delivered by nucleofection) rather than a specific inability to upregulate Nrf2.

Conversely, the commercial activator tBHQ successfully induced Nrf2 activation, illustrated by RT-qPCR of Nrf2 pathway components (Chapter 6). Therefore, while I was unable to demonstrate miRNA targeting as a novel astrocyte-targeted antioxidant therapeutic strategy, I have characterised a simple, disease-relevant astrocyte model for screening Nrf2 activators. Functional assays demonstrated that tBHQ reduced ROS (non-significantly), enhanced viability, and increased the ATP-induced Ca^{2+} response. Rather than screening further Nrf2 activators, the cell model was then used to explore the astrocytic functional effects of the ALS therapeutics riluzole and edaravone, to improve understanding of their mechanisms of action. Edaravone induced similar functional effects to tBHQ but may induce weaker Nrf2 activation (as *NQO1* was the only upregulated Nrf2 pathway gene by RT-qPCR), suggesting that improved Nrf2 activators remain needed for ALS treatment. The increased viability induced by tBHQ and edaravone likely reflects improved astrocyte health, though a possible contribution of increased reactivity-associated proliferation warrants further investigation. Enhanced ATP responses may potentiate excitotoxicity via glutamate release and promote further astrocyte reactivity (Bezzi *et al.*, 2004; Franke *et al.*, 2012), potentially contributing to the limited clinical efficacy of edaravone, especially since purinergic signalling is elevated in ALS patients (Cieślak *et al.*, 2019). This effect of tBHQ is likely due to inhibition of the SERCA Ca^{2+} pump (Uddin & Thomas, 2024), however it should be confirmed that it isn't a limitation of Nrf2 activation, before pursuing novel Nrf2-activating therapeutics. In retrospect, functional astrocyte assays should have included a panel of commercial Nrf2 activators beyond tBHQ to distinguish true Nrf2-driven effects from drug-specific effects.

Riluzole exhibited contrasting effects to tBHQ and edaravone: while it upregulated Nrf2 pathway gene expression, it did so likely via oxidative stress, as indicated by unchanged ROS (despite Nrf2 activity) and reduced viability. Riluzole also did not significantly affect the Ca^{2+} response to either ATP or glutamate. While this means it is unlikely to potentiate excitotoxicity like edaravone, it is also surprising that this known anti-excitotoxic drug does not reduce astrocyte excitability. This may suggest that riluzole is more effective in neurones than in astrocytes, which may partly explain its limited therapeutic efficacy for ALS treatment. Overall, these findings reveal limitations of the current ALS therapeutics, by examining astrocytic effects that have so far been relatively overlooked. These results highlight the need for novel Nrf2 activators that do not promote astrocyte reactivity or excitotoxicity in ALS patients.

During this study, it became apparent that targeting miRNAs to upregulate the Nrf2 antioxidant response was more complex than anticipated, primarily due to challenges in targeting human primary astrocytes. If this study was performed again, optimisation should begin with a well-validated miRNA-target gene pair before testing candidate Nrf2-targeting miRNAs. The inhibitors could also be fluorescently tagged even when delivered by nucleofection, as all

nucleofected cells were assumed to have taken up both GFP plasmid and miRNA inhibitor, however this may not have been the case.

MiRNAs and miRNA-based therapeutics have gained significant attention over the past two decades, with ~55,000 papers published in the last 5 years alone with “microRNA” in the title or abstract. As noted by (DeFrancesco, 2025), miRNA therapeutics carry greater biological risk than other antisense technologies due to their potential to affect hundreds or thousands of mRNA targets. Despite widespread interest, no miRNA therapeutics have reached phase III trials. However, the development of a miR-17 inhibitor for autosomal dominant polycystic kidney disease - backed by substantial investment and promising results - may serve as a valuable case study for advancing miRNA-based therapies (DeFrancesco, 2025). MiRNA therapeutics often fail as miRNAs targets are selected due to altered expression in disease, despite limited understanding of their targets and mechanisms (DeFrancesco, 2025). Arguably the approach of this thesis is therefore preferable by focusing on Nrf2 pathway regulation, rather than the disease-relevance of the inhibited miRNAs. Nonetheless, challenges in Nrf2 upregulation may in part stem from poorly defined mechanisms and off-target effects of the candidate miRNAs.

Although this thesis centres on enhancing astrocytic Nrf2 signalling as a therapeutic strategy for ALS, findings may extend to other diseases and normal physiology. The generated proteomic and sncRNAseq datasets offer valuable insights into astrocyte function and miRNA-regulated pathways. Astrocyte reactivity is common across neurological conditions such as AD, PD and Huntington’s disease, CNS tumours, and injuries induced by stroke or trauma (Ben Haim *et al*, 2015). Despite the heterogeneity of astrocyte reactivity, and the imperfect nature of an FBS-induced reactive astrocyte model for studying human disease, these data may drive hypotheses for future investigations involving these diseases. As for ALS, astrocyte Nrf2 activation has shown neuroprotective effects in Parkinson’s and Huntington’s disease mouse models (Calkins *et al.*, 2010; Chen *et al*, 2009), therefore the functional data revealing the astrocytic effects of tBHQ may be relevant beyond ALS.

7.1 Future directions

In Chapter 3, proteomic analysis revealed ALS-associated proteins differentially expressed during astrocyte reactivity, for example SOD2, PON2 and CAV1. While I focussed on validating the Nrf2 pathway deficit in reactive astrocytes, future work could explore the astrocytic function of these proteins. Following western blot validation, their expression could be assessed in cytokine-induced reactive astrocytes and patient-derived iAstrocytes to determine whether this pattern is broadly relevant to astrocyte reactivity and/or ALS astrocytes. Validated proteins could then be manipulated using siRNAs or commercial activators to evaluate their impact on inflammatory and disease-related phenotypes, and their potential as astrocyte therapeutic targets.

To expand the experimental scope of this thesis, developing an astrocyte-motor neurone co-culture model would enable direct assessment of the effects of therapeutics (for example miRNA inhibitors, TBHQ, riluzole and edaravone) on astrocyte-mediated neurotoxicity. Although not presented here, an indirect method of modelling astrocyte-neurone interactions was attempted by treating mouse primary cortical neurones with human astrocyte-conditioned medium or ADEVs. The quiescent and reactive astrocyte secretomes did not affect axon growth (used as a simple readout of neuronal health) meaning no neurotoxic reactive astrocyte phenotype was identified that may be rescued via Nrf2 modulation, therefore this model was not taken further. Mouse cortical neurones were used for practicality, but future studies should adopt human iPSC-derived astrocytes and motor neurones with ALS-associated gene variants for disease-specific co-culture modelling. A robust co-culture system would support high-throughput screening of Nrf2 activators, for example by the functional assays described in Chapter 6, to identify effective neuroprotective strategies.

In Chapter 4, sncRNAseq analysis of our human astrocyte models identified miRNAs potentially involved in regulating astrocyte reactivity, including miR-206 which was predicted to promote reactivity by targeting NURR1. Although miR-206 expression could not be confirmed in reactive astrocytes via RT-qPCR, future experiments could involve inhibiting miR-206 to assess its impact on astrocyte reactivity and validate NURR1 targeting in reactive astrocytes. The role of NURR1 in ALS-related neuroinflammation could also be explored by measuring its astrocytic expression in patient tissue or patient-derived iAstrocytes, once reliable NURR1 protein detection has been optimised. Additional candidate miRNAs may also be investigated using the morphology assay developed in Chapter 3, to identify any reactivity-associated morphological changes associated with their manipulation.

The sncRNAseq analysis of Chapter 4 also included motif analysis of whole-cell and ADEV-enriched miRNAs, to identify likely RBPs involved in astrocyte miRNA sorting. I identified a possible loss in miRNA sorting activity by ALS-associated RBPs upon astrocyte reactivity, including TDP-43, FUS and hnRNPA1. Ongoing work by our research group (Dr Katherine White) now involves investigating whether ALS-linked *FUS* variants affect miRNA loading into EVs from iPSC-derived astrocytes and motor neurones, aiming to determine if disrupted miRNA sorting contributes to altered cell-cell communication in ALS.

A key aim of this thesis was to explore miRNA targeting to enhance Nrf2 signalling in reactive astrocytes. However as demonstrated in Chapter 5, this was not successful, despite identification of nucleofection as an efficient method of delivering genetic material to human primary astrocytes. Further work should involve ensuring that miRNA inhibitor integrity is not altered by nucleofection, perhaps by RT-qPCR. Additional optimisation of inhibitor concentration and incubation time could also be performed, and perhaps AAV-based delivery explored, which is more viable than nucleofection for therapeutic use.

In Chapter 6, edaravone was found to enhance reactive astrocyte Ca^{2+} responses to ATP, suggesting increased excitability. Given ATP's link to astrocyte reactivity and glutamate release, this may indicate a potentially adverse effect of edaravone. Future studies should measure ATP-induced glutamate release from astrocytes to evaluate the impact of edaravone on excitotoxicity, and its effect on astrocyte reactivity markers or morphology. An astrocyte-motor neurone co-culture model in a compartmentalised microfluidic chamber system would enable direct measurement of the astrocytic effects of edaravone on motor neurone degeneration. This may include astrocytic functions that have not been explored in this thesis such as phagocytosis of synapses, a vital process for synapse pruning and removal of protein aggregates and cellular debris (Choi & Chung, 2025). Dysregulation of this process is likely relevant in ALS patients, for example because phagocytic activity was shown to be increased in the astrocytes of mSOD1 mice (Baker *et al*, 2015; Choi & Chung, 2025).

Chapter 8. References

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Chapter 9. Appendix

Table 9.1. Composition of G5 supplement [Thermo Fisher Scientific].

Components	Concentration (mg/l)	Molar (mM)
Biotin	100	0.41
Basic FGF	0.5	Infinity
EGF	1	Infinity
Human transferrin	5000	Infinity
Insulin	500	Infinity
Hydrocortisone	0.36	9.9 x10 ⁻⁴
Selenite	0.52	0.003

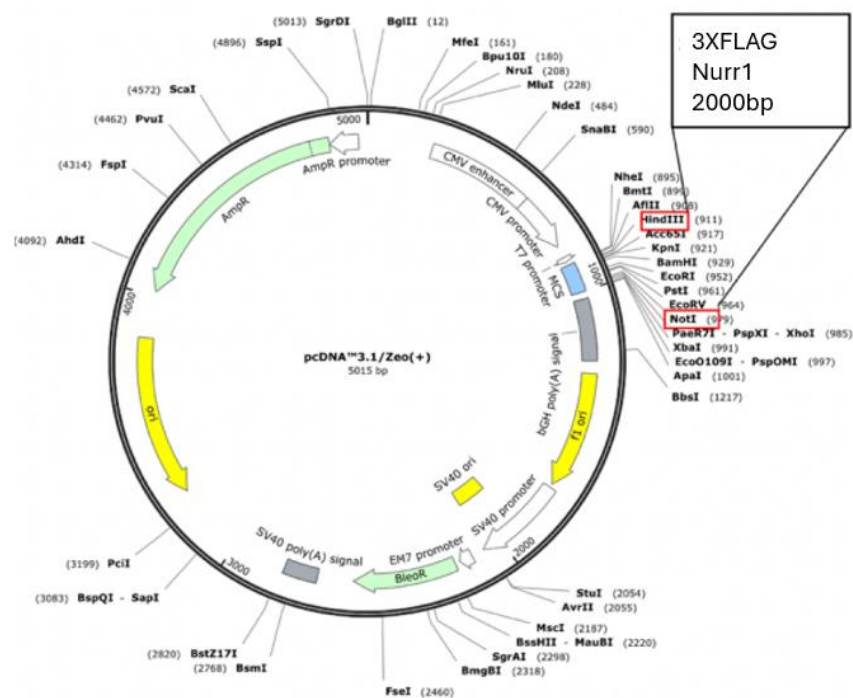


Figure 9.1. NURR1 expression plasmid map. Vector [Invitrogen V86020]. A gift from Nikki De Vivo [University of Nottingham]. Image adapted from (Edey, 2023).

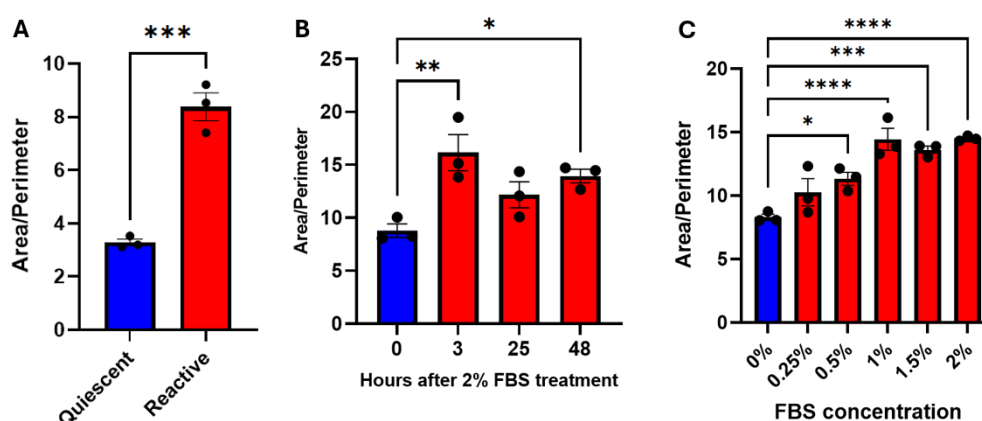


Figure 9.2. Area/perimeter ratio cell measurements can distinguish between human reactive and quiescent astrocytes. FIJI was used to draw around astrocytes in brightfield images using the freehand selection tool, to measure the area/perimeter ratio of each cell. Each datapoint is the average value from each independent experiment. A) Area/perimeter ratio of quiescent and reactive astrocytes. Figure adapted from (White et al., 2024). Welch's t-test, $n=3$. B) Area/perimeter ratio over time after treatment of quiescent astrocytes with 2% FBS in G5 medium. Figure adapted from (White et al., 2024). One-way ANOVA, $n=3$. C) Area/perimeter ratio of quiescent astrocytes after treatment with different concentrations of FBS in G5 medium for 48 hours. One-way ANOVA, $n=3$.

Table 9.2. Nrf2 target gene products detected in a proteomic analysis of quiescent and reactive human astrocytes.

Nrf2 target genes
ABCB6, ACOT7, ACOX1, ATG5, ATG7, BLVRA, BLVRB, CBR1, DNAJB1, EPHX1, FTH1, G6PD, GCLC, GCLM, GPX1, IDH1, NFKB1, NQO1, PGD, PPAT, PRDX1, PSMA1, PSMB5, SQSTM1, TALDO1, TKT

SWATH-MS was used for proteomic analysis of human primary quiescent and reactive astrocytes, with data analysed using StatsPro. The genes shown all passed StatsPro analysis of proteomics batch 1.

Table 9.3. ALS-associated (ALSoD) proteins detected in a proteomic analysis of quiescent and reactive human astrocytes.

Category	Gene symbol
Clinical modifier	ATXN2, ENAH
Definitive ALS gene	ANXA11, FUS, HNRNPA1, OPTN, PFN1, SOD1, TARDBP, TBK1, UBQLN2, VAPB, VCP
Moderate evidence	CHMP2B, DNAJC7, DNMT3A, LMNB1, SARM1, SMN1, SQSTM1
Strong evidence	SCFD1, TUBA4A
Tenuous	ALAD, APEX1, CST3, DCTN1, DYNC1H1, ELP3, ERLIN1, EWSR1, GLT8D1, GRN, HEXA, HNRNPA2B1, ITPR2, KIFAP3, MATR3, NEFL, PARK7, PNPLA6, PON2, PRPH, PSEN1, PVR, RFTN1, SIGMAR1, SOD2, SPAST, SYNE1, TAF15, TIA1, UBQLN1, VRK1
Unassigned	CAV1, CAV2

SWATH-MS was used for proteomic analysis of human primary quiescent and reactive astrocytes, with data analysed using StatsPro. The category of ALS-association according to ALSoD is given (Abel et al., 2012). The genes shown all passed StatsPro analysis of proteomics batch 1.

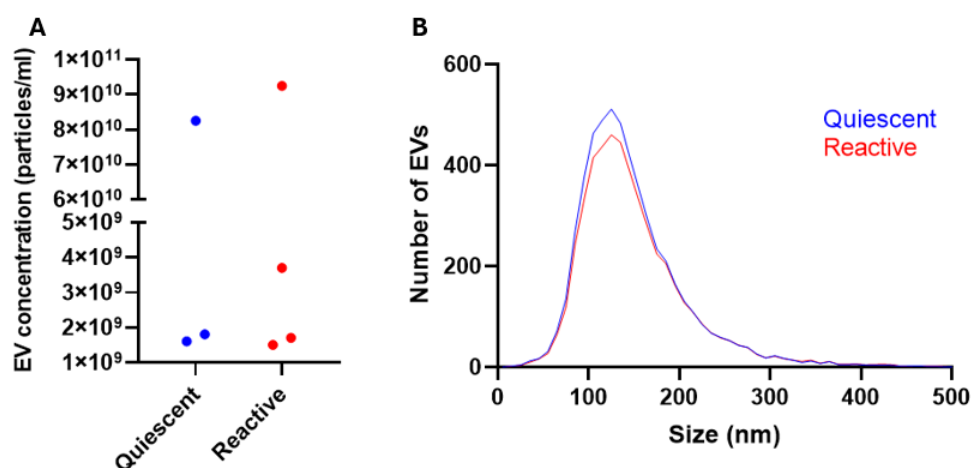


Figure 9.3. Nanoparticle tracking analysis (NTA) of human ADEVs used for *sncRNAseq*. Quiescent and reactive ADEVs were analysed using the Zetaview®. Quiescent $n=3$, reactive $n=4$ (2 replicates combined for one sample). No normalisation has been performed to cell number, as all cells were used for RNA extraction, therefore no statistical tests have been performed. A) Average EV concentration from two technical replicates. SEM bars not possible due to split scale. B) Average size distribution of reactive and quiescent ADEVs across the three biological replicates.

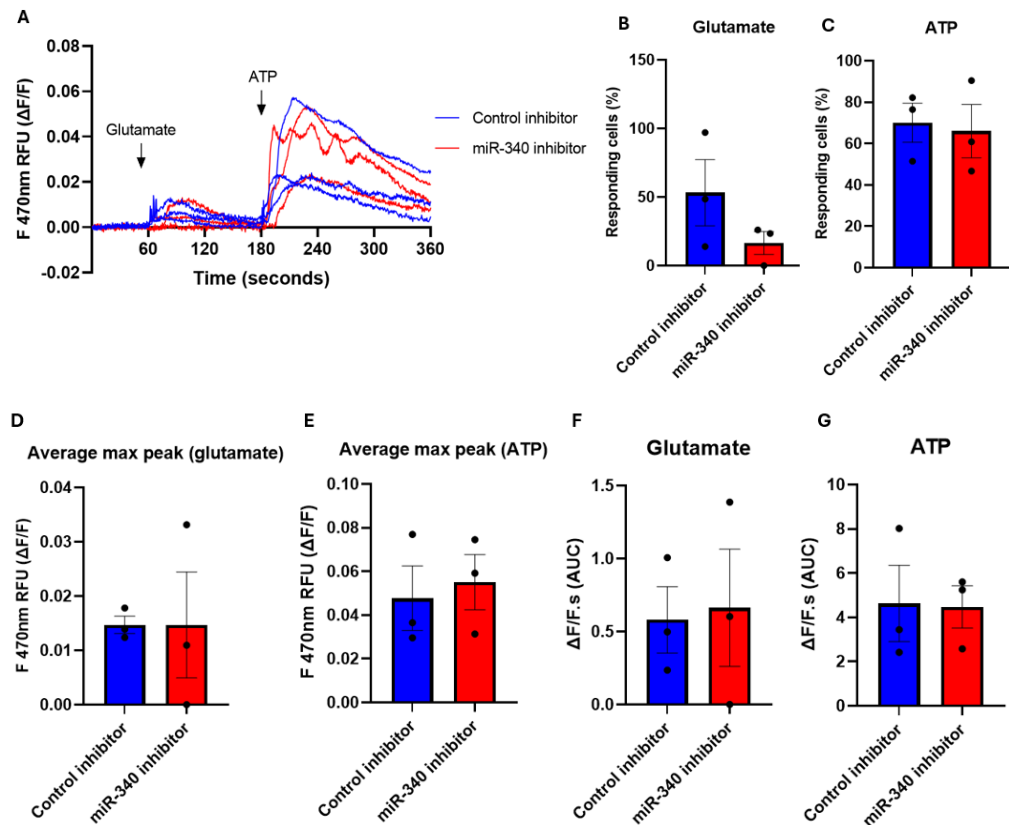


Figure 9.4. Nucleofection of miR-340-5p inhibitor does not affect intracellular calcium concentration of reactive astrocytes after stimulation with glutamate or ATP. Reactive astrocytes were nucleofected with control or miR-340-5p (miR-340) standard inhibitor (~175nM). After 24 hours, calcium imaging was performed using the Fluor-4 calcium indicator. N=3, with one technical replicate each for two biological replicates, while an average of 3 technical replicates was calculated for the third biological replicate. Calcium imaging was then performed using the Fluor-4 calcium indicator (1 μ M). After 1 minute without stimulation, cells were stimulated for 2 minutes with glutamate (200 μ M) then 3 minutes with ATP (300 μ M), followed by 2 minutes with ionomycin (1 μ M) as a positive control. A maximum $\Delta F/F$ value of >0.01 was required to be considered a response and included for analysis. RFU = relative fluorescent units. AUC = area under curve. A) Trace showing Ca^{2+} response to glutamate and ATP stimulation for cells that respond to either neurotransmitter. Ionomycin not included. B+C) Percentage of cells that respond to B) glutamate and C) ATP. The different symbols in Figure B illustrate the variability between biological replicates. D+E) Average maximum (max) Ca^{2+} signal (peak height) of cells responding to D) glutamate and E) ATP. F+G) AUC indicating the total Ca^{2+} signal during the F) glutamate stimulation and G) ATP stimulation. Welch's t-tests used for B-G, no statistical test for A (representative image only).