

Astrocyte Neuropathology in Autism: Role of Neuroinflammation & Glutamatergic Signalling

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LEADING MENTAL HEALTH RESEARCH ACROSS THE LIFESPAN



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Abstract

Alterations in excitatory glutamatergic signalling together with increased astrocytic activation and neuroinflammation have been observed in the brain of individuals with autism. Astrocytes play important roles in developmental corticogenesis and neurogenesis, as well as regulating glutamate receptor signalling and intercellular glutamate levels at the glutamatergic tripartite synapse. The overall aim of my PhD was to investigate astrocytic neuropathology in autism and its potential interaction with disruptions in mGluR5 signalling.

I conducted a post-mortem stereological investigation within the white matter of the dorsolateral prefrontal cortex (DLPFC) to assess density of astrocyte and other glia utilising the optical fractionator. In addition, astrocytic somal size was assessed via the nucleator and total astrocyte process length estimated utilising the spaceballs probe. Using an *in-vitro* approach I then explored the effect of Poly I:C mediated astrocyte activation on mGluR5 glutamatergic signalling. This included assessing levels of the pro-inflammatory markers IL-6 and Rantes using ELISA, gene expression of astrocytic and glutamatergic genes via qPCR, as well as mGluR5 activity using a radioligand binding assay. Finally, I characterised the gene and protein expression of astrocyte markers of human pluripotent stem cells (hPSC) derived astrocytes using qPCR and immunohistochemistry, as well as cytokines levels using ELISA.

The current study revealed no change in astrocyte density or activation morphology within the white matter of the DLPFC in autism versus age matched controls. There was also no alteration in astrocyte cell somal size and total process length. *In-vitro* Poly I:C induced astrocyte activation demonstrated reduced mGluR5 binding and mRNA expression, with disruption to other astrocytic glutamatergic elements. A novel protocol for differentiating

human pluripotent stem cells into astrocytes was developed, with hPSC-derived astrocytes displaying morphology similar to that of primary human foetal astrocytes and expressing mature astrocyte markers at the gene and protein level, as well as having the ability to be activated upon exposure to Poly I:C.

My findings suggest that astrocyte activation within the brain in autism may be less severe than previously appreciated, with the absence of severe astrocytic hypertrophy and increased proliferation not observed. Results from the mechanistic *in-vitro* studies suggest that mGluR5 and glutamatergic signalling dysregulation can occur as a result of astrocyte activation, and that modulation of mGluR5 through positive allosteric modulation may have potential benefits in reversing some of these astrocyte activation mediated glutamatergic disruptions. Finally, our improved protocol for hPSC astrocyte differentiation provides a simple and efficient method to derive mature and functional astrocytes as an *in-vitro* model for autism and other neurological disorders.

Declaration

I Ting Ting Lee hereby declare that the PhD thesis entitled “Astrocyte Neuropathology in Autism: Role of Neuroinflammation and Glutamatergic Signalling” comprises only my original work, except where indicated in the preface. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Due acknowledgement has been made to all other materials used, originated from the work of others. This thesis is within the maximum word limit in length, exclusive of tables, figures, bibliographies and appendices.

Signature : 

Date : 12 December 2017

Preface

The mGluR5 drug exposure study within Chapter 3 of this thesis was carried out in collaboration with Dr. Karen Gregory from Monash Institute of Pharmaceutical Sciences (MIPS), where she conducted the radioligand binding assay, and provided mGluR5 compounds used in the study including MTEP, DHPG and VU.

Within Chapter 4, the adult astrocytes culture was established from brain tissues obtained from temporal lobectomy of patient with temporal lobe epilepsy. This tissue collection was achieved in collaboration with neurologists and neurosurgeons from the Department of Medicine and Surgery, The University of Melbourne and Royal Melbourne Hospital, including Prof Terence O'Brien, Prof Patrick Kwan, Dr Andrew Morokoff and Dr James King.

Materials within Chapter 2 of this thesis have been peer-reviewed and published by Molecular Autism Journal on 8th December 2017. A manuscript based on materials within Chapter 3 has been prepared for submission to Scientific Reports Journal. Chapter 4 contained data that has been presented at the Society for Neuroscience 2017 conference.

Throughout my PhD, the Department of Psychiatry Postgraduate Studentship, the Centre for Neural Engineering as well as the Henry and Rachael Ackman Traveling Scholarship have supported me financially.

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Abbreviations

1H-MRS	proton magnetic resonance spectroscopy
5-HT	serotonin
5-HT1A	serotonin 1A receptor
5-HT2	serotonin 2 receptor
5-HTT	serotonin transporter
ABC	avidin-biotin complex
ACC	anterior cingulate cortex
ACG	anterior cingulate gyrus
ACPD	1-Amino-1,3-dicarboxyclopentane
ADHD	attention deficit hyperactive disorder
ADI-R	autism diagnostic interview-revised
ADOS	autism diagnostic observation schedule
AGP	The Autism Genome Project
AGRE	Autism Genetic Research Exchange
AGS	astrocyte growth supplement
AKT	protein kinase B
ALDH1L1	aldehyde dehydrogenase 1 family member 1
ALDOC	adolase C fructose-biphosphate
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	analysis of variance
APA	viimerican psychology association
AQP4	aquaporin 4
ASD	autism spectrum disorder
ATP	adenosine triphosphate
AUTS2	autism susceptibility candidate 2
BBB	blood brain barrier
BDNF	brain-derived neurotrophic factor
BMP	bone morphogenetic protein
C18orf22	ribosome binding factor A
C1q	complement cascade initializing protein
C3	complement 3
CCL	chemokine (C-C motif) ligand
CD68	cluster of differentiation 68
CDC	centre for disease control
cDNA	complementary deoxyribonucleic acid
CDPPB	3-Cyano-N-1,3-diphenyl-1H-pyrazol-5-yl)benzamide
CE	coefficient of error
CEU	Central European
CHPG	(RS)-2-chloro-5-hydroxyphenylglycine
CHRM1	acetylcholine muscarinic receptor 1
CNS	central nervous system
CNTNAP2	contactin-associated protein-like 2

CNVs	Copy number variants
CNX	connexin
Conc	concentration
CP	cortical plate
CSF	cerebrospinal fluid
CX3CR1	CX3C chemokine receptor 1
CXCL	chemokine (C-X-C motif) ligand
DAB	diaminobenzadine
DALY	disability-adjusted life-years
DHPG	(S)-3,5-Dihydroxyphenylglycine
DIV	days in-vitro
DLPFC	dorsolateral prefrontal cortex
DMEM-AGS	DMEM/F-12 with AGS
DMEM-IHA	DMEM/F-12 with IGF, heregulin β , activin-A
DPP6	dipeptidyl aminopeptidase-like protein 6
dsRNA	double-stranded RNA
DTI	diffusion tensor imaging
E/I	excitatory/inhibitory
EAAT	excitatory amino acid transporter
EB	embryoid bodies
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
EMX2	empty spiracles homeobox 2
FA	fractional anisotropy
FBS	foetal bovine serum
FC	frontal cortex
FG	fusiform gyrus
FGF	fibroblast growth factor
FMR1	fragile X mental retardation 1
FMRP	fragile-X mental retardation protein
FXS	fragile-X-syndrome
GABA	gamma-aminobutyric acid
GAD	glutamic acid decarboxylase
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GDP	gross domestic product
GFAP	glial fibrillary acidic protein
GLT1	glutamate transporter 1
GM-CSF	granulocyte-macrophage colony-stimulating factor
GRIA3	glutamate ionotropic receptor AMPA type subunit 3
GRM5	glutamate metabotropic receptor 5
GS	glutamine synthetase
GWAS	genome wide association studies
HACU	high-affinity choline uptake

hCS	human cortical spheroid
hESC	human embryonic stem cells
HGF	hepatocyte growth factor
hiPSC	human induced pluripotent stem cells
HLA-DR	human leukocyte antigen - antigen D related
HMBS	hydroxymethylbilane synthase
HRP	horseradish peroxidase
IBA-1	ionised calcium-binding adapter molecule 1
IFN- γ	interferon gamma
IGF-1	insulin-like growth factor 1
IGFBP	insulin-like growth factor binding protein
IgG	immunoglobulin
IL	interleukin
iNOS	inducible nitric oxide synthase
IRF	interferon regulatory transcription factor
ISH	in-situ hybridisation
IZ	intermediate zone
KA	kainate
Ki	binding affinity
KO	knockout
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
LTD	long term depression
LTP	long term potentiation
MAPK	mitogen-activated protein kinase
MCP	monocyte chemoattractant protein
MECP2	methyl CpG binding protein 2
MEGF10	Multiple EGF like domains 10
MERTK	Proto-oncogene tyrosine-protein kinase MER
MFC	medial frontal cortex
MFG	medial frontal gyrus
mGluR1	metabotropic glutamate receptor 1
mGluR5	metabotropic glutamate receptor 5
MHC	major histocompatibility complex
MIA	maternal immune activation
MPEP	2-methyl-6-(phenylethynyl)pyridine
MRI	magnetic resonance imaging
mRNA	messenger RNA
MTOR	mammalian target of rapamycin
mTOR	mechanistic target of rapamycin
MZ	marginal zone
nAChR	nicotinic acetylcholine receptor
NAM	negative allosteric modulator
NBM	neurobasal medium
NBM-FEP	neurobasal medium with FGF, EGF, PDGF-AA

NF-κB	nuclear factor kappa-light-chain-enhancer
NFIA	nuclear factor I-A
NGS	normal goat serum
Nkx6.1	NK6 homeobox 1
NMDA	N-nitrosodimethylamine
NMDAR	NMDA receptor
NPCs	neural progenitor cells
OCT4	octamer-binding transcription factor 4
OD	optical density
OFC	orbitofrontal cortex
OLIG2	oligodendrocyte transcription factor
PAM	positive allosteric modulator
PAP	perisynaptic astrocyte process
PAX	paired box protein pax
PAX6	paired-box protein pax-6
PBS	phosphate buffered saline
PC	parietal cortex
PCC	posterior cingulate cortex
pcw	post-conception week
PDD	pervasive developmental disorders
PDD-NOS	PDD not otherwise specified
PDGF	platelet-derived growth factor
PET	positron emission tomography
PFA	paraformaldehyde
PFC	prefrontal cortex
PI	phosphatidylinositol
PI3K	phosphatidylinositol-3-kinase
PLCB1	phospholipase C beta-1
Poly I:C	polyinosinic-polycytidylic acid
PP	pre-plate
PPIA	peptidylprolyl isomerase A
qPCR	quantitative real-time polymerase chain reaction
RHEB	ras homolog enriched in the brain
RIN	RNA integrity number
RNA	ribonucleic acid
ROI	region of interest
RTT	rett syndrome
S100β	S100 calcium binding protein β
SAPAP	SAP90/PSD95 associated protein
SEM	standard error of mean
SFARI	Simons Foundation Autism Research Initiative
SHANK3	SH3 and multiple ankyrin repeat domain
SNPs	single nucleotide polymorphisms
SNVs	single nucleotide variants
SOX	SRY-Box 10

SP	sub-plate
SPECT	single-photon-emission computed tomography
SRS	systematic random sampling
STAT3	signal transducer and activator of transcription 3
TC	temporal cortex
TGF	transforming growth facto
TLR	toll-like receptor
TMB	Tetramethylbenzidine
TNF α	tumor necrosis factor alpha
TSC	tuberous sclerosis
TSP	thrombospondin
TSPO	translocator protein 18 kDa
VEN	von economo neuron
VGLUT1	vesicular glutamate transporter 1
VU	VU0360172
VZ	ventricular zone

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CHAPTER 1 INTRODUCTION

1.1 Autism spectrum disorder (ASD)

ASD comprise a group of complex and highly heritable neurodevelopmental disorders with prevalence rates soaring from 1 in 5000 children in the 1970s (Weintraub, 2011) to 1 in 132 global prevalence reported in the recent years (Baxter et al., 2015), and with estimates of 1 in 100 in Australia (Foundation, 2013). Apart from the higher prevalence rate of 1 in 68 reported by the centre for disease control (CDC) in North America (Christensen et al., 2016), there appear to be minimal regional differences in prevalence of ASD (Baxter et al., 2015). Furthermore, ASD is at least four times more prevalence in male than female (Baxter et al., 2015).

Globally, the burden of ASD was reported at 53-58 disability-adjusted life-years (DALYs) per 100,000 population (Baxter et al., 2015). A report in the United States estimated the economic burden (direct medical, non-medical and productivity cost) of ASD in 2015 to be between USD \$162-\$367 billion, which is as high as 2% of the country gross domestic product (GDP), and exceeded the burden of stroke and hypertension (Leigh & Du, 2015). In terms of family burden, another Western Australia study estimated the cost per family to be AUD \$34,900, with 90% of the burden due to loss of income (Horlin, Falkmer, Parsons, Albrecht, & Falkmer, 2014). ASD is highly detrimental to the daily life of affected children, as well as having a pronounced burden on parents and family members. These expenses include services or interventions by psychologists, speech therapists and special education services. To date, the aetiology of ASD remains unknown, with diagnosis relying solely on clinical interview. While diagnosis can be made as early as 18 months of life, this depends on the severity of symptoms exhibited. Individuals with ASD also frequently present with cognitive deficits, in particular, attention and learning difficulties, as well as other comorbid

conditions including attention deficit hyperactive disorder (ADHD), mood disorders, intellectual disability and language delay (Ronald & Hoekstra, 2011). Given that diagnosis and treatment for ASD are highly variable, understanding the underlying aetiology is crucial to the development of new drug targets for alleviating symptoms, together with providing tools for early detection and patient tailored intervention to achieve a better prognosis.

1.2 Diagnosis

The term autism as was first coined by the Austrian-American psychiatrist Leo Kanner in 1943. Since then, the description has changed from early-onset schizophrenia in the 1950s to infantile autism and autism disorder in the 1980s, before being revised to ASD in 2013 (American Psychiatric, American Psychiatric, & Force, 2013; Kanner, 1943; Weintraub, 2011).

ASD is characterised by core developmental deficits including impairment in social communication, as well as stereotyped and repetitive behaviours. Prior to 2013, The Diagnostic and Statistical Manual of Mental Disorders Fourth Edition, Text Revision (DSM-IV-TR) contained sub-classifications of pervasive developmental disorders (PDD) that included autism/autistic disorder, Asperger's disorder and PDD not otherwise specified (PDD-NOS) (American Psychiatric, American Psychiatric, & Task Force on, 2000). In 2013, the American Psychology Association (APA) introduced DSM-V, which grouped autistic disorder, Asperger's disorder and PDD-NOS under an umbrella diagnosis of ASD, with more stringent criteria for meeting diagnosis (American Psychiatric et al., 2013). Within this thesis, the term "autism" will be used to describe both autism/autistic disorder and ASD from here onwards.

The new diagnosis criteria outline that social communication and interaction deficits need to be persistent across multiple settings, including verbal or nonverbal context. Furthermore, repetitive behaviours should entail repetitive motor movements, adherence to routines or rituals, highly restricted interests, as well as hypo- or hyperactivity to sensory input (American Psychiatric et al., 2013). In the clinical setting, the above-mentioned criteria are commonly observed and scored using the Autism Diagnostic Observation Schedule (ADOS) and Autism Diagnostic Interview-Revised (ADI-R) to determine the diagnosis (Lord et al., 2000; Lord, Rutter, & Le Couteur, 1994). The final diagnosis reached is predominantly at

clinician's discretion due to the wide range of symptoms and severity presentations, including a high possibility of comorbid conditions.

1.3 Genetic basis

1.3.1 Twin and family studies

Autism has a very strong genetic basis, with 77% concordance among monozygotic twins, in which up to 38% is accounted for by genetic heritability and the remainder by environmental factors, as shown in a study of 192 autism twin pairs (Hallmayer et al., 2011). However, a wide range of concordance rates was reported from twin studies over the years, likely as a result of methodological differences. Taking into account 13 twin studies between 1977 and 2015, the concordance rates were estimated to be 45% for monozygotic twins, and 16% for dizygotic twins (Huguet, Benabou, & Bourgeron, 2016). Within the same family, the reported recurrence rates of autism for siblings were as high as 18% (Parellada et al., 2014). A population based study with a sample of just over 2 million children reported that the relative risk of individuals with family history of autism compared to those without, were 153 for monozygotic twins, 8.2 for dizygotic twins and 3.3 for siblings (Sandin et al., 2014).

1.3.2 Syndromic autism: single gene mutation

Monogenic disorders such as Fragile-X Syndrome (FXS), Rett Syndrome (RTT), and Tuberous Sclerosis (TSC), have been shown to have as high as 50% prevalence rate of autism, and are termed syndromic forms of autism (Abrahams & Geschwind, 2008).

Autism is estimated to be present in 21-50% of males with FXS (Ornoy, Weinstein- Fudim, & Ergaz, 2016). FXS is caused by a mutation within the X chromosome, with expansion of the triplet repeats in the promoter region of the gene encoding fragile X mental retardation 1 (FMR1), thus interfering with the synthesis of the protein fragile X mental retardation protein (FMRP), that is encoded by the FMR1 gene. This deficiency results in dysregulation of the glutamatergic, gamma-aminobutyric acid (GABA)ergic, phosphatidylinositol-3-kinase

(PI3K), and mammalian target of rapamycin (mTOR) pathways (Ornoy et al., 2016). Furthermore, several proteins implicated in autism are also known to be regulated by FMRP (Darnell et al., 2011).

RTT is X-linked and typically present in females due to fatality in males with the mutation, while the prevalence for autism is estimated between 25%-40% (Ornoy et al., 2016). In RTT, the disruption in the gene encoding methyl CpG binding protein 2 (MECP2) within the X-chromosome results in disturbance in translation of synaptic proteins, as a result of reduced mTOR and phosphorylated S6 (Ricciardi et al., 2011). Based on an animal model, MECP2 was shown to regulate synaptic maturation and plasticity via PI3K/protein kinase B (AKT)/mitogen-activated protein kinase (MAPK) pathways, and the associated deficit could be rescued by brain-derived neurotrophic factor (BDNF) and insulin-like growth factor 1 (IGF-1) (Castro, Mellios, & Sur, 2013).

TSC is an autosomal dominant disorder caused by mutations in the gene encoding for TSC1 and TSC2, and up to 60% of the population are also diagnosed with autism (Ornoy et al., 2016). TSC1 and TSC2 encode for the protein hamartin and tuberin respectively, which inhibit the mTOR pathway via inhibition of Ras homolog enriched in the brain (RHEB), and the mutation prevents the inhibition of Rheb, which lead to uncontrolled mTOR activation. This excessive mTOR activity affects dendritic protein synthesis, which leads to synaptic abnormalities including altered dendritic spines and glutamatergic signalling (Ornoy et al., 2016).

1.3.3 Idiopathic autism: rare de novo mutation and common variants

Based on the evidence for a strong genetic component to autism, researchers have employed tools such as microarray, whole exome sequencing, whole genome sequencing,

ribonucleic acid (RNA)-sequencing and candidate gene analysis in an attempt to tease out risk contributing genes that are associated with autism. Many genetic variations, including *de novo* and inherited variations, such as copy-number variants (CNVs) and single nucleotide polymorphisms (SNPs), have been found to be associated with autism. However, no single gene contributes to more than 1% of the overall autism cases (Parellada et al., 2014). Among autism patients, *de novo* CNVs are present in 4-7% of the population as opposed to 1-2% among controls, and most CNVs are exclusive to each patient, with a small proportion observed in another (Huguet et al., 2016). Furthermore, individuals with autism were also found to carry more gene disruptive variants than non-affected siblings (Turner et al., 2017). Genes affected by these *de novo* CNVs in autism patients are usually found within pathways of neuronal development and synaptic function (Pinto et al., 2014). As for *de novo* single nucleotide variants (SNVs), the average variant number was 0.73-0.86 in autism patients compared to 0.60 in unaffected siblings, and these SNVs are more likely to be located on paternal chromosome (Huguet et al., 2016; Ronemus, Iossifov, Levy, & Wigler, 2014).

More than 95% of genetic variants in the human genetic pool are common in more than 5% of the population, and are called common variants (Fu et al., 2013; Huguet et al., 2016). Several studies have demonstrated that while rare mutations in genes can contribute to the risk of autism, the additive effect of common genetic variants account for the major source of potential risk for autism and it is likely that the composition of these different variants will differ among individuals (Bourgeron, 2015; Gaugler et al., 2014; Klei et al., 2012; Sanders et al., 2012). Klei et al. reported that 40-60% of autism heritability in families are due to common variants (Klei et al., 2012). Within the same group, Gaugler et al. later estimated the heritability to be mostly due to common variants (52.4%) with a very small proportion of 2.6% due to rare variants (Gaugler et al., 2014). Due to the multi-variants nature of autism genetic composition, researchers constantly face the major challenge of under power in

genetic studies, even in large scaled genome wide association studies (GWAS) (Anney et al., 2012).

The Autism Genome Project (AGP) GWAS in 2705 families investigated over a million SNPs and showed a combined effect of multiple SNPs in case control prediction (Anney et al., 2012). Amongst these SNPs the one with the highest association was rs1718101 located within the gene for contactin-associated protein-like 2 (*CNTNAP2*), that encodes a neuronal transmembrane protein in the neurexin family, involved in axonal differentiation (Anney et al., 2012). Utilising an Affymetrix SNP microarray, Egger and colleagues (Egger et al., 2014) discovered CNVs in genes that have previously been implicated in autism, including *BDNF*, autism susceptibility candidate 2 (*AUTS2*), dipeptidyl aminopeptidase-like protein 6 (*DPP6*), and ribosome binding factor A (*C18orf22*). Using a whole exome sequencing approach, An and colleagues (An et al., 2014) discovered rare variants within important processes involved in neuronal and synaptic development. An RNA-sequencing study on autism brain samples further revealed enrichment of brain-specific enhancer RNAs for genetic variants associated with autism (Yao et al., 2015).

Using SNP data taken from an enriched Central European (CEU) cohort, our group generated an autism genetic predictive diagnostic classifier that achieved a 78% predictive accuracy across three large independent CEU cohorts, based on data from the Autism Genetic Research Exchange (AGRE) and the Simons Foundation Autism Research Initiative (SFARI) (Skafidas et al., 2012). Importantly, SNPs within the gene glutamate metabotropic glutamate receptor 5 (*GRM5*) had the greatest contributing weight in the structure of this classifier. In addition, genes in the glutamatergic pathway were also strongly represented in the 237 SNPs that spanned 146 genes. In relation to the metabotropic glutamate receptor 5 (mGluR5), our genetic association with autism is in keeping with other lines of evidence

demonstrating that *GRM5* knockout mice displayed autism-like features including increased dendritic spine density, microglial activation and cognitive impairment, suggesting a role of mGluR5 in autism aetiology (Chana et al., 2015; Chen, Lu, & Brumberg, 2012; Xu, Zhu, Contractor, & Heinemann, 2009; Zantomio et al., 2015).

Genetic studies with pathway analysis have also identified many other candidate genes for autism, mainly within the clusters of chromatin remodelling, protein synthesis, protein degradation and synaptic signalling pathways (**Figure 1.1**) (Huguet et al., 2016). Other transcriptomic studies using post-mortem brain tissues revealed network modules associated with autism including genes involved in synaptic function, and neuroinflammation (Gupta et al., 2014; Voineagu et al., 2011). Together, these genetic based findings formed the basis of many molecular and mechanistic investigations in autism, especially with evidence appearing to converge on synaptic signalling pathway.

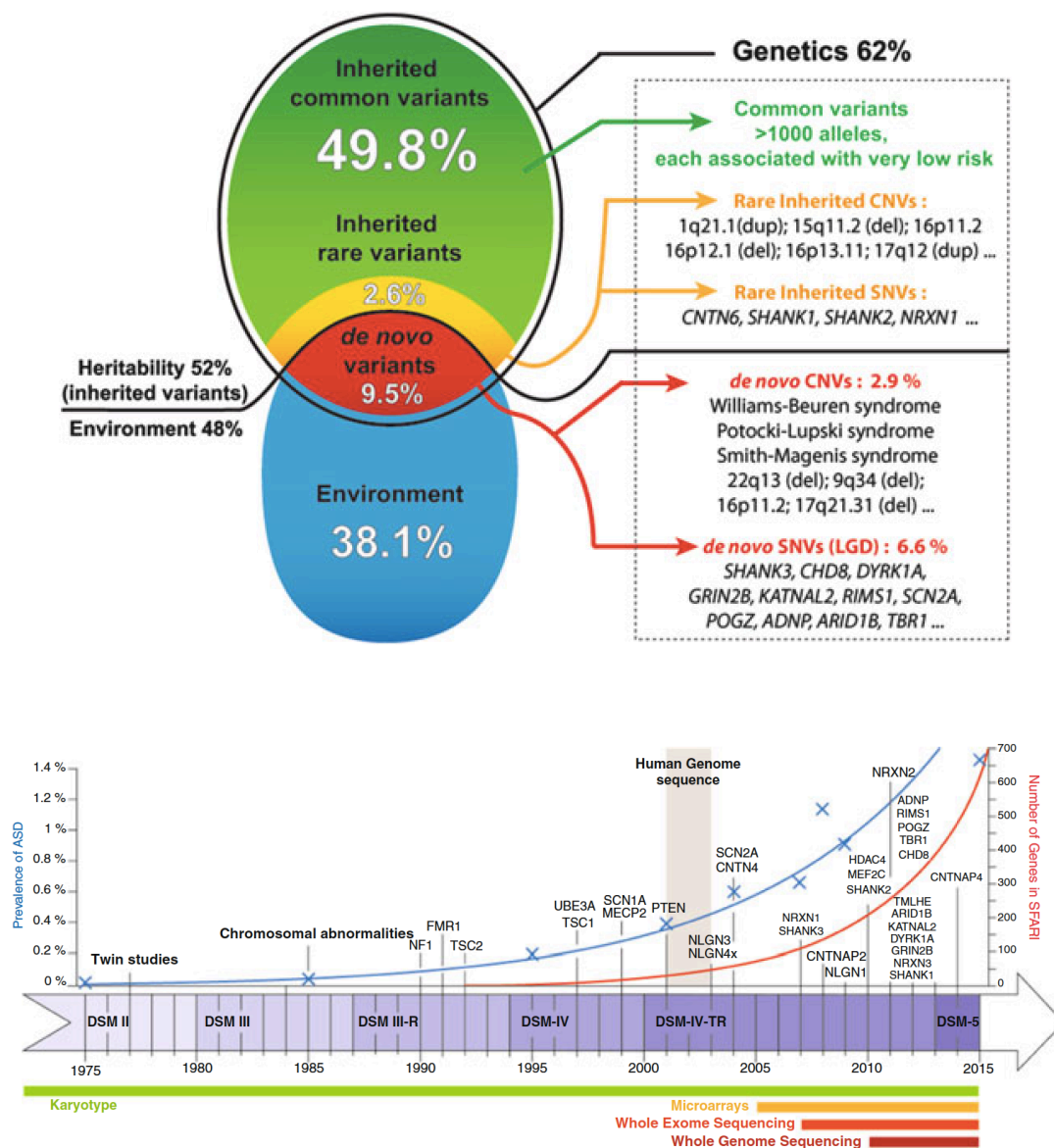


Figure 1.1 | Genetics contribution and prevalence rates of autism

(Top) The risk contribution of genetics and environmental towards autism based on genetic studies on twins and families. Inherited common variants contribute to the majority of liability, followed by rare de novo and inherited variants. **(Bottom)** Prevalence rates of autism from 1975 to 2015, as well as candidate genes identified by genetic studies. (image from figure 1&3 (Huguet et al., 2016))

1.4 Typical neurodevelopment

Given autism is a neurodevelopmental disorder, it is important to understand the typical physiology of brain development to pin-point potential mechanisms and time points that may be associated with the pathophysiology of autism.

Human brain development begins at the third post-conception week (pcw) in the embryonic period and extends through the postnatal period into adulthood (Stiles & Jernigan, 2010). During the embryonic period, gastrulation occurs where a trilaminar structure containing the ectoderm, mesoderm and endoderm is formed, with the ectoderm later developing into the central nervous system (CNS) (Ozair, Kintner, & Brivanlou, 2013). The neural tube contains populations of neural stem cells, which subsequently give rise to neural progenitor cells (NPCs), is the first structure to form in the CNS and serves as the basis for CNS development, beginning to form around the third pcw (Ziats, Edmonson, & Rennert, 2015). Following on, NPCs proliferate from the ventricular zone (VZ) and line up along the adjacent hollow centre of the neural tube, which later develops into the ventricles (Stiles & Jernigan, 2010).

Developmentally, the human foetal period spans from about ninth pcw until birth, and represents the period of rapid development of the CNS where the mature patterning of gyri and sulci are formed (Stiles & Jernigan, 2010). NPCs proliferate in the VZ, then mature into new neurons that migrate outward to their final position in the neocortex, first forming the initial and transient structure pre-plate (PP), which then develops and separates into the marginal zone (MZ) and sub-plate (SP). These neurons settle to form layer 6 of the cortical plate (CP), and commence their final differentiation into mature neurons. Subsequently, further migrating cells arrive and form the more superficial layers of the neocortex in an organised mini-columnar organisation, with the guidance of radial glial cells to arrive in their

final destinations as the brain develops. Radial glial cells from the VZ extend and attach their processes to the pia, which act as scaffolding to facilitate neuronal migration, especially towards the more superficial layers of the CP. This is further aided by neurotrophic signalling molecules to direct cellular movement in a concentration gradient dependent manner, eventually forming the 6 layers of the mature human cortex, while MZ and SP neurons go through apoptosis and disappear (**Figure 1.2**) (Stiles & Jernigan, 2010; Ziats et al., 2015). The neuronal cell bodies make up the grey matter of the cortex, forming distinct regions that consist of different neuronal subtypes and organisation pattern, as a result of various signalling molecules at different time points of development (Valiente & Marín, 2010). One such cascade is the balance between the molecules empty spiracles homeobox 2 (EMX2) and paired box protein pax-6 (PAX6) that exist in the opposite gradient along the anterior-posterior of the brain, where PAX6 is highly expressed in forebrain region while EMX2 is highly expressed in hindbrain region (Hamasaki, Leingärtner, Ringstedt, & O'Leary, 2004). Here, the different concentrations of PAX6 and EMX2 trigger NPCs to switch on/off certain genes in order to direct their final fate corresponding to the brain regions. During the final differentiation process, neurons produce neurotransmitters and neurotrophic factors, as well as extend their processes, axon and dendrites, to establish connections with other cells via synapses. These processes are ensheathed in myelin by oligodendrocytes to enhance transmission of information, and usually densely packed together forming the white matter of the human brain (Stiles & Jernigan, 2010).

During the course of neurodevelopment, a significant number of neurons and their processes are lost as a result of apoptosis as well as synaptic exuberance and pruning. Up to 50% of neurons and their processes are eliminated systematically via these two processes, in order to eliminate excess cells and weaker connections, while enriching for only highly essential, efficient and functional neuronal networks (Buss, Sun, & Oppenheim, 2006).

Similarly, excess glial cells including oligodendrocytes and astrocytes undergo apoptosis based on signalling of nearby neurons to maintain appropriate neuron:glial ratios (McTigue & Tripathi, 2008; Silver & Steindler, 2009). During the early postnatal period, the brain remains plastic, developing and refining connections based on inputs from the major sensory systems, a process termed 'experience dependent' learning (Grossman, Churchill, Bates, Kleim, & Greenough, 2002; Stiles & Jernigan, 2010). Processes such as axonal and synaptic pruning occur as a result of external experience stimuli, which partly influence astrocytic and microglial functioning, as well as the release of neurotrophic factors as the brain mature into adulthood.

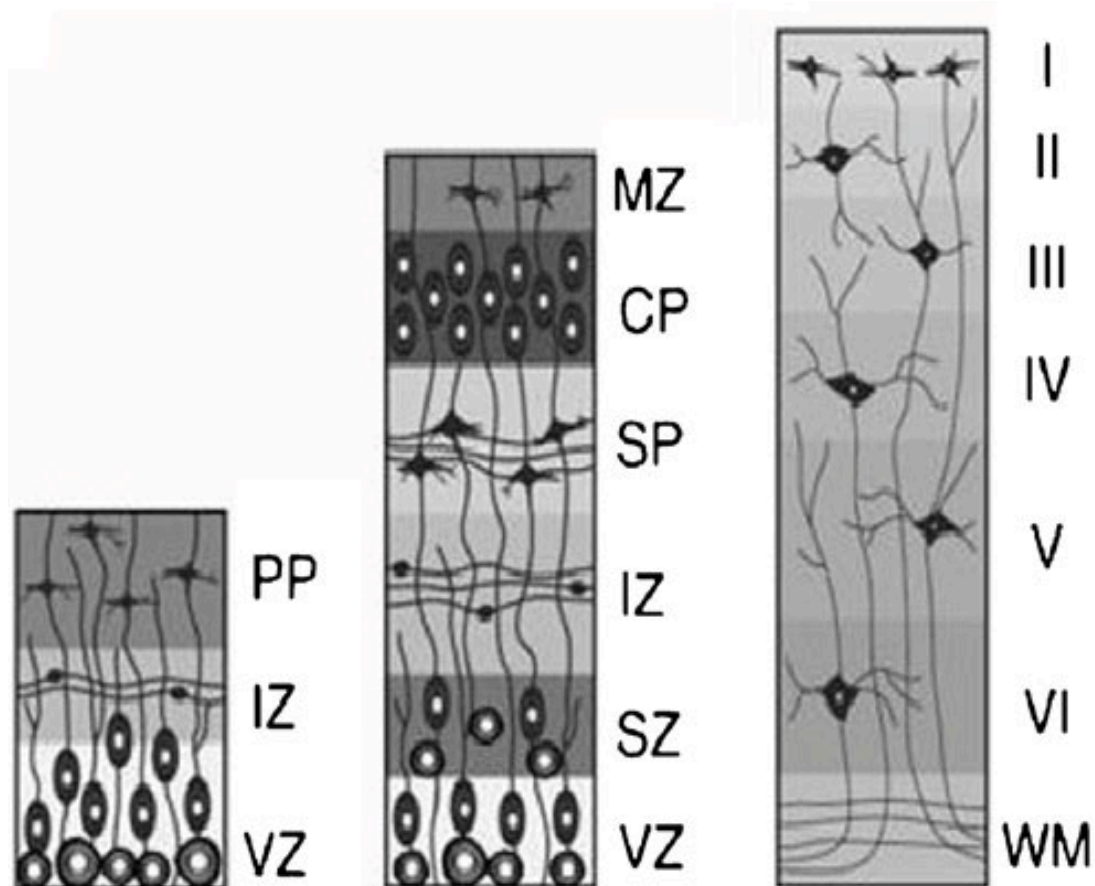


Figure 1.2 | Developmental neuronal migration for corticogenesis

(Left) The first batch of neurons migrating out of the ventricular zone (VZ) to form the preplate (PP).

(Middle) PP further develops into the subplate (SP) and marginal zone (MZ), where cortical plate (CP) starts forming in between the two and further develops into the 6 cortical layers in the mature brain.

(Right) CP develops into 6 layers of mature cortical grey matter, SP and MZ disappear following maturation, the intermediate zone (IZ) develops into mature white matter. Image from Stiles and Jernigan (2010)

1.5 Pathophysiology of autism

Given the early onset, abnormalities within the brain that lead to autism are likely present at birth, although the timing of when these cellular or molecular abnormalities occur during neurodevelopment, and what causes them, remains unknown. Various structural and cellular abnormalities have been identified in the brains of people with autism including increased brain size, reduced neuronal size, increased densities and altered distribution of neurons and glia across different brain regions (Blatt, 2012; Raymond, Bauman, & Kemper, 1996). In addition, the transient structures during neurodevelopment, the MZ and SP, have also been hypothesised to abnormally develop in autism, whereby downregulation of the genes *Reelin* and *TBR1*, which are highly expressed in the MZ and SP, are suspected to potentially contribute to changes and disruption in cortical organisation seen in autism brains (Hoerder-Suabedissen et al., 2013; Ziats et al., 2015). Due to the temporary existence and rapid development of these CNS intermediary structures during prenatal development, in vivo or post-mortem brain investigation to uncover these abnormalities is precluded, and instead must rely on the use of animal models. Nevertheless, numerous neuroimaging and post-mortem investigations into brains of individuals with autism have revealed valuable information regarding the underlying structural abnormalities in autism and how they may relate to pathophysiology of the disorder.

1.5.1 Anatomical/structural abnormalities

Using magnetic resonance imaging (MRI), studies have reported that 90% of individuals with autism between the ages of 2-4 years have larger than average brain volume, with 37% of these individuals being classified as having developmental macrocephaly (Courchesne, 2002; Courchesne et al., 2001; Lainhart et al., 1997). The overall brain enlargement results from increased cerebral cortical grey matter and white matter, as well as increased cerebellar

white matter early in life. These abnormalities diminish in older children and adolescents, mainly due to slow growth rate after the initial overgrowth (Courchesne, 2002). Interestingly, the enlargement was predominantly observed in the frontal cortex, a region responsible for higher cognitive functioning including emotion and social communication (Amaral, Schumann, & Nordahl, 2008; Courchesne, 2002; Courchesne et al., 2001; Courchesne & Pierce, 2005).

An early MRI study of 15 individuals with high functioning autism and age-matched controls reported decreased grey matter volumes in anterior regions (paracingulate sulcus, inferior frontal gyrus) while posterior regions (amygdala, middle frontal gyrus and inferior temporal gyrus) were found to have increased grey matter volume (Abell et al., 1999). Hadjikhani and colleagues later observed cortical thinning in the pars opercularis of the inferior frontal gyrus, inferior frontal cortex, inferior parietal lobule and superior temporal sulcus of individuals with autism. Interestingly, the authors also reported significant correlation between cortical thinning in these areas and autism symptoms severity as measured by ADI-R (Hadjikhani, Joseph, Snyder, & Tager-Flusberg, 2006).

Herbert and colleagues showed, in a cohort of 17 autism individuals and 15 controls, that the increase in brain volume was predominantly contributed by white matter enlargement, but decrease in grey matter volume (Herbert et al., 2003). A diffusion tensor imaging (DTI) study compared 25 adolescent individuals with autism and 25 controls and reported increased fractional anisotropy (FA) in the frontal lobe in autism individuals, and that these are accompanied by age related loss of FA in the autism group as oppose to FA gain in controls (Cheng et al., 2010). Interestingly, this increase in total white matter volume appear

to persist throughout all age range in an independent autism cohort when compared to controls (Lange et al., 2015).

In contrast, several other neuroimaging studies have reported no evidence for overall or regional brain enlargement in autism, suggesting perhaps that changes observed may be restricted to certain brain regions or individuals with autism (Dinstein, Haar, Atsmon, & Schtaerman, 2017; Piven, Arndt, Bailey, & Andreasen, 1996). These reported inconsistencies could also be attributed to differences in methodologies between studies or by the heterogeneity of the disorder and/or age related differences present between sample cohorts (Katuwal et al., 2016). However, it is also possible that macrocephaly describes a subset of individuals with autism, and is not necessarily a hallmark of autism brain pathology, as suggested by a 3-year longitudinal study of 695 individuals that found no significant difference between high risk infants who were later diagnosed with autism versus those who did not develop autism (Zwaigenbaum et al., 2014). A recent longitudinal study of 100 individuals with autism and 56 typical developing controls over an 8 year period reported an increase in whole brain volume in childhood, with subsequent decrease in adolescence and adulthood, potentially explaining the inconsistencies between different brain volumetric studies in autism (Lange et al., 2015).

When presented with fearful stimuli including still images and short videos, individuals with autism failed to activate emotion-processing regions (including amygdala, lateral inferior frontal gyrus, and dorsal premotor cortex) despite normal functioning in their action perception system (activation of posterior superior temporal gyrus, intra parietal sulcus, precentral gyrus and dorsal inferior frontal gyrus) upon presentation of neutral stimuli (Grèzes, Wicker, Berthoz, & de Gelder, 2009). Furthermore, a reduced connectivity was also

observed between the amygdala, lateral inferior frontal gyrus, premotor cortex and superior temporal sulcus among individuals with autism, suggesting a weakened or flattened emotional perception in autism due to hypo-activation and hypo-connectivity in emotion-processing regions (Grèzes et al., 2009).

Hazlett and colleagues investigated a cohort of 59 individuals with autism and 38 controls at 2 years of age, and observed a general increase of cortical thickness as well as disproportionate enlargement of temporal lobe white matter in the autism group (Hazlett et al., 2011). However, no difference in brain growth rate was observed between groups from age 2 to 4 years of age, suggesting brain enlargement in autism, if present, likely occurred prior to the age of 2 (Hazlett et al., 2011). Wolff et al. conducted a DTI study of 92 infants at 6 months of age, of which 28 were later diagnosed with autism at 2 years of age, with prospective analysis demonstrating increased FA at 6 months followed by slower development that normalises and eventually becomes lower than controls at 2 years (Wolff et al., 2012). This suggests that there may be early white matter disruption of axonal and synaptic development, as well as myelination in autism brain as early as 6 months postnatally. While further work is needed to confirm this finding, the first year of life may represent a critical period for detecting autism related brain changes, with an earlier diagnosis than the current system at 18 months of age being highly beneficial to early intervention and improved prognosis. Hazlett and colleagues later reported that increased cortical surface area between 6-12 months might be a critical, early indicator of autism that eventually leads to brain overgrowth and social deficit later (Hazlett et al., 2017).

While neuroimaging studies have provided evidence for gross structural abnormalities such as brain enlargement, inconsistencies in findings potentially related to variations in age,

genetic ancestry, and other methodological biases to a certain extent. Nevertheless, gross brain changes that have been observed within the first 2 years of life coincide with the period where synaptogenesis, myelination and synaptic pruning occur.

1.5.2 Cytoarchitectural abnormalities

The cortical grey matter is formed through migrating neurons from the VZ during early development of the brain, with these neurons guided to their destination by the radial glial guide, and connected within the radially arranged local network of neurons to form minicolumns. It has been postulated that alterations in the characteristics of these minicolumns may contribute to altered cortical thickness and surface area observed in autism brains (Ecker, Schmeisser, Loth, & Murphy, 2017; Pontious, Kowalczyk, Englund, & Hevner, 2008). Furthermore, glial cells such as astrocytes, oligodendrocytes and microglia are also present in the grey and white matter of the brain, and contribute to neurodevelopment, including synaptogenesis and synaptic pruning, as well as maintenance of neuronal health and neurotransmitter signalling (Allen, 2013; Sominsky, De Luca, & Spencer, 2017). Similar to neuronal migration, glial cells migrate to their final locations throughout the brain during development, and undergo terminal differentiation to position their processes around neurons to regulate neuronal activities and maturation (Sloan & Barres, 2014). Therefore, researchers have utilised post-mortem human brain samples to investigate the cellular architecture of both neurons and glia in the autism brain.

Increased number and reduced width of cortical minicolumns have also been reported in the frontal and temporal regions of the autism brain, especially in cortical layer 3 and 5 (Buxhoeveden et al., 2006; Casanova, Buxhoeveden, & Brown, 2002). These were found to

be accompanied by decreased neuronal size and increased neuronal packing density, indicating that the reduced column width was not due to a decrease in the number of cells within each column (Casanova et al., 2006). Together, these suggest that there may be pathological over-production of minicolumns during neurodevelopment and corticogenesis, which may indicate a bias towards local short range connection between minicolumns, while neglecting long range connections that extend into the deeper layers of white matter (Casanova & Trippe, 2009). In contrast, a recent study based on a larger cohort of samples across wider age range reported increased minicolumn width across all brain regions investigated including the primary sensory cortex, primary auditory cortex, orbital frontal cortex and inferior parietal cortex (McKavanagh, Buckley, & Chance, 2015).

Bauman and Kemper in their series of investigations of post-mortem autism brain samples reported increased neuronal cell packing density and reduced cell size in the hippocampus, amygdala, anterior cingulate cortex (ACC), posterior cingulate cortex (PCC), and cerebellum (Bauman & Kemper, 1985; Kemper & Bauman, 1998; Lawrence, Kemper, Bauman, & Blatt, 2010; Raymond et al., 1996; Simms, Kemper, Timbie, Bauman, & Blatt, 2009; Skefos et al., 2014; Whitney, Kemper, Bauman, Rosene, & Blatt, 2008). These are regions known to be involved in learning and memory, cognition and emotion and with developmental deficits in these domains observed within individuals with autism. In contrast, an independent stereological investigation reported a reduced number of neurons within the amygdala and enlargement of cerebellum (Schumann & Amaral, 2006; Scott, Schumann, Goodlin-Jones, & Amaral, 2009). Within the cortex in autism, an increased dendritic spine density has been observed within the frontal, temporal and parietal lobes and is correlated with cognitive impairment and decreased brain weight (Hutsler & Zhang, 2010). Based on a cohort of 7 individuals with autism and 10 controls, a stereological study reported a reduced neuronal

density and size within the fusiform gyrus, but not within the primary visual cortex (van Kooten et al., 2008). Another study utilising 14 brains from individuals with autism and matched controls revealed consistent reductions in neuronal somal size in 14 of the 16 brain regions investigated, with the reductions normalising toward the levels of healthy controls in older samples (Wegiel et al., 2014). Utilising in-situ hybridisation (ISH) with a set of 25 layer and cell type specific markers, Stoner et al. observed localised patches of reduced expression and abnormal pattern of neuronal markers, but not glia, in a cohort of children between the ages of 2-15 with autism, especially in cortical layers 4 and 5 (Stoner et al., 2014). Within layer 5 of the frontoinsular cortex, Santos et al. reported a higher ratio of von Economo neurons (VENs) to pyramidal neurons in individuals with autism compared to healthy controls (Santos et al., 2011). The authors suggested that increased numbers of VENs might underlie the social cognition impairment in autism, given the role that VENs play in emotion regulation (Santos et al., 2011).

Within the white matter, a decrease in large axons responsible for long range communication, and an excessive numbers of thin axons responsible for local communication were observed in the ACC, orbitofrontal cortex (OFC), but not in lateral prefrontal cortex (PFC) of autism brains (Zikopoulos & Barbas, 2010). Recently, a post-mortem case study on a 40-year-old female with autism revealed an abnormal extension of white matter resembling striatopallidal fibers (also known as striatal pencil fibers) into layer 2-6 of the grey matter within the PFC. Detailed immunohistochemical analysis with cell type specific markers revealed that these pencil fibres are made up of glial cells including oligodendrocytes (SRY-Box (SOX)10) and oligodendrocyte transcription factor (OLIG2)), astrocytes (S100 calcium binding protein β (S100 β) and glial fibrillary acidic protein (GFAP)) and microglia (ionised calcium-binding adapter molecule 1 (IBA-1) and cluster of

differentiation 68 (CD68)), but not neurons, which are typically found in the grey matter (Hashemi, Ariza, Lechpammer, Noctor, & Martínez-Cerdeño, 2016).

Investigations of glial populations constitute another important and under researched area of neuropathology in autism. In a stereological study of IBA-1 immunostained brain sections, Morgan et al. observed an increase in microglia density and activation within the dorsolateral prefrontal cortex (DLPFC) of individuals with autism, accompanied by alteration in microglial-neuronal spatial organisation (Morgan et al., 2012; John T. Morgan et al., 2010). However, within the amygdala, no difference in cell numbers and volumes of microglia, oligodendrocytes, astrocytes and endothelial cells was reported in autism (Morgan, Barger, Amaral, & Schumann, 2014). Activation of astrocytes has also been reported in the medial frontal gyrus (MFG), ACC, and cerebellum of the autistic brain, characterised by increased volume of processes, and with this activation particularly evident in the white matter of MFG and anterior cingulate gyrus (ACG) (Vargas, Nascimbene, Krishnan, Zimmerman, & Pardo, 2005).

The cytoarchitectural abnormalities mentioned above, including altered cell densities and spatial distribution, suggest that the neuropathology associated with autism likely occurs during early corticogenesis and may be related to aberrant neuronal and glial migration. Furthermore, the abnormal morphology of reduced neuronal somal size and increased dendritic spine density suggest under-maturation of neurons and under-pruning of excessive synapses, processes that astrocyte and microglia are also known to be actively involved in.

1.5.3 Cellular and molecular neuropathology in autism

Given a lack of gross neuropathological changes in the autism brain it is imperative to understand cellular and molecular changes in order to improve our understanding of the underlying pathophysiology. Higher order cognitive processes such as executive functioning (including tasks related to working memory, attention, and learning), as well as emotional processing are predominantly controlled by the frontal cortex (FC) and amygdala (Logue & Gould, 2014). Furthermore, functions of these systems are mainly driven by excitatory/inhibitory (E/I) signalling mediated predominantly by glutamatergic and GABAergic pathways respectively, with regulatory contribution from dopaminergic, noradrenergic, serotonergic and cholinergic signalling systems (Logue & Gould, 2014). Not surprisingly, gene and protein expression alterations associated with autism have been reported in these brain regions, within the signalling pathways mentioned.

Disturbance of the serotonergic system has been demonstrated in the brain of individuals with autism. A single-photon-emission computed tomography (SPECT) imaging study observed reduced serotonin (5-HT) 2A receptor density in the ACC, PCC, FC and temporal cortex (TC) of subjects with autism, with these reductions also associated with a lower social communication score (Murphy et al., 2006). Another independent positron emission tomography (PET) imaging study also found a reduction in serotonin transporter (5-HTT) binding in the ACC, PCC, and thalamus. These reductions were also found associated with social cognition impairment, as well as obsessive behaviours and interest (Nakamura, Sekine, Ouchi, & et al., 2010).

Within the cholinergic system, BDNF, which is important for cholinergic neuronal functioning, was found to be elevated in the forebrain and blood of individuals with autism via immunoaffinity chromatography and enzyme-linked immunosorbent assay (ELISA) (Nelson et al., 2001; Perry et al., 2001). This finding was reported alongside radio ligand binding assay results showing reduced binding of acetylcholine muscarinic receptor 1 (CHRM1) in the parietal cortex (PC), and reduced nicotinic acetylcholine receptor (nAChR) in the FC and PC of individuals with autism (Perry et al., 2001). However, at the messenger RNA (mRNA) level, nAChR was found to be decreased in the PC but increased in the cerebellum, although a reduction of protein expression was observed in the cerebellum indicating a potential compensatory effect at the mRNA level (Martin-Ruiz et al., 2004).

Apart from the serotonergic system, there is also a large amount of evidence for the imbalance of glutamatergic (excitatory) and GABAergic (inhibitory) neurons and their activities (Rubenstein & Merzenich, 2003). The high comorbidity rate of seizures and epilepsy among individuals with autism also suggest the potential involvement of this E/I imbalance (Keller, Basta, Salerno, & Elia, 2017; Qadir, Obringer, Hageman, & Marcuccilli, 2017). Impairment in the perinatal GABA excitatory to inhibitory switch was suggested to contribute to the disturbance in the balance of E/I signalling and to be associated with hyper connectivity of neuronal networks (Inui, Kumagaya, & Myowa-Yamakoshi, 2017). In the developing brain, GABA is the first neurotransmitter to become functional, and is responsible for early processes including excitatory signalling prior to glutamatergic synapse maturation, as well as non-synaptic transmission roles such as neuronal migration, proliferation and maturation (Represa & Ben-Ari, 2005). Developmental neuroplasticity, synaptic and axonal pruning are also regulated by glutamatergic/GABAergic signalling, and are driven mainly by GABAergic signalling before glutamatergic maturation (Hensch &

Fagiolini, 2005; Nakayama et al., 2012). A large body of studies suggest increased E/I ratio, or suppressed inhibition in autism (Coghlan et al., 2012; Hussman, 2001; Markram, Rinaldi, & Markram, 2007; Rubenstein & Merzenich, 2003), while others reported increased inhibition with a low E/I ratio (Bertone, Mottron, Jelenic, & Faubert, 2005; Gustafsson & Waterhouse, 1997). These differences reported across studies could potentially be contributed by factors such as brain region, age range of cohort and methodological related variations. For instance, some of the studies mentioned above include behavioural studies and neuropathology studies in human subjects, as well as animal studies, therefore the results may not be directly comparable.

An early autoradiographic study based on 4 individuals with autism and 3 matched controls explored receptor binding from 4 neurotransmitter systems. This study observed reductions in GABA-A receptors and benzodiazepine binding sites within the hippocampus, while no alterations were observed with the 6 other receptors investigated in serotonergic (serotonin 1A receptor (5-HT_{1A}), serotonin 2 receptor (5-HT₂)), cholinergic (high-affinity choline uptake (HACU), CHRM1) and glutamatergic systems (N-Nitrosodimethylamine (NMDA) and Kainate (KA)) (Blatt et al., 2001). Other ligand-binding studies in post-mortem autism brain tissue have also reported reduction in GABA-A and B receptors within the ACC, PCC, fusiform gyrus (FG), hippocampus, cerebellum, PC, and FC, together with a reduction in the number of benzodiazepine binding sites (Oblak, Gibbs, & Blatt, 2009; Oblak, Gibbs, & Blatt, 2010, 2011; Oblak, Rosene, Kemper, Bauman, & Blatt, 2011). At the mRNA and protein expression level, GABA A and B receptor subunits were found to be decreased in FC, PC, and the cerebellum (Fatemi, Folsom, Reutiman, & Thuras, 2009; Fatemi et al., 2010; Fatemi et al., 2014; Fatemi, Reutiman, Folsom, & Thuras, 2009), while glutamic acid decarboxylase

(GAD)65 and GAD67, which are responsible for GABA synthesis, were found to be decreased in the cerebellum and PC (Fatemi et al., 2002; Yip, Soghomonian, & Blatt, 2007).

As for excitatory signalling, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor density was found to be decreased via autoradiography in the cerebellum of individuals with autism compared to controls (Purcell, Jeon, Zimmerman, Blue, & Pevsner, 2001). Furthermore, this was accompanied by increased gene expression of excitatory amino acid transporter (EAAT1-2) and AMPA1-3, as well as elevated protein levels of EAAT1-2, AMPA1, and NMDA receptor 1 (NDMAR1) (Purcell et al., 2001). Using western blotting, Shimmura et al. investigated levels of several glutamate-glutamine enzymes within the ACC of 7 subjects with autism and 13 healthy controls, reporting increased protein levels of kidney-type glutaminase but not glutamine synthetase, liver-type glutaminase, glutamate dehydrogenase 1 and 2, indicating disruption in the glutamatergic system may be limited to certain processes in the pathway (Shimmura et al., 2013). Elevated levels of glutamate have also been found within the ACC, amygdala, hippocampus and PC in autism via proton magnetic resonance spectroscopy (1H-MRS) (Bejjani et al., 2012; Page et al., 2006). Additionally, mRNA and protein expression of metabotropic glutamate receptor 1 (mGluR1) have been reported to be increased in autism brains, while mRNA and protein of mGluR5 have been found to be dysregulated in both directions (Blatt, 2012; D'Antoni et al., 2014; A. El-Ansary & L. Al-Ayadhi, 2014). The reduced level of GABA, glutamate/GABA and glutamate/glutamine observed in the brains of individuals with autism compared to controls suggest a possible disruption to the equilibrium of excitatory/inhibitory signalling in autism, possibly leading to excess glutamate and excitotoxicity (Abu Shmais, Al-Ayadhi, Al-Dbass, & El-Ansary, 2012; Afaf El-Ansary & Laila Al-Ayadhi, 2014; Essa, Braidy, Vijayan, Subash, & Guillemin, 2013; Harada et al., 2011). Several other studies investigated peripheral levels of

glutamate and GABA, however, these findings are inconsistent, showing alterations in both directions within the plasma and platelets (Ghanizadeh, 2013; Shimmura et al., 2011; Shinohe et al., 2006)

The disturbance in E/I balance could be attributed to cytoarchitectural abnormalities in autism, where the lack of precise cortical organisation can potentially interfere with neuronal communications, and lead to aberrant neurotransmissions (Dickinson, Jones, & Milne, 2016). Despite numerous studies suggesting alteration in the E/I systems within the brain in autism, some studies showed no difference of E/I balance between autism and control. The E/I imbalance in autism may be restricted only to certain brain regions, and there may be subtypes of autism where distinctive E/I profiles may be present (Dickinson et al., 2016).

1.5.4 Neuroinflammatory abnormalities

Apart from neurotransmission disturbance, the heterogeneous nature of autism could also be linked with neuroinflammation, which can occur as a result of diverse mechanisms. Vargas et al. investigated post-mortem brain samples and cerebrospinal fluid (CSF) of individuals with autism using immunocytochemistry and ELISA (Vargas et al., 2005). This study reported increased astrocyte reactivity in ACG, MFG and cerebellum, particularly in the subcortical white matter, as shown by GFAP immunostaining and western blotting (Vargas et al., 2005). Within the same cohort, microglia activation was also shown in the cerebellum via immunostaining of a microglia surface marker major histocompatibility complex (MHC) class II markers, human leukocyte antigen - antigen D related (HLA-DR). In addition, the accumulation of perivascular macrophages and monocytes in the brain also

suggest chronic neuroinflammation in autism (Vargas et al., 2005). A number of pro- and anti-inflammatory markers were also found elevated within the ACG, MFG and cerebellum (**Table 1.1**), as shown by cytokine protein array and ELISA (Vargas et al., 2005). Within the FC, Li et al. has also reported neuroinflammation as shown by elevated cytokine levels in the autism brains (Li et al., 2009). Peripherally, Masi et al. conducted a meta-analysis based on 17 studies with a total of 746 autism and 595 healthy individuals, and revealed a consistent increase in interleukin (IL)-1 β , IL-6, IL-8, interferon (IFN)- γ , eotaxin, and monocyte chemoattractant protein (MCP)-1 in the plasma/serum of individuals with autism, while transforming growth factor (TGF)- β 1 was found to be decreased (Masi & Quintana, 2015).

Given the evidence for underlying neuroinflammation and neuronal abnormalities in autism, as well as the key roles that astrocytes and microglia play in neuroinflammatory responses and neuronal functioning, the activation of these cells is hypothesised to play a pathophysiological role in autism (Pardo, Vargas, & Zimmerman, 2005). Several post-mortem investigations have demonstrated microglia activation across different brain regions in autism, including the DLPFC, ACC and medial frontal cortex (MFC) (J. T. Morgan et al., 2010; J. I. Rodriguez & J. K. Kern, 2011; Vargas et al., 2005). Microglia activation has also been demonstrated *in vivo* in the cerebellum, midbrain, pons, fusiform gyri, ACC and the orbitofrontal cortex, in a PET study of autism and control subjects using the radiotracer [(11)C]PK11195, which binds to translocator protein 18 kDa (TSPO), a protein known to be elevated during microglial and astrocyte activation (Suzuki et al., 2013). However, this detected signal may also indicate astrocytes activation as reactive astrocytes have also been found to overexpress TSPO as detected by radioligand [(11)C]PK11195 (Dickens et al., 2014; Lavis et al., 2012). Edmonson and colleagues investigated the mRNA expression of post-mortem autism brains and found that microglial-specific markers were elevated in the PFC

while astrocyte marker GFAP was elevated in both PFC and cerebellum (Edmonson, Ziats, & Rennert, 2014). Furthermore, increased GFAP immunoreactivity was also observed in the white matter of the ACC in autism, suggesting a possibility of astrocyte activation and astrogliosis (Crawford et al., 2015a). Utilising double immunocytochemistry with cell specific markers (co-localisation of GFAP for astrocyte and HLD-DR for microglia), MCP-1 and IL-6 were found to be mainly expressed by astrocyte, while TGF- β 1 and insulin-like growth factor binding protein (IGFBP)-1 were found in astrocytes, microglia and Purkinje cells (Vargas et al., 2005). Young et al. further showed that Nuclear factor kappa-light-chain-enhancer (NF- κ B), which is responsible for mediation of immune response and expression of inflammatory factors such as tumor necrosis factor alpha (TNF α), IL-1 β and IL-6, are found to be increased in astrocytes and microglia of the orbitofrontal cortex of autism, particularly in activated microglia (Young, Campbell, Lynch, Suckling, & Powis, 2011). Taken together, the increased levels of pro-inflammatory cytokines seen in autism brain can be linked to activation of astrocyte and microglia, which lead to the activation of their NF- κ B signalling cascade. These also suggest that reactive glial cells may be a source of neuroinflammatory responses seen in the autism brains.

It is of interest to note that there has not been evidence linking peripheral inflammatory response to the neuropathology observed in the brain of autism, suggesting no involvement of the adaptive immune system (Vargas et al., 2005). Some research groups suggest that neuroinflammation observed in autism could be a result of maternal immune activation (MIA), potentially due to autoantibodies and cytokine transfer or through a direct effect of pathogens such as viral or bacterial infections during perinatal period (Scola & Duong, 2017). More recently, evidence points towards imbalance of microbiota associated with MIA as another plausible mechanism of brain inflammatory abnormalities (Buffington et al., 2016;

Hsiao et al., 2013; Madore et al., 2016). Infection during pregnancy and consequent hospitalisation has been consistently found to be associated with increased risk of autism in offspring (Atladóttir et al., 2010; Jiang et al., 2016; Zerbo et al., 2013). This is supported by a myriad of animal studies utilising lipopolysaccharide (LPS; a bacterial endotoxin acting on toll-like receptor (TLR)4) and polyinosinic-polycytidylic acid (Poly I:C; a synthetic double-stranded RNA acting on TLR3) to induce maternal infection during pregnancy, with offspring showing autism traits and features (Meyer, 2014; Scola & Duong, 2017; Young et al., 2016).

The balance between pro-inflammatory and anti-inflammatory cytokines is important in regulating neuronal proliferation, differentiation, connectivity and plasticity, processes known to be disturbed in brains of individuals with autism (Arango Duque & Descoteaux, 2014; Bauer, Kerr, & Patterson, 2007). Nevertheless, it remains unclear if a neuroinflammatory response is the primary insult in autism or a secondary downstream effect of glial activation.

Table 1.1 | Aberrant levels of pro-inflammatory markers in autism

Study	Sample	Increase in autism	Decrease in autism
Vargas et al. 2005	Middle frontal gyrus	TGF- β 1, MCP-2, IGFBP1	
Autism (n=7)	Anterior cingulate cortex	IL-6, IL-10, TGF β 1, MCP-1, MCP-3, Eotaxin, CCL17, CCL22, CCL23, Eotaxin 2, CXCL9, CXCL13, IGF-1, Leptin, IGFBP1, Osteoprotegerin	
Control (n=7)	Cerebellum	TGF- β 1, MCP-1, CCL17, FLT3-ligand	
	CSF	IFN- γ , TGF β 2, MCP-1, IL-8, CXCL10, Angiogenin, VEGF, IGFBP1, IGFBP3, IGFBP4, LIF, FGF4, FGF9, CCL18, Osteoprotegerin, HGF	
Li et al. 2009	Frontal cortex	IL-6, TNF α , GM-CSF, IFN- γ , IL-8	
Autism (n=8)			
Control (n=8)			
Masi et. al. 2015	Plasma/serum	IL-1 β , IL-6, IL-8, IFN- γ , eotaxin, MCP-1	TGF- β 1
Meta-analysis			
17 studies			
Autism (n=743)			
Control (n=592)			

abbreviations: Transforming growth factor (TGF); monocyte chemoattractant protein (MCP); insulin-like growth factor binding protein (IGFBP); interleukin (IL); chemokine (C-C motif) ligand (CCL); chemokine (C-X-C motif) ligand (CXCL); insulin-like growth factor 1 (IGF1); Fms related tyrosin kinase 3 (FLT3); vascular endothelial growth factor (VEGF); leukemia inhibitory factor (LIF), fibroblast growth factor (FGF); hepatocyte growth factor (HGF); granulocyte-macrophage colony-stimulating factor (GM-CSF).

1.6 Pathophysiology of astrocytes in health and autism

Astrocytes are the most abundant glia cell type in the brain, and in their mature state elaborate their fine processes around neuronal synapses to regulate synaptic signalling and neuronal excitation. Despite multiple lines of evidence suggesting astrocyte and microglia activation within the autistic brain, the exact timeline and mechanism remains unknown. One possible scenario is that early neurodevelopmental functioning of astrocytes may be affected, resulting in disruption of their normal roles in CNS development especially cortical organisation and neuronal maturation; their activation could also be a neuroprotective mechanism secondary to other pathogenesis such as microglia activation and pro-inflammatory factors release (Vargas et al., 2005). Therefore, it is imperative to increase our understanding of the developmental stages of astrocytes, their maturation and integration into the neuronal signalling system and how this is altered in autism.

1.6.1 Astrogenesis in neurodevelopment

During neurodevelopment, astrogenesis typically occurs after neurogenesis, following the gliogenic switch, where NSCs in the VZ transition from neurogenesis to gliogenesis to generate radial glial cells or astrocyte precursors (Miller & Gauthier, 2007; Molofsky & Deneen, 2015). Early life astrocytes originate from radial glial cells or astrocyte precursor within the VZ, and once these astrocytes populate the cortex they are responsible for postnatal local astrocyte division (Ge, Miyawaki, Gage, Jan, & Jan, 2012; Ge & Jia, 2016). During this time, factors such as Nuclear Factor I-A (NFIA), Notch, bone morphogenetic protein (BMP) and signal transducer and activator of transcription 3 (STAT3) signalling have been implicated as important elements to promote astrogenesis from radial glia cells, while suppressing neurogenesis (Molofsky & Deneen, 2015; Molofsky et al., 2012). Similar to neuronal progenitor cells, these astrocyte precursors migrate outwards to populate the

neocortex, and can be labelled with early marker EAAT1 (Molofsky & Deneen, 2015). Similarly to their neuronal counterparts, astrocyte migrate in a radial pattern and remain in their columnar organisation within the mature CNS (Tsai et al., 2012). Various transcription factors are also known to regulate astrocyte subtypes in a similar fashion as neuronal subtype differentiation, with levels of paired box protein pax (PAX)6 and NK6 Homeobox 1 (Nkx6.1) regulating subtypes of ventral white matter astrocytes (Deneen et al., 2006). Upon arrival at their final cortical location, the precursors commence the final differentiation and begin to express ion channels, receptors, and mature astrocytic markers including GFAP, S100 β , aldehyde dehydrogenase 1 family member L1 (ALDH1L1), aldolase C fructose-biphosphate (ALDOC), EAAT2 and aquaporin 4 (AQP4) (Clarke & Barres, 2013; Molofsky et al., 2012). Interestingly, astrocytes are influenced by neuronal signalling to promote their maturation, with arborisation found to be decreased in the absence of neuronal vesicular glutamate transporter 1 (VGLUT1) glutamatergic signalling (Morel, Higashimori, Tolman, & Yang, 2014). Amongst the number of astrocytes specific markers, GFAP is very commonly used as a mature astrocyte marker and as a marker of astrocyte activation. However, it is important to note that these markers do not label all populations of astrocytes, for instance GFAP more selectively labels white matter astrocytes as opposed to grey matter astrocytes.

With the maturation of the neocortex and the formation of white matter, astrocytes mature and differentiate into two main subtypes, fibrous and protoplasmic astrocytes. Fibrous astrocytes reside within the white matter, express processes branching directly from the cell body, and are generally less complex compare to protoplasmic astrocytes (Suzuki et al., 2003). While protoplasmic astrocytes are predominantly found in the grey matter, present with processes that branches into many fine processes confined within individual domains (Bushong, Martone, & Ellisman, 2004; Bushong, Martone, Jones, & Ellisman, 2002). Although

highly expressed in white matter fibrous astrocytes, GFAP is very weakly expressed in the grey matter protoplasmic astrocytes and sometimes undetectable via immunostaining as shown in the Protein Atlas (Uhlén et al., 2005; Uhlén et al., 2015). Recent evidence from lineage tracing with retrovirus suggests that astrocytes can originate from different lineages. They can be produced in the VZ and SVZ from both glial progenitors and oligodendrocyte progenitors, while mature protoplasmic astrocytes can also divide in the grey matter, however much more are yet to be unveiled (Tabata, 2015).

1.6.2 Astrocyte promotes neurogenesis and functional maturation

Astrocytes are essential element of functional maturation of synapses. As reviewed by Clarke and Barres, cortical astrocytes are responsible for the formation, maturation and pruning of excitatory glutamatergic synapses, by secreting various signalling molecules including growth factors and extracellular matrix (ECM) proteins (Clarke & Barres, 2013). Upon completion of astrocyte proliferation and expansion, their fine processes continue to elaborate and can connect with up to 100,000 synapses (Bushong et al., 2002; Halassa, Fellin, Takano, Dong, & Haydon, 2007) where they secreted factors such as D-serine (NMDA receptor coagonist), thrombospondin (TSP; ECM protein), and Hevin (ECM protein), and TGF- β 1 for functional maturation (Diniz et al., 2012; Krencik, van Asperen, & Ullian, 2017; Kucukdereli et al., 2011; Xu, Xiao, & Xia, 2010). Synaptic elimination is an important process in neurodevelopment to strengthen appropriate neuronal connections and is facilitated by astrocytes via the C1q classical complement cascade (Chung, Allen, & Eroglu, 2015; Stevens et al., 2007). Similar to microglia, astrocytes have also been shown to be involved in phagocytic activity, via multiple EGF like domains 10 (MEGF10) and proto-oncogene tyrosine-protein kinase MER (MERTK) pathways (Chung et al., 2013; Nguyen et al., 2011).

1.6.3 Astrocyte regulates CNS homeostasis

Once the neuronal connections and network are established developmentally, astrocytes continue to contribute to maintenance of neuronal health and functioning within the CNS. One such role is the recent discovery that astrocytic perivascular channels, the glymphatic system, are responsible for removal of waste from the CNS (including soluble proteins and metabolites) as well as redistributing other compounds such as glucose, lipids, amino acids and neurotransmitters to maintain homeostasis (Jessen, Munk, Lundgaard, & Nedergaard, 2015). Astrocytes are also involved in regulating neurovascular homeostasis, using their endfeet positioned along the vasculature (Nuriya & Hirase, 2016). In addition, astrocyte secreted factors play major roles in the survival and maturation of oligodendrocytes and therefore neuronal myelination (Kiray, Lindsay, Hosseinzadeh, & Barnett, 2016). At excitatory synapses, the extracellular K⁺ and glutamate accumulate following synaptic vesicle release, and are removed by neighbouring astrocytes from the synaptic cleft to avoid excitotoxicity (Cheung, Sibille, Zapata, & Rouach, 2015). Recent evidence suggests that astrocytes release gliotransmitters such as adenosine triphosphate (ATP) and D-serine via Ca²⁺ dependent mechanism, to regulate neurotransmission (Araque et al., 2014).

1.6.4 Astrocytes at the glutamatergic synapse

At the tripartite synapse, astrocytes, together with presynaptic and postsynaptic neurons form a three-way interaction to regulate neuronal signalling at the level of a single synapse or a neuronal network (Popoli, Yan, McEwen, & Sanacora, 2012). The perisynaptic astrocyte processes (PAP) respond to excitatory signalling to regulate their structural plasticity, mainly through the mGluRs and calcium signalling, which in turns contribute to synaptic plasticity and dendritic spine stability (Bernardinelli et al., 2014). Gap-junction subunit Connexin (CNX)30 was shown to be a key player in astrocytic regulation of synaptic plasticity,

modulating synaptic glutamate levels and transport by controlling the proximity of PAP into synaptic clefts (Pannasch et al., 2014).

Astrocytes are also key players in the synthesis of neurotransmitters, including glutamate and GABA from glucose, which cannot be produced in neurons due to the lack of the enzyme pyruvate carboxylase (Schousboe, Bak, & Waagepetersen, 2013; Verkhratsky, Nedergaard, & Hertz, 2015). Furthermore, astrocyte and neurons interact intimately to complete the glutamine-glutamate cycle. Extracellular glutamate is recycled by glutamate transporters, and converted to glutamate by glutamine synthetase (GS) within astrocytes, then transported back to presynaptic neurons (Verkhratsky et al., 2015).

1.6.5 Astrocytes in autism

Neuropathological investigations have provided strong evidence to suggest that astrocytic functions may be perturbed in the brains of individuals with autism, in particular astrocyte-dependent processes such as synaptogenesis and neurotransmitter signalling. An RNA sequencing study discovered genes that are highly related to autism were also enriched for astrocyte markers (Voineagu et al., 2011).

In post-mortem investigations of autism brains, increased GFAP expression has been demonstrated in the FC, PC and cerebellum, indicating astrocyte activation and astrogliosis, however, increased neuronal density has been observed in autism brains (Crawford et al., 2015a; Edmonson et al., 2014; Laurence & Fatemi, 2005). Numerous other astrocytic abnormalities have also been found in the brains of individuals with autism, including

elevated EAAT1, EAAT2 and AQP4 in the cerebellum, as well as elevated CNX43 in the superior FC (Fatemi, Folsom, Reutiman, & Lee, 2008; Purcell et al., 2001). Interestingly, Aida and colleagues (Aida et al., 2015) recently reported that astrocytes-specific glutamate transporter 1 (GLT1) (rodents variant of human EAAT2, responsible of glutamate clearing from the synapse (Popoli et al., 2012)) KO mice exhibits autism-like repetitive behaviours, and that this is ameliorated with treatment of the NMDAR antagonist memantine. In rat primary astrocytes culture, activated astrocytes were found to express lower level of mGluR5 compared to controls (Tilleux, Berger, & Hermans, 2007). Furthermore, Morel and colleagues showed significant reductions of astrocytes domain size, fine processes, and expression of functional protein VGLUT1 in mGluR5 KO mice, suggesting that mGluR5 is involve in the functional maturation of astrocytes via VGLUT1 signalling. Taken together, mGluR5 dysregulation and astrocytic abnormalities in autism as well as the role of mGluR5 in astrocytic functional maturation and its role in synaptic signalling, suggest that astrocytic glutamatergic signalling may play a key role in the underlying neuropathology of autism and warrants investigation.

1.7 Metabotropic glutamate receptor 5 (mGluR5)

1.7.1 mGluR5 signalling

Within minicolumns, the radially arranged neurons preferentially connect with cells of the same lineage where stronger major excitatory connections are established vertically within the column, while weaker long range connections are formed horizontally across multiple columns (Buxhoeveden & Casanova, 2002). Besides disruption of minicolumns, a disruption of the excitatory glutamatergic system is also observed in autism (Zantomio et al., 2015).

Glutamate is the main excitatory neurotransmitter in the brain and signals through a variety of receptors including ionotropic receptors such as NMDAR, KA receptors, AMPA receptors, as well as a set of G-protein coupled mGluRs (Dore et al., 2014). Among these, mGluR5 has been significantly implicated in the pathogenesis of autism. mGluR5 is a class C G-protein-coupled receptor, composed of a large extracellular N-terminal domain containing glutamate binding site, and a 7 transmembrane structure with intracellular C-terminus with signalling via the $G_{q/11}$ pathway (Dore et al., 2014). mGluR5 mediated signalling and Ca^{2+} release also requires the presence of structural cascades including SH3 and multiple ankyrin repeat domain (SHANK) and Homer in their appropriate location (Sala, Roussignol, Meldolesi, & Fagni, 2005). Furthermore, Homer is also responsible for correct localisation of mGluR5 and the assembly of NMDAR/mGluR5 complex in both neurons and astrocytes (Dziedzic et al., 2003; Newell & Matosin, 2014)

mGluR5 is expressed mainly on the cellular membrane of post-synaptic neurons and glial cells, including microglia and astrocytes, distributed throughout the human brain particularly in regions responsible for emotion and cognition, such as the cortex, hippocampus, striatum,

caudate nucleus and nucleus accumbens (Dore et al., 2014). During neurodevelopment, mGluR5 is responsible in regulating synaptogenesis, long term depression (LTD), and neuroinflammation, therefore dysregulation of this receptor could potentially lead to synaptic abnormalities as seen in autism brain (Byrnes, Loane, Stoica, Zhang, & Faden, 2012; Lüscher & Huber, 2010; Piers et al., 2012). Interestingly, genes coding for mGluR5 and Homer1 are highly expressed in developing astrocytes, followed by a drastic decrease as the astrocytes mature, highlighting the key involvement of astrocytic mGluR5 in early postnatal development of glutamatergic synapses (Petrelli & Bezzi, 2018).

1.7.2 mGluR5 dysregulation in autism

In post-mortem studies, mGluR5 has been found to be downregulated in the DLPFC but upregulated in the cerebellum (Chana et al., 2015; Fatemi & Folsom, 2011; Fatemi, Folsom, Kneeland, & Liesch, 2011). In addition, a number of rare mutations associated with autism lie within genes encoding for components downstream of mGluR5 signalling (**Figure 1.3**), such as SH3 and multiple ankyrin domain proteins (*SHANK1*, *SHANK2* and *SHANK3*), as well as Homer protein. These proteins interact with mGluR5 and NMDAR to mediate long-term potentiation (LTP) and LTD (D'Antoni et al., 2014). Furthermore, mGluR5 knockout (KO) mice have been shown to exhibit features of autism such as impaired short term spatial learning and hyperlocomotion (Gray, van den Buuse, Scarr, Dean, & Hannan, 2009; Xu et al., 2009).

In syndromic autism, the Fragile-X mouse model *Fmr1* KO mice exhibit autism like behaviours such as attentional dysfunction and impaired social cognition, as well as increased mGluR5 signalling and increased LTD. Interestingly, mGluR5 antagonists (2-methyl-6-(phenylethynyl)pyridine (MPEP), fenobam, AFQ056, and STX107) were shown to reverse

some of these autism-like behaviours, with disrupted signalling and LTD also salvaged in mGluR5 KO mice (Dolen et al., 2007; Provenzano, Zunino, Genovesi, Sgado, & Bozzi, 2012). On the other hand, the tuberous sclerosis mouse models (*tsc2*^{+/-}) also show reduced mGluR5-mediated LTD, but this was rescued by exposure to mGluR5 agonists 3-Cyano-N-1,3-diphenyl-1H-pyrazol-5-yl)benzamide (CDPPB) (Auerbach, Osterweil, & Bear, 2011). The strong association of mGluR5 in the syndromic autism led to a great interest in the receptor as a drug target for autism. Various positive and negative allosteric modulators (PAM and NAM) have been developed including two NAMs fenobam and mavoglurant that have been tested clinically (Pop, Gomez-Mancilla, Neri, Willemsen, & Gasparini, 2014). Despite the lack of efficacy of mavoglurant in Fragile X clinical trials, the robust evidence of the involvement of mGluR5 in syndromic and non-syndromic autism suggests it remains a highly potential therapeutic target (Berry-Kravis et al., 2016; Mullard, 2015)

Work within our group has demonstrated a consistent line of evidence showing the involvement of mGluR5 in the pathophysiology of autism. In the autism genetic classifier, mGluR5 was found to have the strongest weight in the risk genetic profile (Skafidas et al., 2012). Furthermore, a significant reduction in mGluR5 mRNA, along with its downstream elements SHANK3 and phospholipase C beta-1 (PLCB1) were observed in the DLPFC of individuals with autism utilising microarray data. In a stereological analysis of mGluR5 immunostaining, we also observed a reduction of neuronal mGluR5 in the DLPFC of an independent cohort of autism samples. In addition, increased number of microglia has been shown by our group in the mGluR5 knockout mice utilising stereological quantitation of a microglia marker IBA-1 (Chana et al., 2015). These findings are in line with the evidence of increased microglia density and activation in the post-mortem DLPFC of autism patients (J. T. Morgan et al., 2010). In addition, the mGluR5 agonist (RS)-2-chloro-5-hydroxyphenylglycine

(CHPG) has also been found to alleviate neurotoxicity associated with microglia activation (Loane, Stoica, Pajooresh-Ganji, Byrnes, & Faden, 2009).

Although microglia associated glutamatergic abnormalities have been widely studied, evidence on the role of astrocyte driven neuroinflammation and related glutamatergic disturbance is sparse. mGluR5 is commonly expressed in glutamatergic synapses and has a key role in astrocyte-mediated neuronal health and neuroplasticity. Given it is also intimately involved in astrocyte physiology and in neuroinflammation, it is therefore important to investigate the role of astrocytic mGluR5 relative to the neuropathology of autism in light of being a potential therapeutic target.

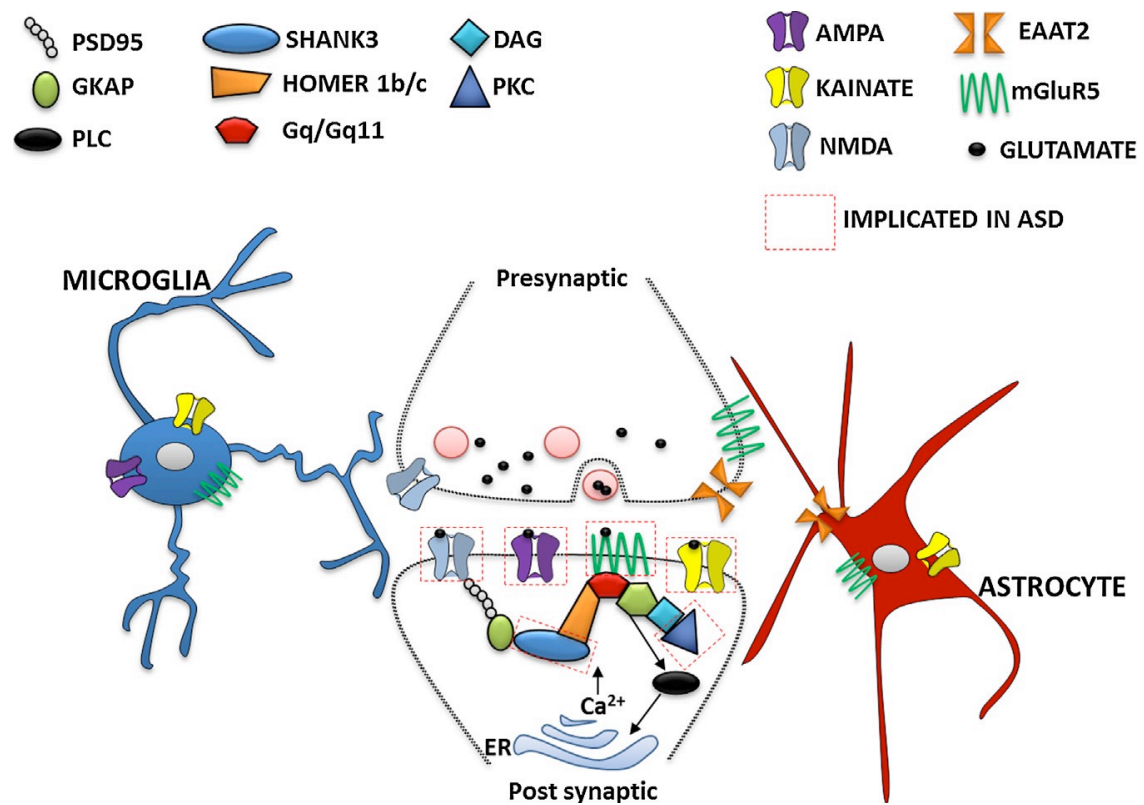


Figure 1.3 | Location of synaptic glutamate receptors and proteins implicated in autism.

Diagram illustrating glutamate receptors and downstream signalling elements that have been implicated in autism, including SHANK3 and Homer. (Figure from Zantomio et al. (Zantomio et al., 2015))

1.8 Human pluripotent stem cell models of autism

Recent advances in stem cell technologies has allowed the reprogramming of human somatic cells into pluripotent stem cells, which can then be differentiated into various cell types, including neurons and astrocytes. These somatic cells can be obtained from fibroblasts, blood cells, and dental pulp cells, which can be easily sampled with minimally invasive procedures (Goorha & Reiter, 2017; Lorenzo, Fleischer, & Bachiller, 2013; Okita et al., 2013). The use of iPSCs marked a significant milestone in biomedical research where the generation and maturation of cells representative of different stages of neurodevelopment, including the perinatal period, could be modelled. Compared to *in-vitro* models with genetic modification, hiPSC lines carry the true genetic composition of autism probands, and are therefore able to represent the vast heterogeneity of autism-associated variants.

Idiopathic autism derived hiPSC lines have been shown to recapitulate the phenotype of patients with macrocephaly where their NPCs were found to have a higher proliferation (Marchetto et al., 2017). Neurons derived from RTT hiPSCs were also shown to express cellular phenotypes such as reduced somal size and excitatory synapses, reduced dendritic spines and lower Ca^{2+} activity, as well as aberrant synaptic transmission (Marchetto et al., 2010). Furthermore, these hiPSCs can be differentiated using small molecules and proteins, into specific subtypes, including excitatory and inhibitory neurons. These include dopaminergic, and serotonergic neurons as well as astrocytes, microglia, and Purkinje cells. Unlike a complex neural system within the human brain consisting of various cell types that are interconnected, hiPSC can be differentiated into homogenous cell populations. This allows for a targeted investigation of specific systems, such as glutamatergic, GABAergic, serotonergic or dopaminergic neurons, independently in the context of autism pathophysiology, as well as interaction of two or more systems when co-cultured.

Various hiPSC-derived models of autism have been characterised over the past decade. FXS hiPSC-derived neuronal cells were reported to have smaller somal size and reduced neurite number and length, as well as reduced neural activity (Doers et al., 2014; Sheridan et al., 2011; Teliás, Kuznitsov-Yanovsky, Segal, & Ben-Yosef, 2015). While RTT hiPSC-derived neurons also showed reduced synaptic complexity, somal size, and spine density, as well as decreased spontaneous firing (Cheung et al., 2011; Djuric et al., 2015; Kim, Hysolli, & Park, 2011; Marchetto et al., 2010; Zhang et al., 2016). Idiopathic autism hiPSCs derived from patients carrying the TRPC6 gene mutation, which encodes for a Ca^{2+} channel important for spine formation, also display characteristics such as decreased dendritic arborisation, number of spines and synapses, as well as reduced calcium signalling, which can be rescued with IGF-1 treatment (Griesi-Oliveira et al., 2015). Furthermore, another study reported higher cell proliferation rates, as well as a bias for inhibitory neurons in autism hiPSC-derived cells compared to controls (Mariani et al., 2015). Recently, Marchetto and colleagues also showed that idiopathic autism derived NPCs have increased proliferation rates and reduced synaptogenesis that could be rescued by IGF-1 (Marchetto et al., 2017). This suggests that there may be a common IGF-1 related pathway disturbance in both syndromic and idiopathic autism.

1.9 Overall aim

In the developing brain, astrocytes play many key roles, including the neurogenesis and synaptogenesis. Furthermore, astrocytic mGluR5 is essential in regulating glutamatergic functioning at the tripartite synapses, by maintaining appropriate extracellular glutamate levels, by reuptake and recycle of glutamate from the synaptic cleft. Given the evidence for both astrocyte activation and disruption to glutamatergic functioning in autism, an investigation focusing on the involvement of astrocytes and the interrelationship between these two potential pathophysiological pathways is warranted.

The studies conducted within this PhD thesis aimed to improve understanding of astrocytic neuropathology in autism, particularly the role of neuroinflammation in mediating astrocyte activation and the impact of glutamatergic signalling, in particular mGluR5, in regulating astrocyte activation and function. In addition, the characterisation of astrocytes differentiated from human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) will explore the potential utility of this *in-vitro* model in future investigation of autism neuropathology, as well as other brain disorders.

CHAPTER 2 A stereological investigation of astrocyte within the white matter of dorsolateral prefrontal cortex (DLPFC) in autism

2.1 Aim

Given evidence for astrocyte activation and white matter disruptions in the brain in autism, this study aimed to assess astrocyte density and morphology, including somal size and arborisation within the white matter of the DLPFC in autism using stereological methods, and GFAP utilised as a cellular astrocyte marker.

2.2 Hypotheses

We hypothesise that there will be increased astrocyte density and activation related hypertrophy morphological changes within the white matter of DLPFC in autism. These morphological changes will include increased cell somal size and process length.

2.3 Publication

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Molecular Autism

SHORT REPORT

Open Access



No preliminary evidence of differences in astrocyte density within the white matter of the dorsolateral prefrontal cortex in autism

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Abstract

Background: While evidence for white matter and astrocytic abnormalities exist in autism, a detailed investigation of astrocytes has not been conducted. Such an investigation is further warranted by an increasing role for neuroinflammation in autism pathogenesis, with astrocytes being key players in this process. We present the first study of astrocyte density and morphology within the white matter of the dorsolateral prefrontal cortex (DLPFC) in individuals with autism.

Methods: DLPFC formalin-fixed sections containing white matter from individuals with autism ($n = 8$, age = 4–51 years) and age-matched controls ($n = 7$, age = 4–46 years) were immunostained for glial fibrillary acidic protein (GFAP). Density of astrocytes and other glia were estimated via the optical fractionator, astrocyte somal size estimated via the nucleator, and astrocyte process length via the spaceballs probe.

Results: We found no evidence for alteration in astrocyte density within DLPFC white matter of individuals with autism versus controls, together with no differences in astrocyte somal size and process length.

Conclusion: Our results suggest that astrocyte abnormalities within the white matter in the DLPFC in autism may be less pronounced than previously thought. However, astrocytic dysregulation may still exist in autism, even in the absence of gross morphological changes. Our lack of evidence for astrocyte abnormalities could have been confounded to an extent by having a small sample size and wide age range, with pathological features potentially restricted to early stages of autism. Nonetheless, future investigations would benefit from assessing functional markers of astrocytes in light of the underlying pathophysiology of autism.

Keywords: Astrocytes, Glia, Autism, Dorsolateral prefrontal cortex (DLPFC), Cell density, White matter

Background

Autism is a highly complex and heritable neurodevelopmental disorder with prevalence rates soaring from 1 in 5000 children in the 1970s [1] to 1 in 132 global prevalence reported in recent years [2]. The majority of post-mortem brain studies in autism have focused on uncovering neuronal abnormalities; however, recent

evidence has demonstrated alterations in both microglial and astrocytic markers within the autistic brain [3]. Microglia and astrocytes are known to have crucial roles in neurodevelopment, including regulation of synapse formation as well as neuroinflammatory and immune pathways. As both synaptogenesis disruption and neuroinflammation are reported in autism, a role for microglial and astrocyte involvement in the pathophysiology of autism has begun to receive an increasing amount of attention [3–5].

Astrocytes represent the most abundant glia cell type in the brain, and in their mature state, form tripartite

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synapses together with presynaptic and postsynaptic neurons in a three-way interaction to regulate neuronal signaling via neurotransmitter/gliotransmitter receptors and ion channels at the level of a single synapse or a neuronal network [3, 6–8]. Astrocytes have also been shown to play a critical role in oligodendrocyte survival and maturation, including influencing the ability of oligodendrocytes to myelinate axons [9, 10]. In addition, glial fibrillary acidic protein (GFAP), a major structural component of astrocytes, has been shown to be necessary for the integrity of CNS white matter as well as long-term maintenance of myelination [11]. This is of interest given post-mortem studies have revealed axonal thinning within the white matter in individuals with autism as well as reduced myelin thickness in the anterior cingulate cortex (ACC) and prefrontal cortex (PFC) [12]. While neuroimaging studies have shown accelerated maturation of white matter within the frontal cortex [13, 14], increased gray and white matter volume in the frontal and temporal regions [15–18], as well as hyper-connectivity in prefrontal, amygdala, and temporal regions [18, 19]. Along with the ACC, the dorsolateral prefrontal cortex (DLPFC) is responsible for higher level executive functioning, including attention, emotional processing, learning and memory, domains that are impaired in individuals with autism [20]. This is evidenced by several studies reporting impairments in individuals with autism compared to controls when performing tasks known to be mediated by the DLPFC, including poor performance in the Wisconsin Card Sorting Test (WCST), as well as decreased functional activity as measured by magnetic resonance spectroscopy (MRS) and functional magnetic resonance imaging (fMRI) [21–23].

Evidence for astrocyte dysfunction have also come from a post-mortem brain investigation in autism demonstrating increased mRNA levels of *GFAP* as well as the excitatory amino acid transporter 1 (*EAAT1*) via microarray profiling within the cerebellum, with upregulation confirmed at the protein level via western blotting [24]. Subsequently, Vargas et al. demonstrated increased GFAP in the cerebellum, middle frontal gyrus, and ACC within individuals with autism compared to controls [25]. This was followed by Laurence and Fatemi's results, also demonstrating increased protein levels of GFAP in the superior frontal, parietal and cerebellar cortex in autism [26]. Further support for prefrontal and cerebellar changes were demonstrated by a quantitative real-time polymerase chain reaction (qPCR) investigation showing increased *GFAP* expression in both regions in autism compared to controls [27]. In addition, Crawford et al. demonstrated increased GFAP at the protein level via western blotting within the white matter of the anterior cingulate gyrus, but not in the gray matter [28]. In contrast to increased GFAP levels within the brain in

autism, Morgan et al. demonstrated no difference in density and size of astrocytes between autism and controls following a stereological study of the amygdala [29]. Other astrocytic abnormalities have also been observed in the brains of individuals with autism, including reduced protein levels of aquaporin 4 (AQP4) within the cerebellum together with elevated connexin 43 (CNX43) seen in the superior frontal cortex [30].

Astrocytes are known to be intimately involved in the inflammatory response within the CNS [31], with growing evidence implicating neuroinflammation in the pathophysiology of autism, especially via dysregulation of neurogenesis and neuronal physiology related to activation of microglia and astrocytes [5, 32, 33]. Astrocyte activation in autism has been suggested to occur due to observations of astrogliosis in the cerebellar white matter and subcortical white matter within the frontal lobe [25–27]. Given that astrocytes are known to be involved in neurodevelopment, potential changes in their activation state and/or functioning due to inflammation during development might have consequences for disrupted connectivity and neuropathological changes in autism [34]. Activation of astrocytes and the subsequent pro-inflammatory cytokines release, including interleukin 6 (IL-6), tumor necrosis factor alpha (TNF α), and interleukin 1B (IL-1B), has also been observed in autism within the brain as well as peripherally in the cerebrospinal fluid [25, 35].

Given the growing body of evidence for astrocytic involvement in autism, and their known functioning in maintaining myelin, together with disruptions in myelin integrity seen within the brain of individuals with autism, a more detailed investigation of astrocytic neuropathology within the white matter is warranted. The aim of this investigation was to assess astrocyte density and morphology within the white matter of the DLPFC in autism compared to age- and gender-matched controls using GFAP as a cellular astrocyte marker. This was achieved using the optical fractionator, nucleator, and spaceballs probes to investigate density, somal size, and overall process length respectively.

Methods

Brain tissue

Formalin-fixed DLPFC tissue blocks restricted to BA9 and BA46 from individuals with autism ($n = 11$) and controls ($n = 10$) were provided by the NICHD Brain and Tissue Bank for Developmental Disorders (NBTBD), Baltimore, Maryland upon request (University of Melbourne ethics approval ID: 1339835.1). Tissue was matched as closely as possible for age between autism (range = 4–51 years) and controls (range = 4–46 years). Tissue blocks were serially sectioned using a Leica VT1000s Vibratome (Leica Biosystems, Germany) in

phosphate-buffered saline (PBS) at 50 μ M and stored in PBS with 0.5% sodium azide at 4 °C. Six samples (3 autism, 3 controls) were excluded from analysis due to poor tissue morphology following immunohistochemistry, thereby reducing the number of cases available to 8 autism and 7 controls (Table 1).

Immunohistochemistry

Four sections from each subject were chosen via systematic random sampling (every 14 sections) spanning the entire tissue blocks and processed for immunohistochemistry using a Vectastain ABC Kit (Vector Laboratories, USA) and a rabbit polyclonal antibody against GFAP (DAKO, Denmark) [1:4000]. For detailed methods, please refer to Additional file 1.

Astrocyte density and morphological measurement

All density quantitation was performed using a $\times 100$ oil immersion objective lens (numerical aperture = 1.4) and a Nikon Eclipse 80i (Nikon Instruments, Japan) together with StereoInvestigator 11 (MBF Biosciences, USA) as described previously [33]. A pilot study was conducted to determine sampling parameters of probes including disector depth, size of sampling contour, and numbers of sampling fields in order to achieve coefficient of error (CE) below 0.05 [36]. Three regions of interest defined by 1 mm $\times$ 2 mm (optical fractionator and nucleator) or 1 mm $\times$ 1 mm (spaceballs) were drawn using $\times 4$ objective lens within the white matter for each section and systematic random sampling (SRS)

grid applied for data collection. Within StereoInvestigator, we utilized the optical fractionator probe to estimate astrocyte and glia density, with simultaneous application of the nucleator probe for astrocyte somal size estimation. The spaceballs probe was used to estimate total astrocyte process length. For detailed methods, please refer to Additional file 1.

Statistical analysis

All measures were tested for normality using a Shapiro-Wilk test, with Pearson or Spearman correlations, carried out between our outcome measures, age, and post-mortem interval (PMI). Any significant correlation at $p < 0.05$ were considered as covariates during analysis for group differences. A general linear model was used to test differences between groups for normally distributed measures while a Mann-Whitney U test was utilized for non-parametrically distributed measures.

Results

Identification of adjustment variables

Shapiro-Wilk normality tests demonstrated that nucleator area ($p = 0.023$) was not normally distributed within the autism group; however, all other measures including age, post-mortem interval (PMI), astrocytes density, negative glia density, total glia density, spaceballs length, and IOD were normally distributed. No significant correlations were observed (Table 2).

Table 1 Brain tissue demographics

Sample ID	Age		Cause of death	Sex	PMI (h)	Diagnosis
	Years	Days				
5308	4	182	Skull fractures	M	21	ASD
5144	7	55	Complications from cancer	M	3	ASD
4899	14	126	Drowning	M	9	ASD
5403	16	266	Cardiac arrhythmia	M	35	ASD
4999	20	274	Cardiac arrhythmia	M	14	ASD
5578	35	294	Accident	M	22	ASD
5115	46	135	Complications of pseudomyxoma peritonei	M	29	ASD
5137	51	91	Pneumonia	M	72	ASD
1185	4	258	Drowning	M	17	Control
1500	6	320	Multiple injuries	M	18	Control
917	14	217	Accident/multiple injuries	M	10	Control
1158	16	63	Cardiomegaly	M	15	Control
4916	19	47	Accident/drowning	M	5	Control
4593	33	24	Cardiac arrhythmia	M	8	Control
1936	46	35	Arteriosclerotic cardiovascular disease	M	13	Control

Table 2 Statistical analysis of normality test, correlation test, and group comparison between autism and control

Diagnosis	Shapiro-Wilk normality test			Correlation age		Correlation PMI		Group differences (ASD vs control)		
	Statistic	df	Sig.	Pearson (<i>r</i>)	Spearman (rho)	Pearson (<i>r</i>)	Spearman (rho)	Mean	SE	<i>p</i> value
Age										
ASD	0.907	8	0.335					24.612	5.803	0.604 ^a
Control	0.907	7	0.374					20.092	6.204	
PMI (h)										
ASD	0.864	8	0.133					25.625	5.673	0.132 ^a
Control	0.954	7	0.766					12.286	6.065	
Population (all glia)										
ASD	0.950	8	0.715	0.205 (<i>p</i> = 0.463)		−0.390 (<i>p</i> = 0.150)		2,401,704.83	121,494.58	0.415 ^a
Control	0.873	7	0.196	−0.267 (<i>p</i> = 0.337)				2,351,803.21	129,883.17	
Population (astrocyte)										
ASD	0.969	8	0.894			0.168 (<i>p</i> = 0.549)		100,669.51	8557.62	0.202 ^a
Control	0.978	7	0.949			−0.392 (<i>p</i> = 0.149)		117,513.34	9148.48	
Population (other glia)										
ASD	0.951	8	0.718	0.219 (<i>p</i> = 0.433)				2,401,035.25	124,249.39	0.376 ^a
Control	0.868	7	0.178					2,234,289.88	132,828.19	
Nucleator (area)										
ASD	0.791	8	0.023		−0.093 (<i>p</i> = 0.742)	−0.118 (<i>p</i> = 0.676)		92.475	4.556	0.397 ^b
Control	0.866	7	0.170					87.423	4.870	
Spaceballs length (all) (um)										
ASD	0.901	8	0.298	−0.289 (<i>p</i> = 0.296)		−0.206 (<i>p</i> = 0.461)		51,228,155.8	6,563,846.09	0.923 ^a
Control	0.982	7	0.970					52,176,610.3	7,017,046.63	
IOD (mean)										
ASD	0.938	8	0.591	−0.681 (<i>p</i> = 0.007)		−0.517 (<i>p</i> = 0.059)		143.083	4.159	0.066 ^c
Control	0.922	7	0.487					156.127	4.809	

^aGeneral linear model^bNon-parametric independent sample *t* test^cGeneral linear model with covariate "age"

Astrocyte and total glial densities

No significant differences in the density of astrocytes were seen between autism and controls (autism = $10.07 \times 10^4 \pm 85.58 \times 10^2$, control = $11.75 \times 10^4 \pm 91.48 \times 10^2$ (mean $\pm$ SEM); $p = 0.202$, Fig. 1a). In addition, no group differences were seen for GFAP negative glia (autism = $24.01 \times 10^5 \pm 12.42 \times 10^4$, control = $23.34 \times 10^5 \pm 13.28 \times 10^4$ (mean $\pm$ SEM); $p = 0.376$, Fig. 1b), or total glia (autism = $24.02 \times 10^4 \pm 12.15 \times 10^4$, control = $23.52 \times 10^4 \pm 12.99 \times 10^4$ (mean $\pm$ SEM); $p = 0.415$, Fig. 1c).

Astrocyte somal size and process length

As somal sizes were not normally distributed within the autism group, we employed a Mann-Whitney U non-parametric test to compare differences between autism and control groups. No significant difference between autism and controls were seen for somal area of white matter astrocytes (autism = $92.475 \pm 4.56 \mu\text{m}^2$, control = $87.423 \pm 4.87 \mu\text{m}^2$ (mean $\pm$ SEM); $p = 0.397$, Fig. 2a). Furthermore, spaceballs analysis showed no significant difference between autism and control group for total length of astrocytes processes in the white matter (autism = $51.23 \times 10^6 \pm 65.64 \times 10^5 \mu\text{m}$, control = $52.18 \times 10^6 \pm 70.17 \times 10^5 \mu\text{m}$ (mean $\pm$ SEM); $p = 0.923$, Fig. 2c).

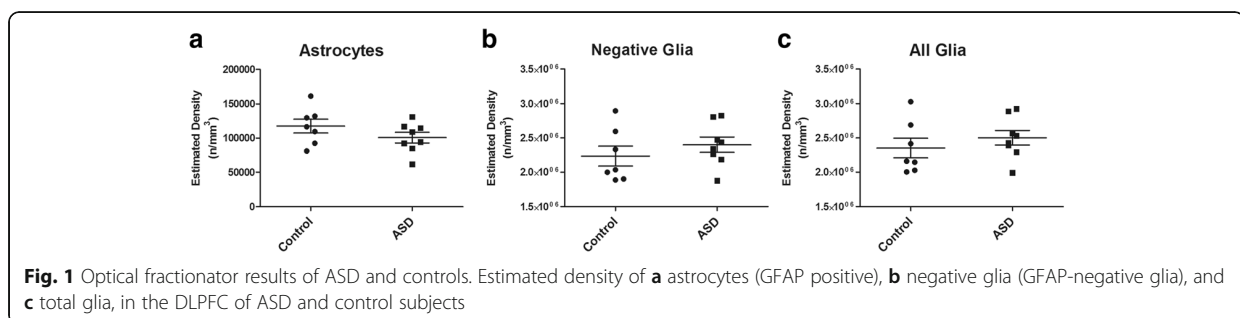
Discussion

This study provides the first investigation of astrocyte density, morphology, and activation status within the white matter of the DLPFC in autism. Based on previous evidence demonstrating increased GFAP within the brain in autism, we hypothesized that we would observe an increased density of astrocytes in the white matter of the DLPFC of individuals with autism. In contrast, our results demonstrate no difference in the density and cellular morphology of GFAP-positive astrocytes in the white matter of the DLPFC of autism when compared to age-matched controls. Our findings are in keeping with recent studies demonstrating no alteration in astrocyte number and astrocyte somal size in the amygdala in autism, as well as no increase in GFAP mRNA and protein in the anterior PFC and ACC [28, 29].

During severe reactive astrogliosis, a marked increase in astrocytic size, arborization, GFAP expression, and proliferation are observed [37, 38]. Therefore, the current study investigated the population of GFAP-positive astrocytes along with other glial cells using StereoInvestigator to obtain an estimation of astrocyte and glial density, as well as astrocytic somal size and process length. Our results provide support for no alteration in astrocyte or glial density in the autism brain, consistent with Morgan et al. reporting no alteration of astrocyte numbers in autism within the amygdala [29]. In addition, our results demonstrated no evidence for increased astrocytic reactivity within the DLPFC white matter of individuals with autism compared to controls, as indicated by no changes in morphology, including cell somal size and process length. Similar to our findings for astrocyte somal size, Morgan et al. also found no evidence for differences in astrocyte somal size between autism and controls within the amygdala [29].

Our lack of findings for alterations in astrocyte density and activation status within the DLPFC white matter in autism represent an absence in gross astrocyte morphological changes in this brain region. However, another possibility is that any astrocyte activation may be present at a less pronounced level and that our employed strategies for investigating activation may have not been sensitive enough to detect such changes. This is in keeping with a lack of stark neuropathological changes observed in the autism brain. Yet another possible explanation is that astrocytes measured for our investigation may have resolved their reactivity and activation status, with morphological changes potentially taking place much earlier in disease progression [38]. Given the early developmental nature of autism and likely heterogeneity in autism pathophysiology, the latter two possibilities are equally likely to potentially explain the lack of astrocyte reactivity within our samples. Our results therefore highlight the need for more sensitive measures of assessing astrocyte activation as well as potentially assessing temporal and regional changes in astrocyte activation.

Despite a lack of evidence in our study to suggest activation of astrocytes, their contribution to the pathophysiology of autism might still be very significant.



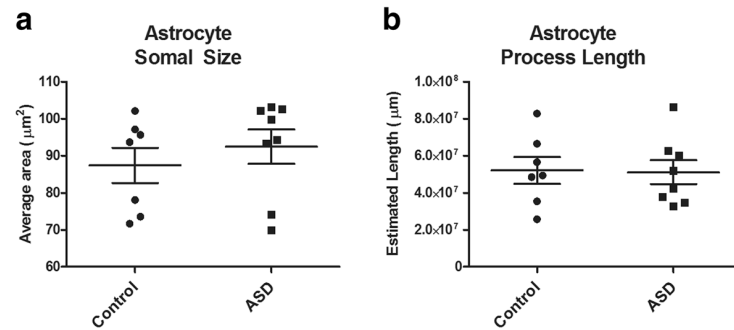


Fig. 2 Astrocyte morphology estimates. Astrocytes reactivity measures of **a** cell somal area via nucleator probe, and **b** cell processes length using spaceballs probe

Given astrocytes play a central role in regulating the functions of other cell types in the brain, any loss or gain of function of astrocytes could have a direct or indirect effect on brain physiology. Astrocytes contribute to synaptic functioning, especially clearing of glutamate via transporter EAAT2. A disturbance of this function could potentially be detrimental to neuronal health and functioning due to excitotoxicity caused by excess glutamate within the extracellular space [39]. Developmentally, astrocytes contribute to synaptic pruning by actively removing synapses via MEFG10 and MERTK pathways [40], or by tagging synapses for elimination by microglia via upregulation of C1q protein, loss of this function could lead to over- or under-pruning of synapses as observed in autism [41]. Within the white matter, disturbance of astrocytic physiology could also lead to perturbation of myelination, which has also been observed in autism [42, 43]. Therefore, potential functional alterations in astrocytes due to neuroinflammation and change in their activation may warrant a more detailed investigation in an attempt to tease out the mechanistic roles of astrocytes in the neuropathology of autism.

Due to the limitation of post-mortem brain samples available, autism studies including the current one are often limited by sample size. This also contributed to a relatively large age range within the cohort investigated. Consequently, our lack of astrocyte abnormalities in the current study could to a certain extent have been masked by an age-related effect, whereby abnormalities present in early developmental stages may be normalized later in life. This is particularly interesting given age-related cortical thinning has been reported within the temporal and parietal cortices of autism brain during adolescence and early adulthood, despite evidence of cortical overgrowth earlier in life [44]. In addition, findings from several longitudinal neuroimaging studies also suggest that brain abnormalities such as overgrowth is pronounced within the first few years of life and subsequently found to normalize towards controls during aging [45–47]. Furthermore, it is not

uncommon for individuals with autism to have comorbid neurological disorders such as epilepsy, which may additionally represent a confounding factor, with potential brain changes due to an underlying neurodegenerative disease. With regard to epilepsy, this would be particularly pertinent for our investigation as individuals with epilepsy are known to have increased astrocyte numbers and activation [48].

For this study, we employed a strict protocol of sampling whereby we ensured the CE for all cases was below 0.05, in attempt to increase our precision in estimating density, size, and process length measurements. As the spaceballs probe was used to estimate all astrocyte processes present within the ROI regardless of the presence of the cell body, our results reflect more global increases in astrocyte length as opposed to arborization characteristics of individual astrocytes. Nevertheless, our data provides a reliable 3D estimate of overall length of astrocyte processes as a measure of potential astrocyte activation. Future studies may benefit from investigating an extended suite of astrocyte markers, including aldehyde dehydrogenase 1 family member L1 (ALDH1L1), glutamate synthetase (GS), and S100 calcium-binding protein B (S100B), to obtain a more complete perspective on structural and functional astrocyte abnormalities in the autism brain.

Conclusions

In conclusion, our results suggest that gross morphological changes in astrocyte density and activation status are not present in the DLPFC white matter. This could suggest a lack of astrogliosis, but does not rule out astrocytic abnormalities, that may be present in the absence of gross morphological changes. In addition, regional differences in the reactivity of astrocytes in the brain in autism may also be present and should be considered. Further post-mortem investigations within the autism brain should be aimed at assessing astrocyte abnormalities utilizing more functional markers of astrocytes to further understand the role that

astrocytes play in the pathophysiology of autism and their impact on other cell types in the brain including neurons, microglia, and oligodendrocytes, with these cells also likely contributing to the underlying etiology of the disorder.

Additional file

Additional file 1: Supplemental methods. (DOCX 12083 kb)

Abbreviations

ABC: Avidin-biotin complex; ACC: Anterior cingulate cortex; ALDH1L1: Aldehyde dehydrogenase 1 family member L1; AOI: Area of interest; AQP4: Aquaporin 4; CE: Coefficient of error; CNS: Central nervous system; CNX43: Connexin 43; DAB: Diaminobenzidine; DLPFC: Dorsolateral prefrontal cortex; EAAT1: Excitatory amino acid transporter 1; GFAP: Glial fibrillary acidic protein; GS: Glutamate synthetase; IL-1B: Interleukin 1B; IL-6: Interleukin 6; IOD: Integrated optical density; MRI: Magnetic resonance imaging; NGS: Normal goat serum; PBS: Phosphate-buffered saline; PFC: Prefrontal cortex; qPCR: Quantitative real-time polymerase chain reaction; S100B: S100 calcium binding protein B; SEM: Standard error of mean; SRS: Systematic random sampling; TNF α : Tumor necrosis factor alpha

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TL conducted the experiment and contributed to study design, data analysis, and manuscript writing. ES contributed to study design, data analysis, and manuscript editing. MD, DZ, and CP contributed to study design and manuscript editing. IE and GC contributed to study design, acquisition of samples, data analysis, and manuscript writing. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Brain tissue for this study was provided by the NICHD Brain and Tissue Bank for Developmental Disorders (NBDT), Baltimore, Maryland (supported by NICHD contract #HHSN275200900011C, Ref. No. N01-HD-9-0011; University of Melbourne ethics approval ID: 1339835.1), with informed consent from donors and/or families.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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2.4 Additional Materials and Methods

2.4.1 Immunohistochemistry

Free-floating sections were incubated in 2% hydrogen peroxide (Chem-Supply, Australia) in 100% methanol (Chem-Supply, Australia) for 20 minutes at room temperature. Following removal of excess methanol and hydrogen peroxide through washing in phosphate buffered saline (PBS), sections were microwaved in 1X citrate buffer [0.01% hydrochloric acid] (Sapphire Biosciences, Australia) at 1000W for 3 minutes, then at 500W for another 3 minutes in order to aid antigen retrieval. Sections were then incubated in blocking solution consisting 3% normal goat serum (NGS) in PBS containing 0.1% Triton-X100 (Sigma, USA) for 1 hour at room temperature, followed by incubation with a rabbit polyclonal antibody against GFAP (DAKO, Denmark) [1:4000] in 1.5% normal NGS in PBS containing 0.1% Triton-X100 overnight at 4°C. Sections were washed three times in excess PBS, followed by incubation in a biotinylated secondary anti-rabbit immunoglobulin G (IgG) 1.5% normal NGS in PBS containing 0.1% Triton-X100 for 2 hours at room temperature. Sections were then washed again three times in excess PBS and then incubated in Avidin-Biotin Complex (ABC) for 1 hour at room temperature. Sections were washed again in PBS three times and immunostaining visualized using a ImmPACT diaminobenzadine (DAB) peroxidase substrate (Vector Laboratories, USA). Following washing in PBS and distilled water, sections were counterstained using haematoxylin (Vector Laboratories, USA) and mounted onto Menzel-Glaser Superfrost® Ultra Plus microscope slides (Thermo Scientific, Germany) and allowed to air dry overnight. Sections were then gradually dehydrated in graded ethanol (Chem-Supply, Australia), and then in xylene (Chem-Supply, Australia), followed by permanent mounting using

DPX mountant (Sigma Aldrich, USA). GFAP stained astrocytes for autism and control sections are shown in **Figure 2.1**.

2.4.2 Stereological quantitation

2.4.2.1 Identification of regions of interest (ROI)

Regions of interest (ROI) were chosen within the DLPFC where the pia matter, grey matter, and white matter could be clearly identified. Using Stereo Investigator 11, three contours defining our ROI 1mm x 2mm (x- and y- axes) (optical fractionator & nucleator) or 1mm x 1mm (spaceballs) were drawn using a 4x objective lens within the white matter for each section, using the edge of white/grey matter as a reference point (**Figure 2.2**). Due to the different sizes, regions, and composition on grey/white matter of tissue blocks and sections, the pilot study determined the largest possible ROI that could be achieved across all sections and cases. ROI contour traces were overlaid between subsequent sections and cases together with a constant grid size maintained as a pre-requisite within the optical fractionator to enable computation of Gunderson's coefficient of error (CE). Following delineation of our ROIs, StereoInvestigator 11 then systemically overlaid optical disectors representing our individual fields for sampling.

2.4.2.2 Identification of cells

Astrocytes, negative glia and neurons were identified with criteria as described in previous studies (Chana et al., 2015). Briefly, astrocytes were identified by positive GFAP staining in the membrane, cytoplasm and nucleus as described in the human protein atlas (Pontén, Jirström, & Uhlen, 2008; Uhlén et al., 2015). Negative glia was identified via a rounded nucleus, lack of visible cytoplasm and the presence of heterochromatin. Neurons were identified based on their larger size, counterstained nucleus and visible nucleolus.

2.4.2.3 Optical fractionator

Our pilot analysis revealed that our processed and mounted sections had an average thickness between 19.5µm to 24.7µm, with the lowest single-point thickness recorded at 16.8µm. An optical disector depth of 12µm was chosen based on our lowest z-axis measure, and to allow for a 2µm upper guard zone, in order to eliminate z-axis bias due to unevenness of the sections. Pilot analysis revealed that approximately 40 sampling fields were required to obtain a CE below 0.05 (Gundersen, Jensen, Kieu, & Nielsen, 1999). An optical disector of 70µm x 70µm x 12µm (x,y, and z-axes) was employed (**Figure 2.3**), giving a total disector volume of $58.8 \times 10^3 \mu\text{m}^3$ per sampling field. Disectors where more than 50% of the sampling field did not contain any tissue, due to tear or hole in section, blood vessels (to minimize bias of potentially sampling more or less blood vessels and perivascular astrocytes, due to plane of sectioning), and outside of the ROI contour were excluded to prevent bias in estimation of cell counts. Estimation of densities of GFAP

positive astrocytes, GFAP negative glia, and total number of glia were then calculated for our ROI of 1mm x 2mm for each case.

2.4.2.4 Nucleator

Somal size of positively identified cells were measured using the nucleator probe with 6 random isotropic rays spanning the soma radiating from the center and marked manually at the edges. This was performed simultaneously within the optical fractionator workflow, with average somal size estimated for each section and sample (**Figure 2.4**). Nucleator measurements for all samples had CE's lower than 0.005.

2.4.2.5 Spaceballs

The spaceballs probe within Stereo Investigator 11 was employed to estimate the length of astrocyte processes for our region of interest. Pilot analysis revealed that approximately 30 systematically random sampling (SRS) fields were required to collect measurements with CE lower than 0.05. Using the spaceballs probe, a virtual hemisphere was embedded within the tissue and each transection of astrocytes processes with the outline of hemisphere was recorded (**Figure 2.5**) and the total astrocyte process lengths within the 30 SRS fields in the region of interest estimated. Sampling field was excluded if more than 50% of virtual hemisphere overlay contained no tissue.

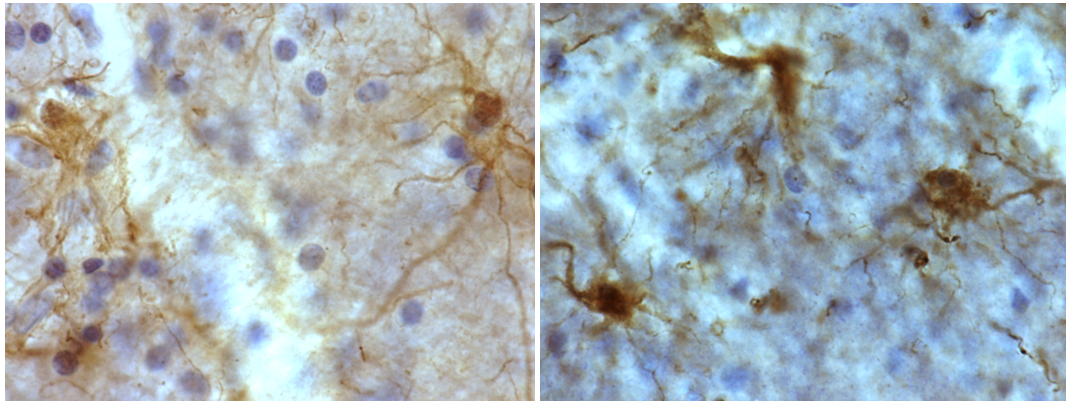


Figure 2.1 | GFAP astrocytes in autism and control brain sections.

Images of GFAP positive astrocytes in the brain sections of (**left**) autism case 5144, and (**right**) control case 1185. Images acquired at 100x objective.

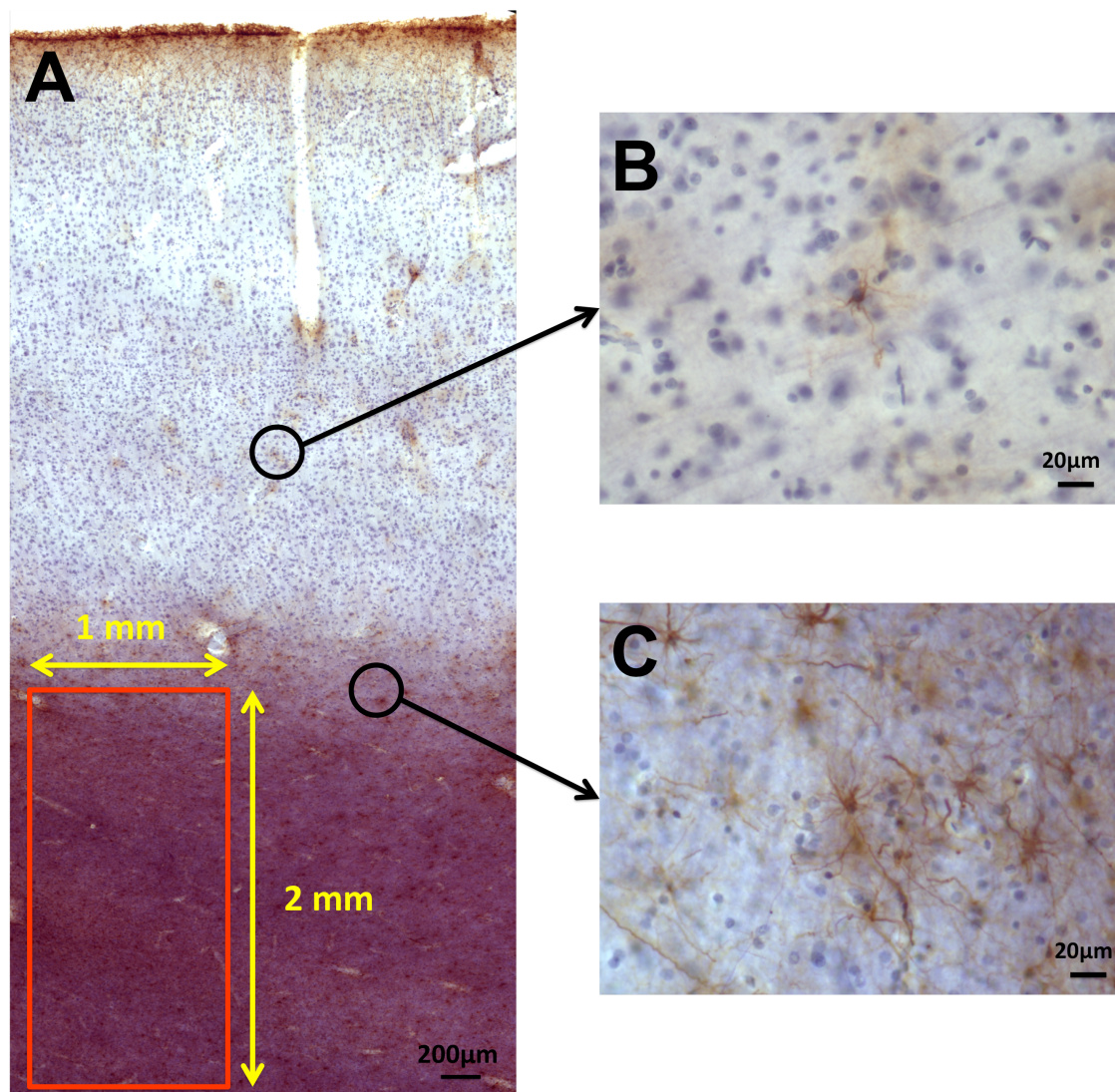


Figure 2.2 | Representation of region of interest (ROI) sampling

(A) Tiled images captured at 10x objective, from control sample 4916, showing GFAP staining in radial glial cells near the pia, as well as grey and white matter astrocytes. A ROI was drawn with 1mm width across the grey and white matter border, extending 2mm into the white matter. 40 sampling fields were generated within the ROI using SRS method within Stereo Investigator 11. **(B)** Enlarged grey matter area from tiled image showing GFAP positive astrocyte at 40x objective. **(C)** Enlarged white matter area from tiled image showing GFAP positive astrocytes at 40x objective.

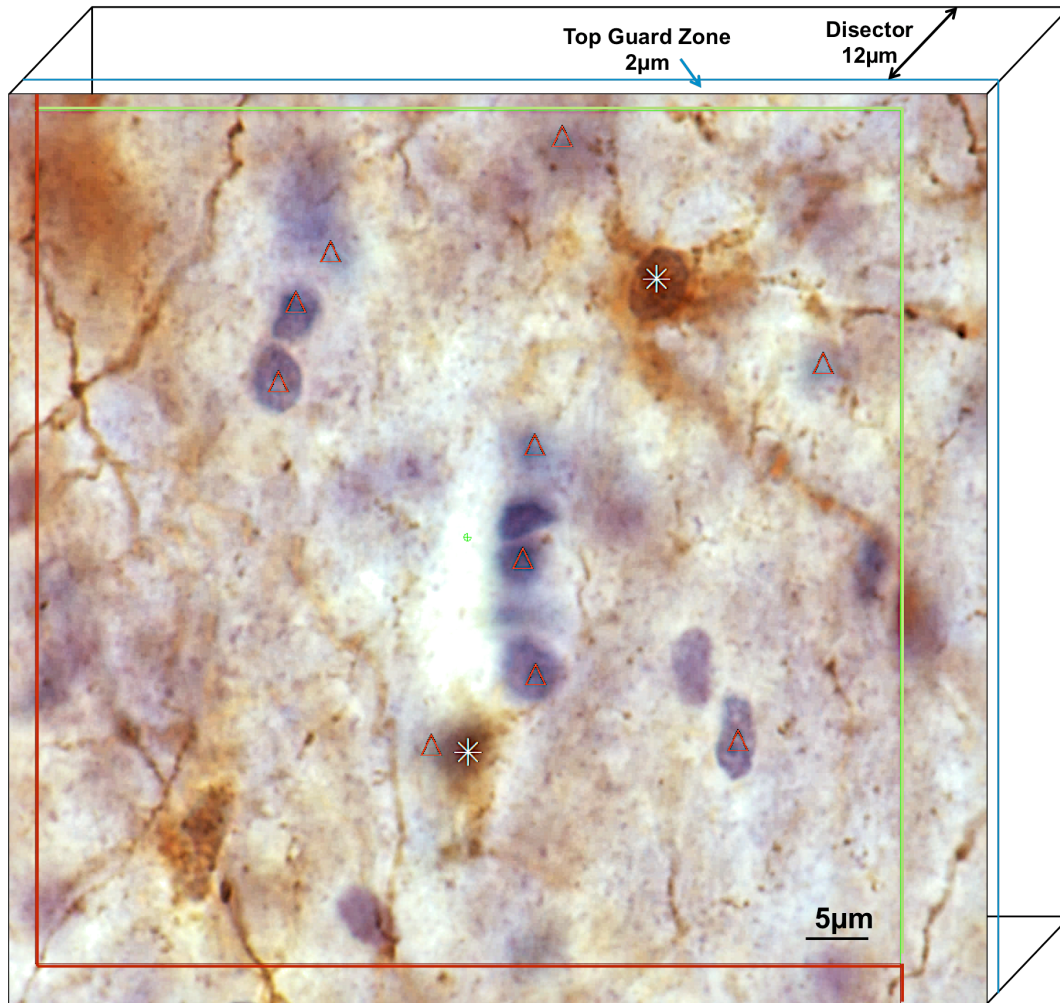


Figure 2.3 | Representation of optical disector

Optical disector represented by a rectangular cuboid of $70\mu\text{m} \times 70\mu\text{m} \times 12\mu\text{m}$ (width x height x depth), defined by 2 inclusion lines (green), 2 exclusion lines (red) on the x- and y-axes, center point of disector marked by green dot, as well as disector depth (black line with double arrowhead) of $12\mu\text{m}$ on the z-axis. Top guard zone (blue arrow and lines) of $2\mu\text{m}$ was employed to account for bias due to unevenness of tissue. Astrocytes (white asterisk) were identified via a GFAP stained cell body, processes, and nucleus. Any white matter neurons were identified via the presence of a single nucleolus, larger appearance and non-spherical shape as well as counterstain in the cytoplasm. Other glial cells were identified via their smaller nucleus, spherical appearance, and the presence of heterochromatin as well as a lack of visible cytoplasm (red triangle). Cells were only counted if nucleus (glia) or nucleolus (neurons) were in focus within the disector. Images captured using a 100x objective (n.a. = 1.4).

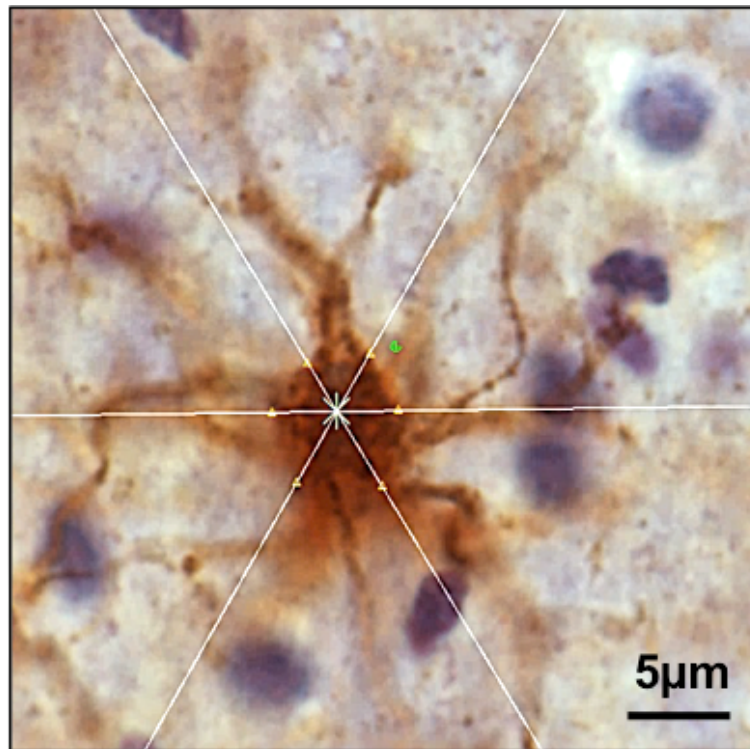


Figure 2.4 | Nucleator probe

Nucleator probe with 6 isotropic rays radiating from the centre of the cell soma. Investigator tags edges of cells so that software can measure the diameters and hence use the information to estimate somal size. 100x objective (n.a. = 1.4).

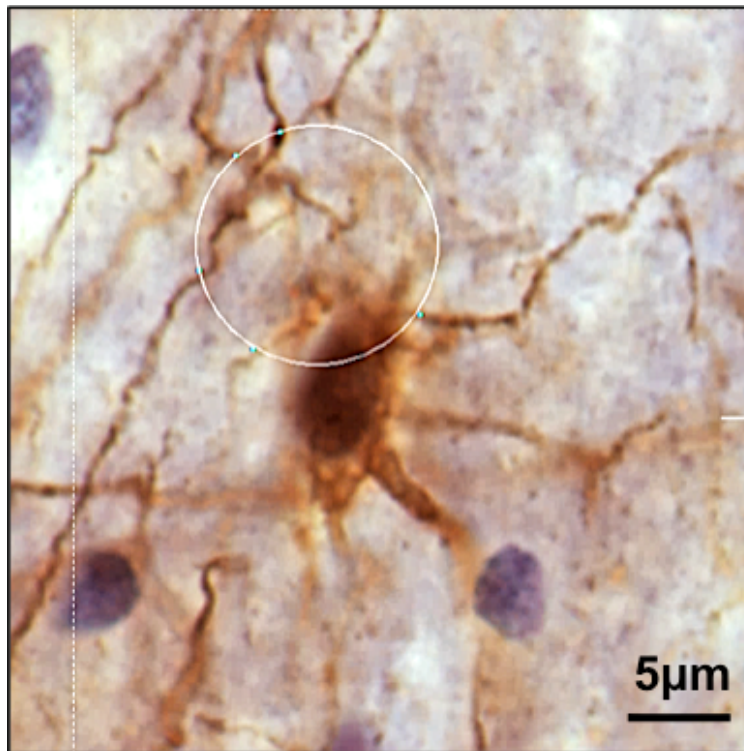


Figure 2.5 | Spaceballs probe

Spaceballs probe using hemisphere, any intersection point between the outline of hemisphere and astrocytes processes in focus were marked, regardless of the presence of cell body. Image captured at 100x objective (n.a. = 1.4).

2.5 Additional discussion

While GFAP is commonly used as a marker of astrocyte activation, it is important to note that increased GFAP expression is not necessarily indicative of increased number of astrocytes. Although increased astrocyte proliferation is commonly observed in severe astrogliosis, the morphological and physiological changes may not be as prominent in the case of mild/moderate astrogliosis (Sofroniew & Vinters, 2010). Furthermore, as a large proportion of mature astrocytes in the healthy CNS do not express detectable levels of GFAP, activation may lead to increased GFAP expression in these cells, which is independent of astrocyte proliferation (Sofroniew & Vinters, 2010). The lack of increased GFAP labelled astrocytes as seen in our study may suggest that astrogliosis in autism brain is not as severe as previously appreciated, and that the much debated neurodegeneration may not be present (Kern, Geier, Sykes, & Geier, 2013). It is also crucial to recognise that GFAP can be absent in the PAP, and that the process length measures reported within this study is only representative of GFAP-positive astrocyte processes. Therefore, future studies would benefit from quantitating the process length astrocytes labelled with another marker that is present in the PAP. Nevertheless, our findings present an informative comparison of the process length of GFAP-positive astrocytes in the autism versus healthy brain.

Our post-mortem stereological study was conducted in a cohort with relatively large age range, although the autism (age range= 4-51 years) and control (age range= 4-46 years) groups were age-matched. Given the small sample size (autism=8 and control=7), the wide age range could have contributed to masking any astrocyte abnormalities, especially if changes were potentially restricted to early stages of the disorder within the first few years of life and then normalise in later years. Although restricted to tissues from the DLPFC, our cohort contained samples from BA46 and BA9 due to availability from the brain bank. This

led to variation in brain section sizes and composition of grey/white matter across the sample cohort, which could potentially contribute to masking positive findings. To address this, we ensured that the stereological study was conducted with strict sampling criteria and low CE in order to minimise variability related errors. This is a common challenge faced by autism researchers due to the limited availability of autism post-mortem brain samples, it is also an issue that can only be resolved with further collection of brain tissue and increasing cohort sizes available for investigation. Medication is not commonly used for autism, although they could be prescribed for other comorbid conditions such as ADHD or epilepsy. Medication histories of the subjects in the current study are not available, therefore the potential confounding effect could not be investigated. This is yet another challenge of post-mortem studies, where valuable information may not be readily available.

Future stereological studies should explore the potential astrocyte abnormalities using different markers, such as S100 β , ALDH1L1 or EAAT1, in order to label a more complete population of astrocytes. In addition, these should be repeated in the grey matter, as well as other brain regions involved in cognitive function and/or have been implicated in autism such as the PFC, ACC, amygdala and hippocampus. Moreover, an investigation focusing on the co-expression of mGluR5 and astrocyte marker within the same cohort would also add some depth into the understanding of the potential relationship between astrocyte activation and mGluR5 signalling.

CHAPTER 3 Investigation into the reciprocal interaction of astrocytic activation on mGluR5 signalling.

3.1 Background

Astrocytes play an important role in regulating pre- and post-synaptic glutamate receptors as well as glutamate levels at synapses to prevent excitotoxicity caused by accumulation of extracellular glutamate (Yang et al., 2009). Following reuptake of glutamate from the synaptic cleft, by PAP, astrocytes convert glutamate to glutamine, and then transport it back to neurons to maintain an ongoing neurotransmitter cycle (Tani et al., 2014). mGluR5 receptors, which are found in abundance at the PAP, have been shown to regulate the functional maturation of astrocytes, as evidenced by findings of reduced process arborisation and expression of glutamate transporter GLT1/EAAT2 following inhibition of mGluR5 via pharmacology or genetic approaches during early developmental stage (Morel et al., 2014).

At the glutamatergic synapse, astrocytic mGluR5 is responsible for synaptic glutamate detection, with receptor activation leading to an increase of Ca^{2+} in astrocytic processes, inducing different astrocytic responses, including exocytotic release depending on neighbouring neuronal activity (**Figure 3.1**) (Panatier & Robitaille, 2015). When glutamate binding occurs, mGluR5 activation stimulates phosphoinositide (PI) hydrolysis as well as subsequent Ca^{2+} channel activation and influx, which can lead to the downstream release of gliotransmitters such as glutamate, D-serine, and ATP (Loane, Stoica, & Faden, 2012; Miller, Romano, & Cotman, 1995). During sustained synaptic activity, astrocytes can also release glutamate, which acts on extrasynaptic ionotropic or metabotropic receptors, to regulate neuronal synchrony, excitability and synaptic transmission (Haydon & Carmignoto, 2006;

Panatier & Robitaille, 2016). In addition, astrocytic D-serine (a co-agonist of NMDA receptors, that is selectively synthesised in astrocyte) is important for regulating neuroplasticity, where it can bind to the glycine site to induce LTP, while a lack of it can induce LTD (Haydon & Carmignoto, 2006). Interestingly, astrocytic mGluR5 activation can also lead to the release of purine, which could suppress or trigger synaptic transmission. Briefly, astrocytic ATP is hydrolysed in the extracellular space to adenosine, which can activate neuronal presynaptic P2, A1 and A2 receptors and lead to an increase in GABAergic inhibition (Bowser & Khakh, 2004; Panatier & Robitaille, 2016).

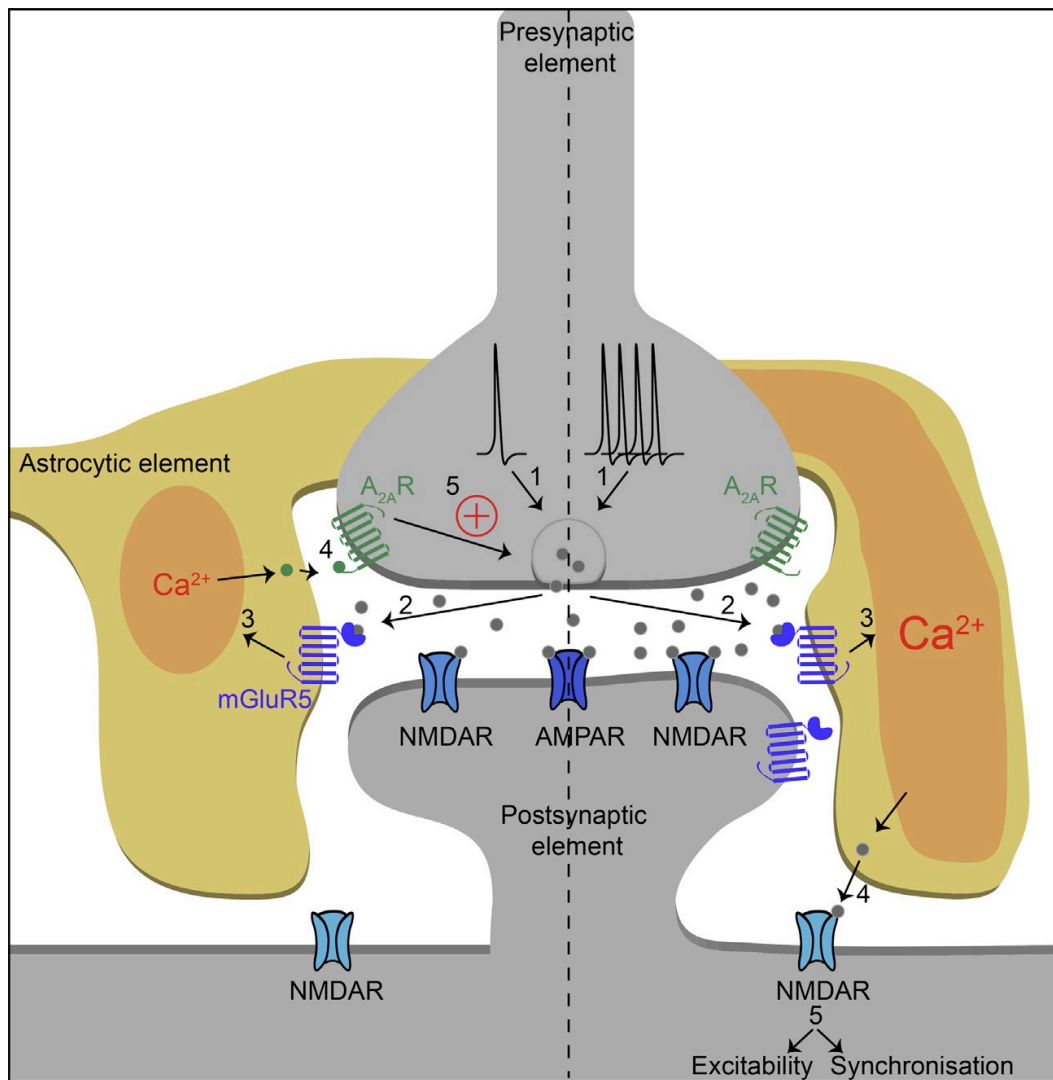


Figure 3.1 | Model of mGluR5-dependent neuronal-astrocyte interaction at glutamatergic synapses.

(Left) At the synapse, single action potential causes glutamate release that activates astrocytic mGluR5s, which then lead to a local increase of Ca^{2+} in the astrocytic process and induces purine release. Astrocytic purine can act on presynaptic A2A receptor to trigger or A1 receptor to suppress synaptic transmission. **(Right)** Sustained presynaptic potentials lead to an intense glutamate release and astrocytic mGluR5 activation, causing a global Ca^{2+} increase in astrocytes process and triggering glutamate release from astrocytes. Astrocytic glutamate appears to be specific to extra synaptic NMDAR and function to modulate neuronal excitability and synchrony. (Figure from Panatier & Robitaille (2015))

Apart from regulating gliotransmitter release, mGluR5 can also modulate the expression and activity of glutamate transporters, such as EAAT1 and EAAT2, in order to maintain synaptic glutamate concentration (Loane et al., 2012). In line with this, reactive rat astrocytes exposed to the mGluR5 agonist (S)-3,5-Dihydroxyphenylglycine (DHPG) displayed increased GLAST/EAAT1 and GLT1/EAAT2 activities, that was attenuated by the mGluR5 antagonist MPEP and GLT1 blocker (Vermeiren et al., 2005).

Gene expression of astrocytic mGluR5 has been shown to increase in the early postnatal period, and then decrease with age, with mRNA levels in adulthood significantly lower compared to early life as shown in a mouse study (Morel et al., 2014). Mature astrocytes express only about 20% of the concentration of mGluR5 found in neurons, however astrocytic mGluR5 has been shown to have high binding efficiency and receptor activation relative to receptor density, initiating downstream signalling sufficient to maintain astrocyte physiological functioning (Balázs et al., 1997). Following prolonged exposure to the agonist 1-Amino-1,3-dicarboxycyclopentane (ACPD), astrocytic mGluR5 undergoes a desensitisation stage where a reduction of protein expression and activity of mGluR5 is observed. However, receptor internalisation has not been observed and the desensitisation effect is reversible upon removal of agonist. This suggests that in pathological conditions where excessive glutamate release is present, astrocytic mGluR5 undergoes a reduction in expression and activity, although still maintaining partial functioning despite receptor desensitisation (Balázs et al., 1997).

In addition, growth factors such as FGF, epidermal growth factor (EGF), or TGF α have also been shown to increase the expression of mGluR5 in astrocytes, as well as enhanced receptor activity as shown by increased phosphatidylinositol (PI) hydrolysis following agonist

exposure (Loane et al., 2012; Miller et al., 1995). Astrocytes exposed to these growth factors were also shown to display hypertrophic morphological changes of reactive astrocytes, suggesting that mGluR5 dysregulation may also be associated with astrogliosis (Miller et al., 1995). Together, this evidence indicates that astrocytes can respond to external stimuli such as growth factors, which in turn can affect their reactive state and mGluR5 glutamatergic signalling, however, the exact mechanism remains unclear.

Viral infection has been implicated in various neuropsychiatric disorders including schizophrenia, bipolar disorder and autism. Double-stranded RNA (dsRNA) is a signature of viral infection recognised by TLR3 to induce an innate immune response in astrocytes such as activation and astrogliosis. Poly I:C, a synthetic analogue of dsRNA, is commonly used to simulate viral infection in-vitro or in-vivo. While LPS did not trigger activation in human astrocytes in-vitro, Poly I:C has been shown to induce astrocyte reactivity as characterised by increased secretion of pro-inflammatory cytokines, including IL-6, TNF α , Rantes, and IFN β (Bilbo, Block, Bolton, Hanamsagar, & Tran, 2018; Scumpia, Kelly-Scumpia, & Stevens, 2014; Tarassishin, Suh, & Lee, 2014). Furthermore, decreased astrocytic functioning has also been reported following Poly I:C induced activation, evidenced by decreased gene expression of GLAST/EAAT1 and glutamate uptake activity (Scumpia, Kelly, Reeves, & Stevens, 2005). Interestingly, increased protein expression of mGluR5 has been reported following brain injury caused by needle insertion (Ferraguti, Corti, Valerio, Mion, & Xuereb, 2001). This is supported by another mouse study of prenatal Poly I:C exposure where offspring were found to have elevated plasma levels of pro-inflammatory cytokines and increased protein expression of mGluR5 (Arsenault, St-Amour, Cisbani, Rousseau, & Cicchetti, 2014). Although other findings have instead shown a downregulation of mGluR5 expression following astrocyte activation with TNF α , IL-1 β or LPS-exposed microglial conditioned media,

suggesting that the mechanisms and responses of astrocyte activation may be more complicated than previously appreciated (Aronica, Gorter, Rozemuller, Yankaya, & Troost, 2005; Tilleux et al., 2007).

In autism brain, mGluR5 has been found to be upregulated in the cerebellum and downregulated in the DLPFC (Chana et al., 2015; Fatemi et al., 2011). This is accompanied by evidence of astrocyte activation, including elevated levels of neuroinflammatory markers, as well as astrocyte markers EAAT1, EAAT2 and GFAP (Crawford et al., 2015b; Edmonson et al., 2014; Laurence & Fatemi, 2005; Purcell et al., 2001).

Given astrocytes play key roles at the tripartite synapse in regulating physiological glutamatergic signalling and neuroplasticity, as well as the body of work demonstrating the crosstalk between astrocyte activation and mGluR5 signalling, it is pertinent to examine and understand the reciprocal interaction between mGluR5 modulation and astrocytic activation. With the co-occurrence of astrocyte activation and glutamatergic disruption shown within the autism brain, it is also logical to consider the potential effect of neuroinflammation and subsequent alteration in astrocytic mGluR5 activity and how this relates overall to the neuropathology of autism.

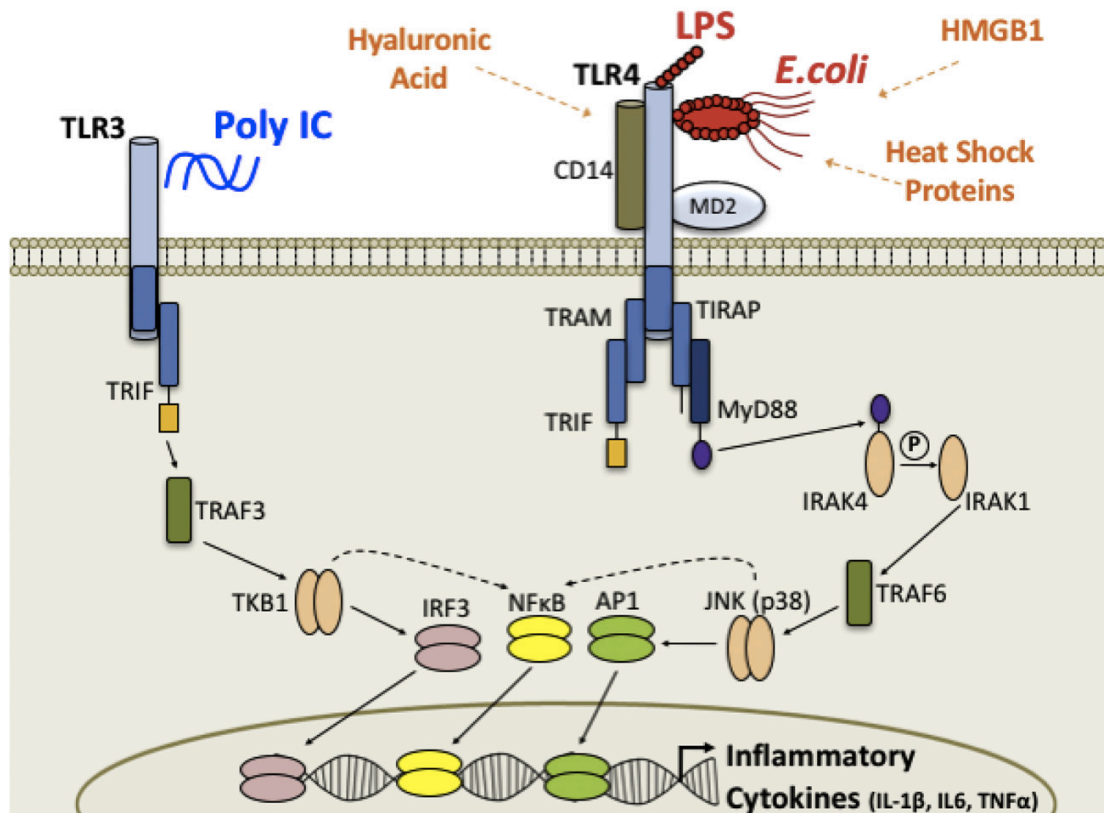


Figure 3.2 | Toll-like receptors signalling of astrocyte.

Poly I:C and LPS are recognised by TLR3 and TLR4 respectively, which triggers the downstream interferon regulatory factor 3 transcription factor (IRF3) or nuclear factor kappa-light-chain-enhancer of activated B cells transcription factor (NFκB) signalling to induce the release of pro-inflammatory cytokines (figure from Bilbo et al. (2018)) .

3.2 Aim

Based on the evidence of alterations in mGluR5 signalling and astrocyte activation in autism, this study aimed to investigate if there is an interaction between mGluR5 functioning and activation of astrocytes as well as whether exposure to compounds that can modulate mGluR5 provide protection following neuroinflammatory insults.

3.3 Hypotheses

Based on the findings of both astrocyte activation and reduced mGluR5 expression within the autism brain, as well as the interrelationship between astrocyte activation and mGluR5 signalling as presented in the literature, we hypothesise that there will be a reduction in expression and signalling activity of mGluR5 following astrocyte activation. We further hypothesise that increasing mGluR5 function via positive allosteric modulators may have a protective effect against neurotoxicity associated with astrocyte activation.

3.4 Materials and methods

3.4.1 Culturing of primary human foetal astrocytes

Primary human foetal astrocytes lines were established from brain tissue kindly donated by consenting patients undergoing pregnancy terminations, between gestational ages of 14-16 weeks, at Dr Marie Maroondah Reproductive Health Clinic, Victoria, Australia. Ethics approval was granted by the University of Melbourne (ID: 1135373) covering tissue collection for research use. Establishment of astrocytes was carried out as previously described (Trillo-Pazos, McFarlane-Abdulla, Campbell, Pilkington, & Everall, 2000). Briefly, tissue was mechanically disaggregated to single cell suspension, and seeded in T175 culture flask at 3×10^5 cells/ml in DMEM/F-12 containing foetal bovine serum (FBS) [10%], L-glutamine [1mM] and gentamicin sulphate [20µg/ml] (all reagents from Life Technologies, USA). Media change was performed weekly, and culture was passaged at least twice prior to experiments to remove any contaminating neurons. These cultures were shown to express astrocyte markers (**Figure 3.3**).

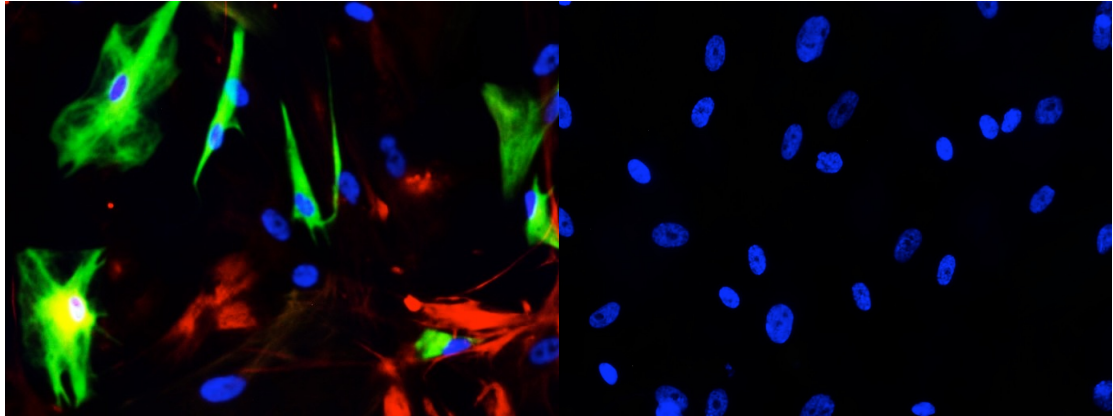


Figure 3.3 | Primary human astrocyte cultures express astrocyte specific markers

Immunocytochemistry images show that our astrocyte cultures express (Left) astrocyte markers GFAP (green) and S100B (red), however (Right) they do not express IBA-1 (green).

3.4.2 Astrocyte activation

Astrocytes were detached using TrypLE Select 1X (Life Technologies, USA), and then seeded onto 6-well plates at a density of 250,000 cells/ml and allowed to plate down overnight. The following day, astrocytes were exposed to TLR3 ligand Poly I:C [100ng/ml] as an activation agent or an equivalent amount of sterile distilled water (vehicle). The concentration of Poly I:C was decided based on a study demonstrating that Poly I:C concentration as low as 100ng/ml is sufficient to activate TLR3 and astrocyte reactivity, as well as to minimise potential toxicity associated with higher concentrations (Friboulet et al., 2010). Time points of 24 hours and 7 days exposure were chosen in order to model acute and chronic activation respectively. All experiments were carried out using multiple subsequent passages of cells from the same preparation of primary human astrocytes (same donor), the exposure conditions were conducted technical triplicates.

3.4.3 Modulating mGluR5 activity

Stock solutions of mGluR5 compounds Dihydroxyphenylglycine (DHPG) [100mM], VU0360172 (VU) [10mM] and 3-((2-Methyl-4-thiazolyl)ethynyl)pyridine (MTEP) [10mM] were prepared in DMSO according to manufacturer's recommended reconstitution concentration. Cells were exposed to Poly I:C [100ng/ml] in the presence of mGluR5 agonist DHPG [120mM], PAM VU [4.3µM], NAM MTEP [0.33µM], or vehicle [equivalent amount of DMSO] with concentrations determined based on known affinity estimates at 30x binding affinity (K_i) to achieve close to 100% receptor occupancy (Gregory et al., 2012). All mGluR5 compounds and their binding affinity details were provided by Dr. Karen Gregory from Monash Institute of Pharmaceutical Sciences (MIPS).

3.4.4 Characterisation of activation profile and glutamatergic functioning

3.4.4.1 Lactate dehydrogenase (LDH) toxicity assay

CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, AUS) was used to determine the cytotoxicity associated with activation agents. Briefly, 50µl of culture media was harvested for each sample, and pipetted in duplicates into 96 well plates, followed by 50µl of LDH substrate mix and incubated at 30 minutes in the dark at room temperature. The reaction was terminated by adding 50µl of stop solution, and absorbance measured using a SpectraMax 190 micro-plate reader (Molecular Devices, USA) at 490nm.

3.4.4.2 Enzyme-linked immunosorbent assay (ELISA)

Following exposure of cells to experimental conditions media was collected to carry out ELISA for pro-inflammatory markers (ELISakit.com, AUS). A pilot study examined the levels of IL-6, Rantes, TNF α , IL-1 β , IFN β , and based on the results, we chose to focus on IL-6 and Rantes as these markers were shown to be elevated in Poly I:C exposed astrocytes compared to controls. Briefly, duplicates of standards (100µl) and samples (100µl) were incubated in the sample plate for 2 hours at room temperature. Following 4 washes with wash buffer, 100µl of biotin labelled antibody was incubated in each well for 2 hours at room temperature. After another 4 washes, 100µl of streptavidin-horseradish peroxidase (HRP) was incubated in each well for 45 minutes at room temperature. A final 5 washes was carried out prior to incubation of wells in 100µl of Tetramethylbenzidine (TMB) substrate for 15 minutes at room temperature, followed by 50µl of stop solution. The optical density was then determined using a SpectraMax 190 micro-plate reader (Molecular Devices, USA) at 450nm.

3.4.4.3 Gene expression

Total RNA was extracted using a Purelink RNA Minikit (Life Technologies, USA) as per manufacturer's instruction. RNA quality was analysed using a TapeStation (Agilent Genomics, USA) before converting to cDNA using SuperScript IV Reverse Transcriptase (Life Technologies, USA) according to manufacturer's instruction. All RNA samples used in this study had a RIN above 8. Quantitative real-time polymerase chain reaction (qPCR) was performed using Taqman gene expression mastermix and Taqman gene expression assays (Life Technologies, USA) including S100 β (Hs00902901_m1), GFAP (Hs00909233_m1), ALDH1L1 (Hs01003842_m1), EAAT1 (Hs00904823_g1), EAAT2 (Hs01102423_m1), VGLUT1 (Hs00220404_m1), GRM5 (Hs00921949_m1), SHANK3 (Hs01393541_m1), glutamate ionotropic receptor AMPA type subunit 3 (GRIA3) (Hs01557466_m1), as well as reference genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Hs02758991_g1), peptidylprolyl isomerase A (PPIA) (Hs04194521_s1) and hydroxymethylbilane synthase (HMBS) (Hs00609297_mq). qPCR was performed with 20ng of cDNA template per reaction. mRNA expression data (threshold cycle, Ct) was transformed to fold change using the 2^{-ddCt} method based on the arithmetic mean of reference genes and control samples (Livak & Schmittgen, 2001). Statistical analysis of gene expression levels in different samples was performed using a student t-test in IBM SPSS Statistics (IBM, USA).

3.4.4.4 mGluR5 radioligand binding assay

The radioligand binding assay was conducted by Dr Karen Gregory. Changes in mGluR5 binding activity were assessed in intact cells as previously described (Gregory et al., 2012). For 7-days exposure, human primary foetal astrocytes were exposed to Poly I:C [100ng/ml] for 6 days in complete culture media, then for an additional day in glutamine-free culture media with Poly I:C [100ng/ml] to avoid desensitisation of mGluR5 binding. Cells

were seeded at 250,000/well and incubated with a range of [3H]methoxyPEPy concentrations (0.5 nM to 60 nM) for 1 hour at room temperature with agitation in Hanks' buffered saline solution; nonspecific binding was determined with 10 μ M MPEP. Assays were terminated by three washes with ice-cold 0.9% Sodium Chloride. Samples were then incubated with UltimaGold scintillant for 2 hours prior to radioactivity determination with a TriCarb liquid scintillation analyser (PerkinElmer Life and Analytical Sciences). The maximum number of binding sites (Bmax) was calculated using nonlinear regression in Graphpad Prism (GraphPad Software, USA) based on specific and non-specific binding output.

3.5 Results

3.5.1 Activation of primary human foetal astrocytes

3.5.1.1 LDH toxicity assay

LDH assay was conducted as a measure of potential cytotoxicity due to exposure to Poly I:C. LDH levels increased significantly in Poly I:C exposed cells at 24 hours (Optical density (OD)=0.658, $p=0.001$) compared to non-exposed controls (OD=0.597), but not at 1 week Poly I:C exposure (OD=0.683, $p=0.089$) compared to controls (OD=0.593) (**Figure 3.4**).

3.5.1.2 ELISA for pro-inflammatory markers

ELISA of IL-6 and Rantes was used as an indicator astrocyte activation. IL-6 was increased in culture media of cells exposed to Poly I:C at 24 hours (Concentration, Conc=456.21pg/ml, $p=0.005$) and 1 week (Conc=134.30pg/ml, $p=0.001$) compared to controls at 24 hours (Conc=8.95pg/ml) and 1 week (Conc<3.9pg/ml) respectively. Rantes was increased in the culture media of cells exposed to Poly I:C at 24 hours (Conc=494.39pg/ml, $p=0.01$) and 1 week (Conc=161.42pg/ml, $p=0.015$) compared to controls at 24 hours (Conc<3.9pg/ml) and 1 week (Conc=6.12pg/ml) respectively (**Figure 3.5**). It is interesting to note that the levels of increase in both IL-6 and Rantes were greater at 24 hours compared to 1 week time point, suggesting that cytokine release may be greater in acute pathogenic exposure than chronic exposure.

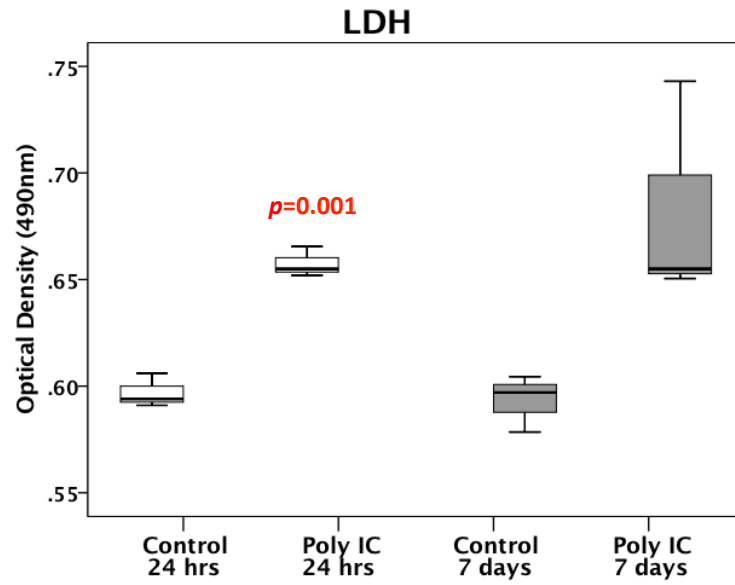


Figure 3.4 | LDH toxicity assay

LDH Toxicity assay revealed an increase in LDH levels following 24 hours Poly I:C exposure, but not with 1 week exposure. Error bars represent standard error of mean (SEM).

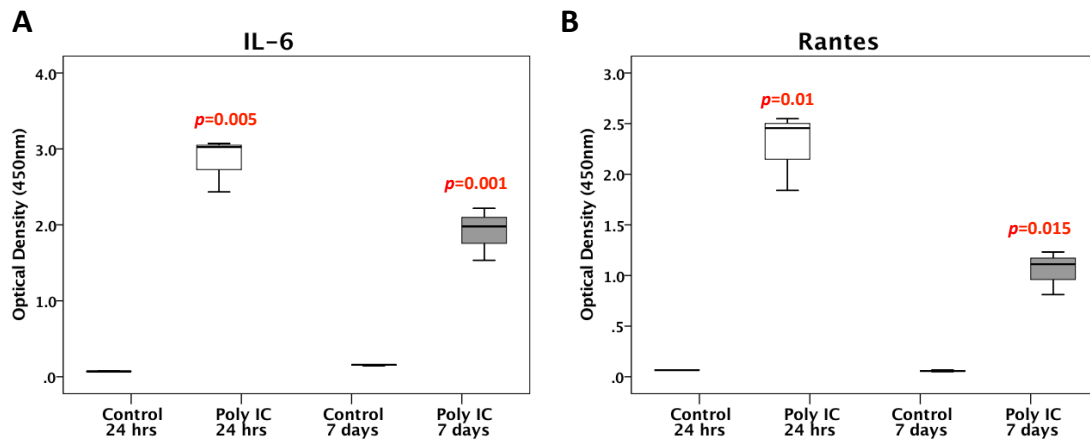


Figure 3.5 | ELISA of IL-6 and Rantes

ELISA to assess concentration of pro-inflammatory markers in culture media of cells exposed for 24 hours and 7 days. Data was analysed using univariate analysis of variance with LSD multiple comparison. Both **(A)** IL-6 and **(B)** Rantes increased significantly in culture media of cells exposed to Poly I:C for 24 hours and 1 week compared to controls. Error bars represent SEM.

3.5.1.3 Gene expression

The levels of all 3 reference genes were shown to be not significantly different across all the treatment groups, and therefore are stable controls appropriate for use in the calculation of the fold changes.

Gene expression levels of all astrocyte structural markers were downregulated in Poly I:C treated cells at 24 hours timepoint, including GFAP (fold change, $FC=0.390$, $p=7\times10^{-6}$), S100 β ($FC=0.424$, $p=0.002$), and ALDH1L1 ($FC=0.358$, $p=0.001$). This is also consistent for cells exposed to Poly I:C for 1 week, GFAP ($FC=0.355$, $p=1.5\times10^{-4}$), S100 β ($FC=0.383$, $p=0.001$), and ALDH1L1 ($FC=0.435$, $p=3.2\times10^{-4}$) (**Figure 3.6**).

As for glutamatergic elements, EAAT1 was upregulated in Poly I:C treated cells at 24 hours ($FC=4.342$, $p=7.3\times10^{-5}$) and 1 week ($FC=2.917$, $p=2.6\times10^{-5}$). Whereas EAAT2 was downregulated at 24 hours ($FC=0.137$, $p=2\times10^{-6}$) and 1 week ($FC=0.194$, $p=6.1\times10^{-7}$). VGLUT1 was also downregulated at 24 hours ($FC=0.429$, $p=0.003$) and 1 week ($FC=0.503$, $p=0.006$). GRM5 was downregulated following 24 hours exposure ($FC=0.765$, $p=0.021$), with a trend decrease following 1 week exposure that did not reach significance. GRIA3 was consistently downregulated following 24 hours ($FC=0.912$, $p=0.007$) and 1 week ($FC=0.685$, $p=0.03$) Poly I:C exposure (**Figure 3.7**).

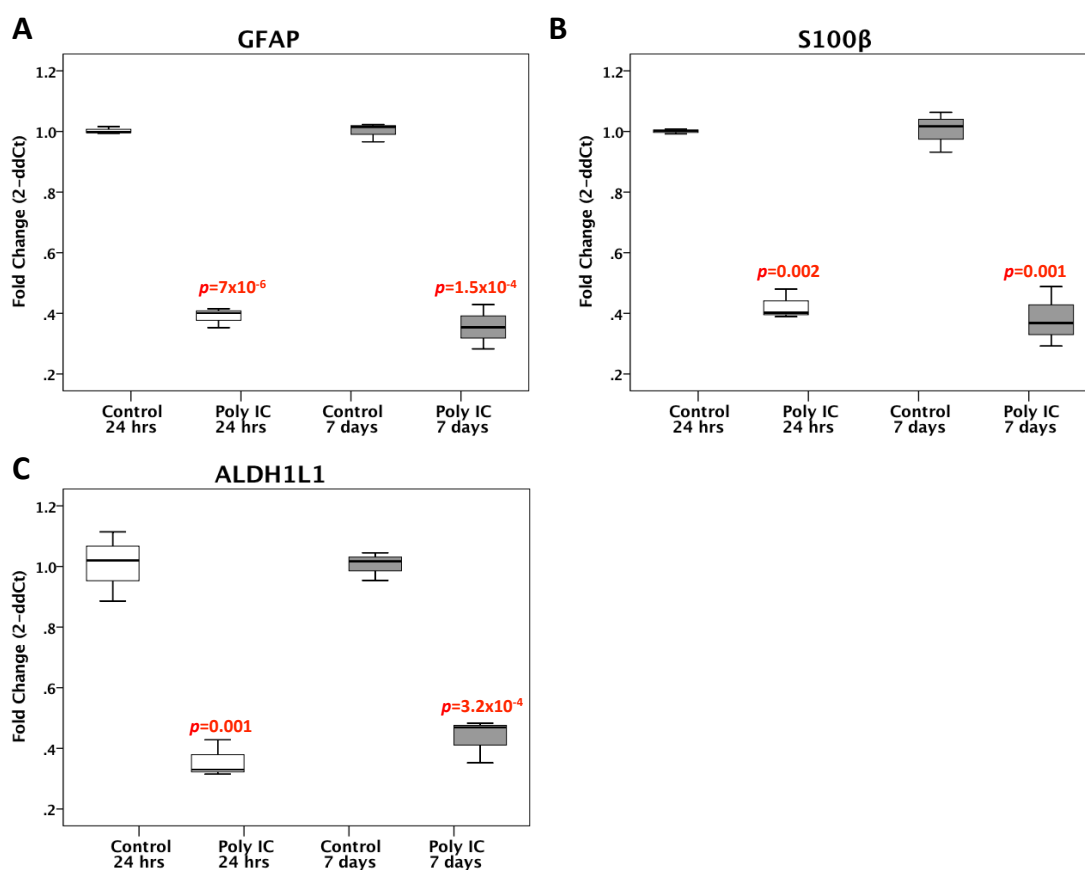


Figure 3.6 | Gene expression fold changes of astrocyte cytoskeletal markers

Gene expression changes of primary human foetal astrocytes exposed to Poly I:C for 24 hours and 7 days compared to controls using one-way ANOVA. We observed a reduction of mRNA expression of astrocyte cytoskeletal markers (A) GFAP, (B) S100β, (C) ALDH1L1. Error bars represent SEM.

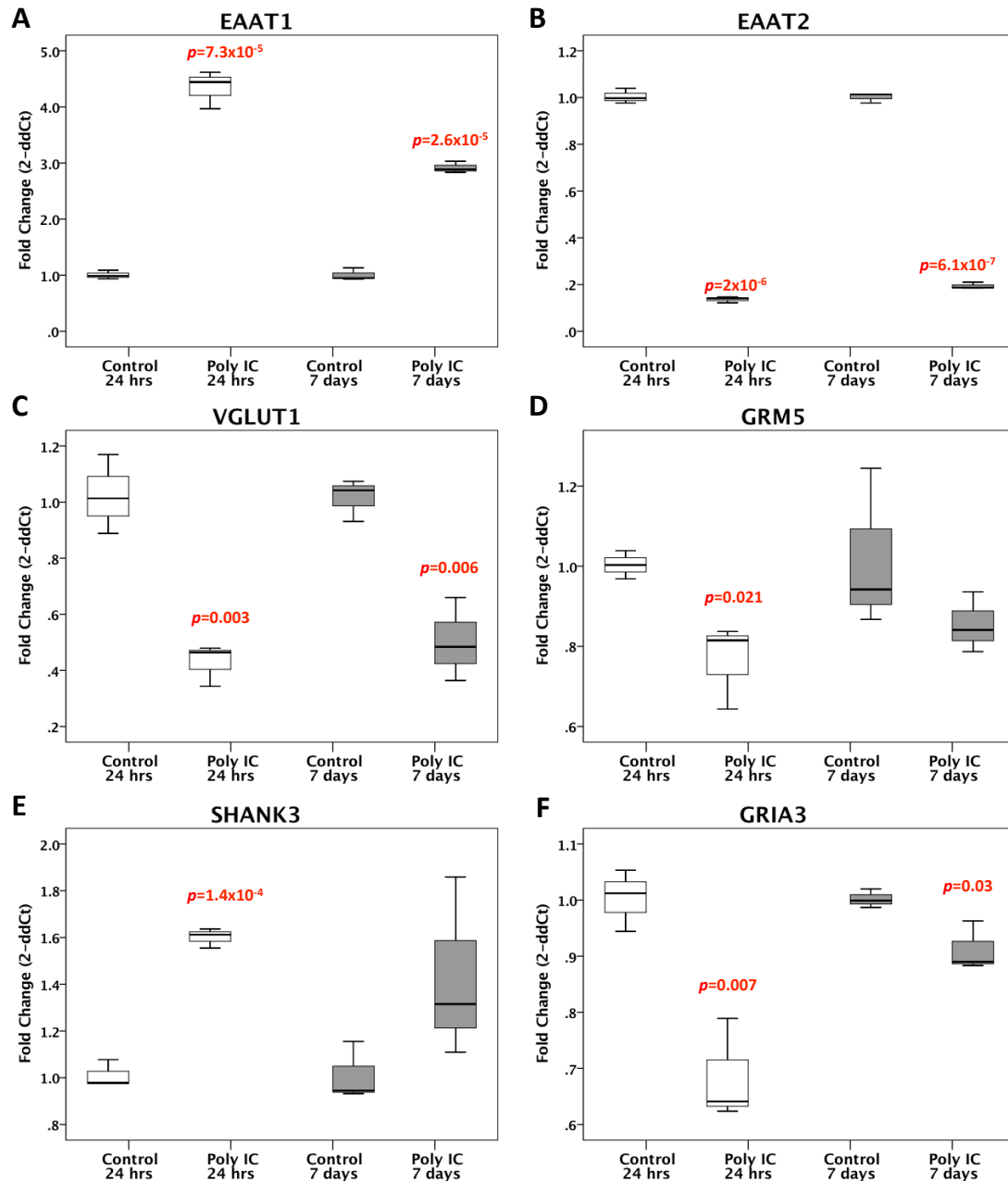


Figure 3.7 | Gene expression fold changes of glutamatergic markers

Gene expression changes of primary human foetal astrocytes exposed to Poly I:C for 24 hours and 7 days compared to controls using one-way analysis of variance (ANOVA). We observed increased expression of (A) EAAT1, reduced expression of (B) EAAT2 and (C) VGLUT1. mRNA expression of (D) GRM5 was downregulated, as well as disruption of other glutamatergic signalling elements (E) SHANK3 and (F) GRIA3. Error bars represent SEM.

3.5.1.4 mGluR5 radioligand binding assay

A radioligand-binding assay was conducted in order to assess mGluR5 activity in the exposed cells. Binding assays revealed that activation of astrocytes using Poly I:C reduced mGluR5 binding at 7 days ($B_{\max}=12.38$) compared to controls ($B_{\max}=137.2$) but no difference was observed at 24 hours between PIC ($B_{\max}=198.4$) and controls ($B_{\max}=220.3$) (**Figure 3.8**).

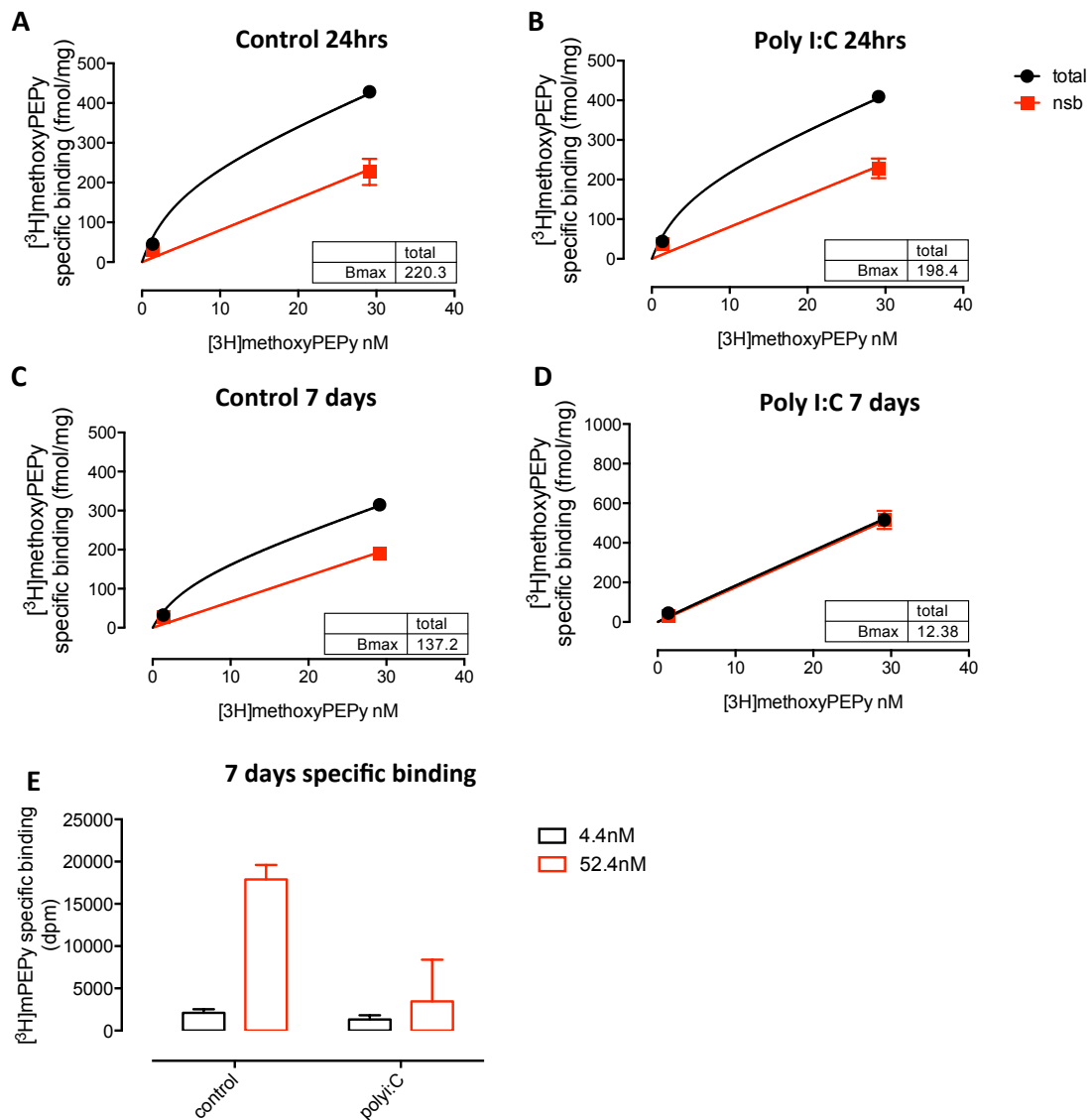


Figure 3.8 | mGluR5 [3H]mPEPy radioligand binding assay

mGluR5 [3H]mPEPy radioligand binding assay in primary human foetal astrocytes at 24 hours timepoint, (A) controls, (B) Poly I:C, and 1 week timepoint (C) controls, (D) Poly I:C. (E) comparison of specific binding (52.4nM) and non specific binding (4.4nM) between controls and Poly I:C treated primary human foetal astrocytes. nsb=non specific binding (Data and images generated by Dr. Karen Gregory, MIPS)

3.5.2 Astrocytic mGluR5 modulation with activation

Given previous data showing only depletion of mGluR5 binding activity following 7 days exposure to Poly I:C but not 24 hours exposure, subsequent co-exposure experiments focused on 7 days Poly I:C exposure.

3.5.2.1 LDH toxicity assay

Results indicated LDH levels increased significantly in culture media of astrocytes exposed to Poly I:C (OD=0.573, $p=0.035$), Poly I:C with DHPG (OD=0.578, $p=0.013$), and Poly I:C with VU (OD=0.576, $p=0.020$) for 7 days when compared to non-exposed controls (OD=0.546). Culture media from cells concurrently exposed to Poly I:C and MTEP (OD=0.548, $p>0.05$) showed no LDH toxicity compared to controls. Cells with concurrent mGluR5 drugs and Poly I:C exposure showed no difference in LDH toxicity when compared to cells exposed to Poly I:C only. (**Figure 3.9**).

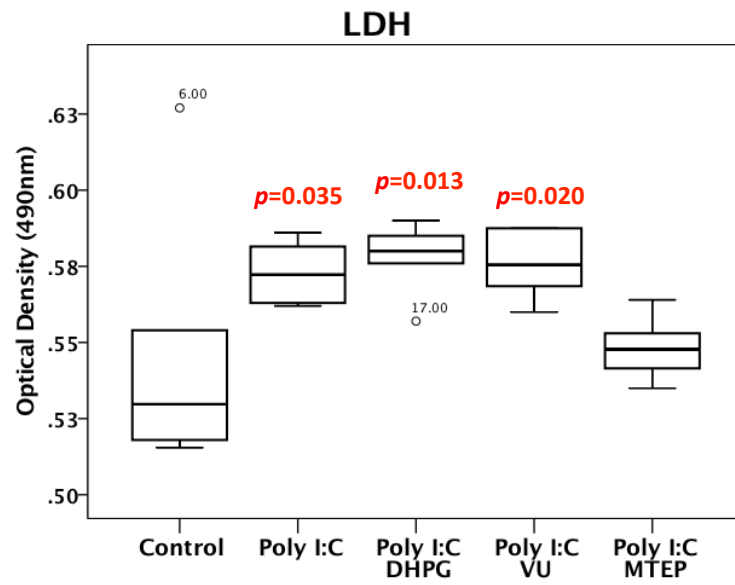


Figure 3.9 | LDH toxicity assay for Poly I:C and drugs exposure

LDH toxicity assay showing increased LDH toxicity in culture media of cells exposed to Poly I:C alone, Poly I:C with DHPG and Poly I:C with VU, but not in Poly I:C with MTEP. Error bars represent SEM.

3.5.2.2 ELISA for pro-inflammatory markers

IL-6

There was no difference between levels of IL-6 within culture media of cells exposed for 7 days to Poly I:C (OD=0.054, $p>0.05$), Poly I:C with DHPG (OD=0.0568, $p>0.05$), Poly I:C with VU (OD=0.050, $p>0.05$), Poly I:C with MTEP (OD=0.0618, $p>0.05$) when compared to controls (OD=0.0547). When compared to cells exposed to Poly I:C alone, cells with concurrent exposure to DHPG, VU and MTEP also showed no significant difference (**Figure 3.10**).

Rantes

Rantes was increased in culture media of cells exposed for 7 days to Poly I:C (OD=2.134, $p=4.2\times 10^{-11}$), Poly I:C with DHPG (OD=2.117, $p=4.5\times 10^{-11}$), Poly I:C with VU (OD=2.334, $p=1.7\times 10^{-11}$), Poly I:C with MTEP (OD=2.239, $p=2.6\times 10^{-11}$) when compared to controls (OD=0.0572). Cells exposed to Poly I:C and VU ($p=0.016$) had significantly higher level of Rantes when compared to Poly I:C only, while concurrent DHPG and MTEP exposure were not significantly different to Poly I:C only exposure (**Figure 3.10**).

3.5.2.3 mGluR5 radioligand binding assay

The mGluR5 binding assay revealed a trend decrease in mGluR5 binding at 7 days when exposed to Poly I:C ($B_{\max}=49.75$) and Poly I:C with MTEP ($B_{\max}=39.07$). Reduced mGluR5 binding was observed with concurrent exposure of Poly I:C with DHPG ($B_{\max}=27.38$) and Poly I:C with VU ($B_{\max}=1.713$) when compared to controls ($B_{\max}=49.75$) (**Figure 3.11**).

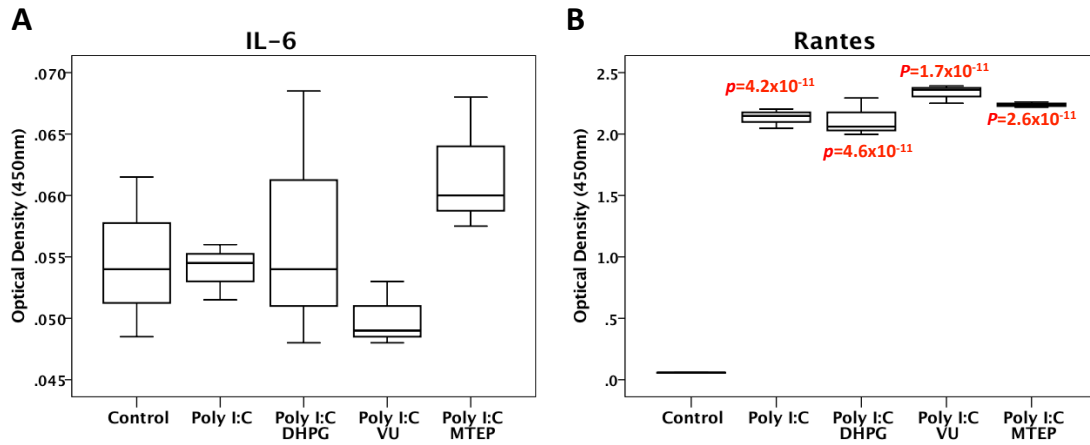


Figure 3.10 | ELISA of IL-6 and Rantes for Poly I:C exposure and drugs exposure

ELISA to assess concentration of pro-inflammatory markers in culture media of cells exposed for 7 days, data analysed using univariate analysis of variance with LSD multiple comparison. **(A)** Rantes increased significantly in culture media of cells exposed to Poly I:C, Poly I:C with DHPG, Poly I:C with VU, and Poly I:C with MTEP when compared to controls. **(B)** IL-6 was not significantly different between culture media of cells exposed to Poly I:C, Poly I:C with DHPG, Poly I:C with VU, and Poly I:C with MTEP compared to controls. Error bars represent SEM.

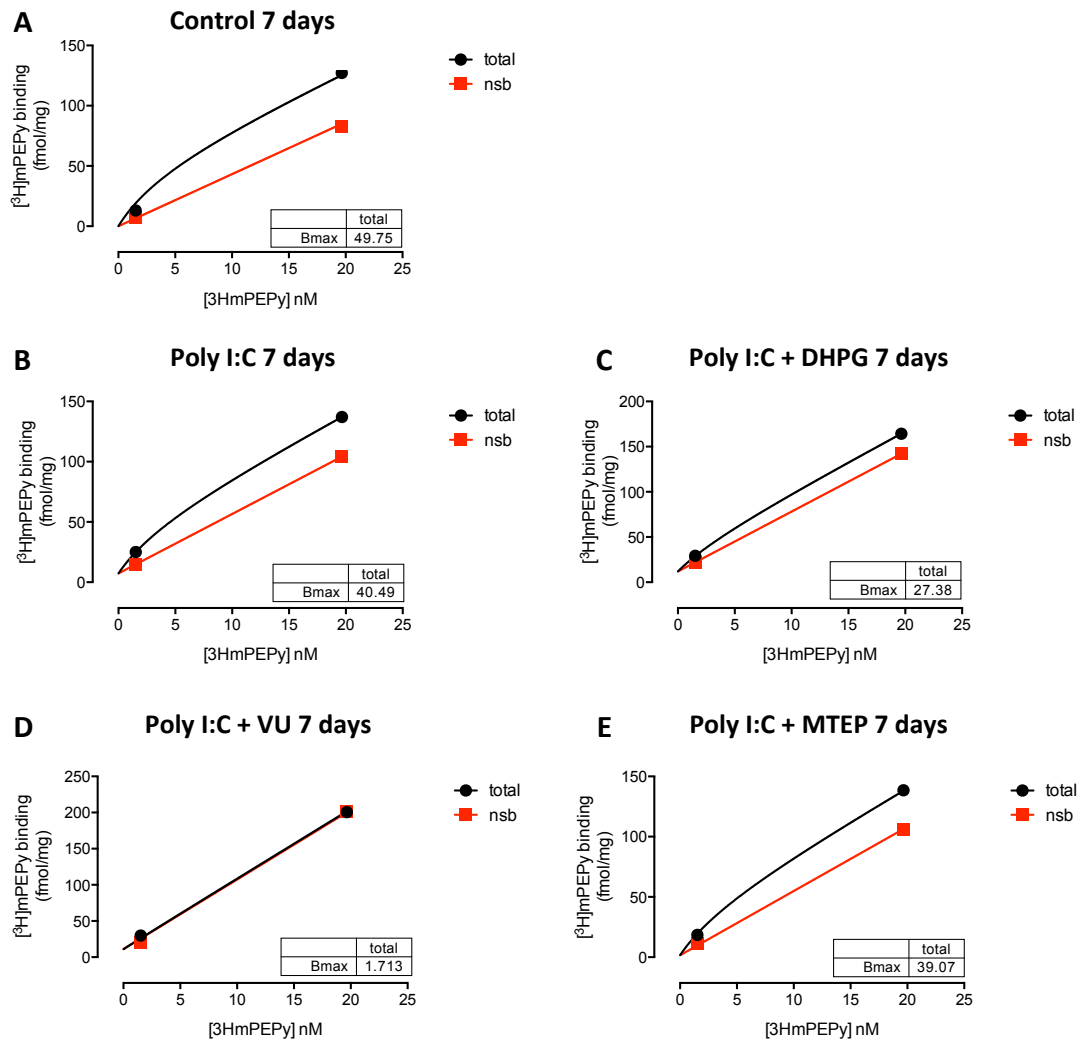


Figure 3.11 | mGluR5 [3H]mPEPy radioligand binding assay for Poly I:C and drugs exposure

mGluR5 [3H]mPEPy radioligand binding assay in primary human foetal astrocytes at 7 days timepoint, (A) control, (B) Poly I:C (C) Poly I:C with DHPG, (D) Poly I:C with VU (E) Poly I:C with MTEP (Data and images generated by Dr. Karen Gregory, MIPS).

3.6 Discussion

This study showed a reduction of mGluR5 gene expression and binding activity following astrocyte activation induced by the TLR3 ligand Poly I:C. Activation was characterised by elevation of IL-6 and Rantes in the culture media, however activation status of TLR3 was not assessed directly. In addition, decreased gene expression of astrocyte structural markers including GFAP, S100 β and ALDH1L1 were also observed together with a disruption in other elements of the glutamatergic pathway, including upregulation of SHANK3 and GRIA3. Whereas glutamate transporter EAAT2 was downregulated, accompanied by upregulation of EAAT1 and VGLUT1. Our preliminary mGluR5 modulation experiments showed that only Rantes increased in cultures but not IL-6, following Poly I:C exposure. The concurrent exposure of VU with Poly I:C lead to an increase in Rantes compared to Poly I:C alone, and no differences for levels of LDH and IL-6. There was also no significant difference between concurrent MTEP or DHPG for levels of LDH, IL-6 and Rantes. mGluR5 binding activity was reduced in cells exposed concurrently to DHPG and VU compared to Poly I:C only, while no difference was observed for cells exposed to MTEP. This was accompanied by significantly diminished overall mGluR5 binding signal across all conditions when compared to previous experiments.

Initially, we considered the utilisation of LPS and Poly I:C, which activates TLR4 and TLR3 respectively, to induce reactivity in astrocytes. However, Holm and colleagues reported that compared to microglia, astrocytes express very low levels of TLR4 with low binding activity to LPS, and that TLR4 driven astrocytic activation relies predominantly on the presence of microglia and its secreted factors (Holm, Draeby, & Owens, 2012). Based on this and our aim to investigate neuroinflammatory mechanisms specific to astrocyte mGluR5, we decided to focused on utilising Poly I:C to induce astrocyte activation. In their activated states, both

astrocytes and microglia release pro-inflammatory cytokines, including TNF α , IL-1 β , IL-6, IL-8 IFN γ , and MCP-1, some of which have also been found to be elevated in the brain, plasma and serum of individuals with autism (Li et al., 2009; Masi & Quintana, 2015). Within our study, astrocytes exposed to Poly I:C contained elevated levels of IL-6 and Rantes compared to controls, in the absence in LDH toxicity, indicating astrocyte activation without eliciting toxicity. However, a high variability of LDH toxicity is observed in culture exposed to Poly I:C for 7 days, this could potentially contribute to the lack of significant differences when compared to control despite the higher level of LDH. Moreover, it is important for future studies to clarify if this increase level of LDH is direct effect of Poly I:C or a feedback from elevated levels of cytokines such as IL-6 and Rantes.

Our findings showed reduced mRNA expression of astrocyte structural markers GFAP, S100 β and ALDH1L1 following exposure to Poly I:C. These changes are counterintuitive to what we expected, given that an increased expression of structural markers should generally be seen with hypertrophic morphology of activated astrocytes. These results could have been a consequence of mRNA levels not always reflecting protein levels, and could also potentially indicate an active process of mRNA to protein translation of these structural elements. Conversely, reduced GFAP, S100 β and ALDH1L1 may also represent a decrease in structural astrocytic proteins, which could be indicative of a reduced astrocyte health, as well as reduced capacity to carry out their normal functioning. Therefore, future work should investigate the protein levels of these structural markers in order to elucidate the potential mRNA versus protein discrepancies, which would also aid in furthering understanding of the potential mechanisms involved in astrocyte activation and related morphological changes.

Our data shows reduced mGluR5 signalling and reduced mRNA expression of GRM5 following astrocyte activation, indicating a potential link between astrocyte activation and glutamatergic signalling. This is also in line with evidence showing astrocyte activation and mGluR5 dysregulation within the autism brain, and is suggestive that dysregulation of mGluR5 signalling observed in autism may not be limited to neurons, but also present in astrocytes (Chana et al., 2015; Edmonson et al., 2014; Fatemi et al., 2011). Furthermore, both astrocyte activation and glutamatergic disruption may constitute aetiological events that contribute to neuroinflammation and neuropathology within the autism brain. In addition, we also observed upregulation of other glutamatergic signalling elements, including SHANK3 and GRIA3. These could potentially be a mechanism to compensate for reduced mGluR5 signalling. Interestingly, a number of SHANK3 mutations, as well as the dysregulation of SHANK3 expression have been shown to contribute to the severity of cognitive deficits, and are found to exist in ~1% of patients with autism (Leblond et al., 2014). Together with PSD95, SAP90/PSD95 associated protein (SAPAP) and Homer, SHANK3 plays a key role in the formation and regulation of a functional link between NMDARs and mGluRs at glutamatergic synapses (Sheng & Kim, 2000). A myriad of animal models with SHANK3 mutations and deletions also present with autism like behaviours, including excessive/repetitive self-grooming and impaired social behaviours (Monteiro & Feng, 2017). Although contradictory to decreased expression or complete deletion of SHANK3 observed in autism, our data nevertheless provide an association between disruption of SHANK3 and astrocytic activation associated neuroinflammation. Furthermore, the reduced expression of SHANK3 in the autism brain could be restricted to neurons, or could be driven by a mechanism that requires the presence of other brain cell types such as microglia or oligodendrocytes, which are not present in our cultures as shown by immunostaining. These findings also revealed that neuroinflammation related glutamatergic disruption in neurons seen within the autism brain can also be present in astrocytes, particularly dysregulation of

mGluR5 and SHANK3. Our findings emphasise the need for further work to take into consideration the active involvement of astrocytes in glutamatergic signalling, and their potential contribution to neuroinflammation related pathophysiology pathways in autism.

The mRNA of astrocytic glutamate transporter, EAAT2, responsible for the majority of glutamate clearing was found to be significantly downregulated following Poly I:C induced astrocyte activation, likely indicative of a reduced extracellular glutamate clearing capacity. Together with the observed upregulation of EAAT1, this could suggest a shift in glutamate clearing responsibility from EAAT2 to EAAT1 as a neuroprotective mechanism, an observation that is also reported in traumatic brain injury induced astrocyte activation where EAAT1 was shown to be increased by immunohistochemistry, especially around the site of lesion (Beschoner et al., 2007). Therefore, the potential neuroprotective effect of EAAT1 should be taken into consideration in the future in order to ascertain whether modulation of EAAT1 or EAAT2 could potentially alleviate astrocyte activation and correct any glutamatergic imbalance that could be associated with neuroinflammatory response and toxicity.

Contradictory to our results, a similar study of acute (24 hours) Poly I:C exposure in rat primary astrocytes revealed a reduction in mRNA expression of EAAT1 and glutamate reuptake (Scumpia et al., 2005). The authors attributed the downregulation of EAAT1 glutamate transport to $\text{TNF}\alpha$, which has been shown to suppress NF κ B driven promoter binding and mRNA synthesis of EAAT1 (Wang et al., 2003). It is important to note that human and rodent astrocytes may have distinct activation mechanisms, with Poly I:C being shown to induce both NF κ B-dependent cytokines ($\text{TNF}\alpha$, inducible nitric oxide synthase (iNOS), IL1 β , IL-6) and interferon regulatory transcription factor (IRF)3-dependent cytokines

(IFN β , CXCL10, Rantes, IL-27) in mouse astrocytes but in human astrocyte activation is mainly driven by IRF3-dependent cytokines (Tarassishin et al., 2014). Within our study, astrocyte activation was characterised mainly by increased release of IL-6 and Rantes, while TNF α was not found elevated for both acute and chronic Poly I:C exposure. This could potentially explain the contradictory results of our EAAT1 upregulation compared to downregulation reported by Scumpia et al. (Scumpia et al., 2005).

Apart from neurons, astrocytes have also been shown to be capable of glutamate release via VGLUT1, triggered by mGluRs activated Ca²⁺ release (Bezzi et al., 2004). Our results showed downregulation of VGLUT1 mRNA following astrocyte activation, potentially another neuroprotective mechanism to compensate for the reduced glutamate clearing due to downregulation of the major glutamate transporter EAAT2. With reduced expression of VGLUT1, the vesicular release of gliotransmitters, especially glutamate could be limited, in order to regulate synaptic glutamate levels and minimise excitotoxicity. Taken together, dysregulation of glutamatergic signalling components including EAAT2, EAAT1 and VGLUT1 may represent downstream effects of reduced mGluR5 activity and mRNA expression, and modulating mGluR5 activity could potentially correct these disruptions.

Neuroinflammation has been reported across different brain regions in autism including the FC, ACC and cerebellum, as well as in CSF, plasma and serum (Li et al., 2009; Masi & Quintana, 2015; Vargas et al., 2005). These neuroinflammatory changes are driven in part by astrocyte and microglia reactivity, via TLR signalling. Microglia have both pro- and anti-inflammatory roles, including the M1 state where they attack infectious or foreign cells by engulfing and phagocytosing them, as well as releasing pro-inflammatory cytokines. Following the initial M1 activation, microglia then enter the M2 activation state to perform

their anti-inflammatory roles, including removal of cellular debris and repair in order to restore homeostasis (Kern, Geier, Sykes, & Geier, 2015; Tang & Le, 2016). Developmentally, microglia are also responsible for synaptic pruning, while microglial abnormalities could potentially lead to over/under connectivity of the brain as well as imbalance of E/I signalling as seen in autism brain (Juan I. Rodriguez & Janet K. Kern, 2011). Reactive astrocytes also contribute to the neuroinflammation process, mainly via the release of pro-inflammatory cytokines and recruitment of microglia, as well as potentially the disruption in their physiological functioning, including disruption to glutamatergic signalling. However, certain astrocyte activation mechanisms are partly facilitated by microglial-secreted factors, especially TLR4 mediated astrocyte activation that only occurs in the presence of microglia (Holm et al., 2012; Kern et al., 2015). While activation and inflammatory responses were observed in our astrocyte cultures exposed to Poly I:C (TLR3 ligand), it would be interesting for future studies to investigate this effect in the presence of microglia conditioned media to ascertain if microglia can facilitate TLR3 driven astrocyte reactivity and inflammation. Furthermore, a direct measure of TLR3 activity would add more strength to the fact that cytokine release was related to TLR3 activation.

When concurrently exposed to the mGluR5 agonist (DHPG), PAM (VU) and NAM (MTEP) or vehicle, we found that concentration of IL-6 within the media was not different to controls, while Rantes remained elevated in all Poly I:C treated groups. An increased level of LDH toxicity was also observed. This could potentially be due to the effect of DMSO exposure, which can be toxic to cells, or as a result of the downstream effect of mGluR5 modulation itself. Nevertheless, the specific cause of LDH toxicity observed here should be further investigated in future studies. Unexpectedly, mGluR5 binding activity across all conditions, including controls/vehicles also showed much weaker signal compared to previous

experiments. While the lack of IL-6 elevation in our work could be explained by studies that suggest potential anti-inflammatory properties of DMSO in suppressing IL-6 production, this is yet to be confirmed in the CNS and/or in astrocytes (Elisia et al., 2016; Hollebeeck et al., 2011). However, other studies have reported that DMSO at concentration as low as 0.5% could contribute to mitochondrial damage, disruption of glutamatergic functions, as well as astrocyte toxicity and reduced cell viability (Yuan et al., 2014; Zhang et al., 2017). Although DMSO is commonly used as a vehicle, especially for non water-soluble compounds, further work is required to elucidate the exact effect of DMSO on astrocyte health and glutamatergic functions. Therefore, it is important to take into consideration that DMSO may be confounding some effects related to Poly I:C and mGluR5 compounds in the current study.

The data presented within this study showed the potential link between TLR3 astrocyte activation and disturbance to the glutamatergic system, especially mGluR5. However, these experiments are yet to be repeated so that these preliminary results can be validated. In particular, future studies should reconsider the experiments involving mGluR5 modulators, whereby the effect of DMSO needs to be taken into consideration, and the activation status of astrocytes to be better confirmed by measuring the activity of TLR3 directly. To better understand the effect of astrocyte disturbance on the neuronal pathophysiology within the autism brains, co-culture of astrocytes exposed to different conditions here, with neuronal cultures should be considered in future studies.

Our findings demonstrate that during TLR3 driven astrocytic activation, mGluR5 signalling and mRNA expression are reduced. This is accompanied by a reduction in EAAT2 expression that is suggestive of a reduced capability to clear extracellular glutamate. We also observed

upregulation of EAAT1 and downregulation of VGLUT1, potentially a mechanism by which accumulation of extracellular glutamate and associated excitotoxicity can be minimised. Our findings point to mGluR5 signalling being important for regulating astrocyte activation and maybe relevant to the aetiology of autism, where decreased expression of mGluR5 and increased astrocyte activation have been previously reported. The results also support a role of mGluR5 in regulating astrocyte activation and maybe important in the context of reducing astrocyte activation for therapeutic benefit in autism and other neurological disorders.

CHAPTER 4 A comparative investigation of human pluripotent stem cell-derived astrocytes in relation to primary human foetal and adult astrocytes

4.1 Background

Within the CNS, astrocytes can be found throughout all brain regions (Ge & Jia, 2016; Gonzalez-Perez, Lopez-Virgen, & Quinones-Hinojosa, 2015). Astrocytes express different levels of cell type specific markers at different maturation stage, including GFAP, S100 β , EAAT1 and EAAT2, GS, as well as the recently discovered marker ALDH1L1. These cells generally have non-overlapping cellular domains and can be categorized into two main subtypes: protoplasmic astrocytes reside within the cortical grey matter and have main processes initializing from the cell soma, then branching into fine processes; fibrous astrocytes are located within the cortical white matter and their morphology is characterized by many long fine processes projecting directly from the cell soma (Khakh & Sofroniew, 2015; Molofsky & Deneen, 2015). Astrocyte processes form end feet that ensheath blood vessels and provide metabolic support to endothelial cells, thus helping to maintain tight junctions that ensure an intact blood brain barrier (BBB). Astrocytes carry out important roles and functions during development of the CNS, including promoting synapse formation and regulating neurotransmission (Gonzalez-Perez et al., 2015). Astrocytes are also responsible for the reuptake and recycling of released neurotransmitters from the synaptic cleft, including the major excitatory neurotransmitter glutamate, thereby maintaining synaptic plasticity and intracellular homeostasis (Benjamin Kacerovsky & Murai, 2016; Gonzalez-Perez et al., 2015; Verkhratsky et al., 2015). Under the circumstances of CNS injury or infection, astrocytes become activated and stimulate the innate immune system

and neuroinflammation via secretion of cytokines and chemokines (Gonzalez-Perez et al., 2015; Verkhatsky et al., 2015).

Developments in stem cell technology have enabled the generation of human embryonic stem cells (hESC) and induced pluripotent stem cells (hiPSC) from human embryos and adult human somatic cells (such as fibroblasts) respectively, as well as directing their differentiation into a variety of brain cell types, thus providing *in-vitro* models to investigate brain disorders without the need for invasive biopsy collection (Takahashi et al., 2007). However, the phenotypic and functional characterization of these cells and comparison with native brain derived neurons and glia is warranted in order to give greater validity and translation to their use.

Krencik and colleagues were one of the first groups to successfully differentiate astrocytes from hESC and hiPSC with a high efficiency (Krencik, Weick, Liu, Zhang, & Zhang, 2011; Krencik & Zhang, 2011). Their method requires 120-180 days *in-vitro* (DIV) before expression of the mature astrocytic marker, GFAP, was observed. Other studies have since developed protocols with improved differentiation efficiency, to generate mature astrocytes (Chandrasekaran, Avci, Leist, Kobolak, & Dinnyes, 2016; Juopperi et al., 2012; Lafaille et al., 2012; Mormone, D'Sousa, Alexeeva, Bederson, & Germano, 2014; Roybon et al., 2013; Serio et al., 2013; Shaltouki, Peng, Liu, Rao, & Zeng, 2013; Sloan et al., 2017). Notably, the majority of published protocols derived astrocytes via the embryoid bodies (EB) neural induction method, while Roybon et al. (2013) was the only group to successfully differentiate astrocytes via a monolayer neural induction, achieving 100% of cells expressing the astrocyte developmental marker S100 β and 70% expressing the mature astrocyte marker GFAP from 90 DIV. Amongst the highest efficiency protocols to date, human astrocytes were

generated from NPCs within 28-35 days, with majority of cells expressing GFAP, S100 β , EAAT1, and GS (Jones, Atkinson-Dell, Verkhatsky, & Mohamet, 2017; Mormone et al., 2014; Shaltouki et al., 2013; Tcw et al., 2017). This was all achieved via the EB neural induction method, with defined media at the final differentiation stage to enrich for astrocytes. Some studies further utilized fluorescent-activated cell sorting to select for glial progenitors and astrocytes using the respective cell surface markers, thereby filtering out any contaminating cell types that are present during the differentiation process. Our initial screening approach was similar to a recently published study, where a selection of defined media were chosen based on previously published protocols, and tested on their ability to generate astrocytes from NPCs (Tcw et al., 2017). TCW et al. conducted a highly comprehensive investigation including screening 11 differentiation media, and a series of phenotypic characterization and comparison against primary human foetal astrocytes by examining mRNA expression, protein expression, neuroinflammatory response, phagocytic capacity, and calcium activity. Furthermore, Sloan et al. compared the transcript levels astrocyte markers within their 3D human cortical spheroid (hCS) derived astrocytes to human primary foetal and adult astrocytes via RNA-seq and qpCR (Sloan et al., 2017). The hCSs derived astrocytes were shown to have glutamate uptake and phagocytosis activities, as well as promoting neuronal calcium signalling (Sloan et al., 2017). Our study builds and extends on this investigation by comparing our monolayer NPC derived astrocytes against both human primary foetal astrocytes and adult astrocytes. Given it is known that morphology and expression of astrocytes are altered between foetal, juvenile and adult human brain tissue such a comparison is warranted (Zhang et al., 2016). In addition, for this investigation we also assessed the ability to maintain our NPC derived astrocytes in long-term culture, including stability of mature astrocyte marker expression.

Here we describe an improved, simplified, and efficient protocol for deriving mature astrocytes via a monolayer neural induction method within 42 days, from hESCs or hiPSCs (Denham & Dottori, 2011). Upon trialing various defined media for the final differentiation stage, we found that media used for culturing human primary foetal astrocytes are optimal for deriving astrocytes from both hESC and hiPSC glial progenitors. These cells visually represented morphology consistent with astrocytes and were shown to express mature astrocyte structural and functional markers, including GFAP, S100 β , EAAT1, EAAT2 and ALDH1L1. This study presents a direct comparison of hESC and hiPSC derived astrocytes with both primary human foetal and adult astrocytes for key astrocytic markers at the gene expression level. In addition, we provide a comparison of activation markers between our stem cell derived astrocytes and human foetal astrocytes. In doing so, this investigation provides important comparative information that moves the field one step closer to being able to derive astrocytes from stem cells that can truly re-capitulate mature astrocytic expression markers as well as functionality. This work has important implications for providing better translational models for neurological disorders where astrocytes are thought to play causative roles.

4.2 Aim

This study aimed to develop an efficient protocol to differentiate astrocytes from hPSC, as well as to characterise these astrocytes as compared to primary human foetal and adult astrocytes.

4.3 Hypotheses

We hypothesise that our hPSC-derived astrocytes resembles primary human foetal astrocytes morphologically, and express astrocyte markers. We further hypothesise that these hPSC derived astrocytes can be activated with Poly I:C and display similar activation profile to primary human foetal astrocytes.

4.4 Materials and methods

4.4.1 Human hESCs and hiPSCs maintenance

Cell lines hESC H9 (WA-09, WiCell) and hiPSC CERA007 were maintained in TeSR E8 complete media (StemCell Technologies, USA) in a feeder-free condition, on vitronectin (StemCell Technologies, USA) coated culture surface. The cells were passaged using ethylenediaminetetraacetic acid (EDTA) [0.5 μ M] twice weekly. CERA007 hiPSCs were generated using skin fibroblasts from a 36 year old healthy male by the episomal method as described previously (Hernandez et al., 2016). These studies complied with approvals from University of Melbourne Human Ethics Committee (#154384 and #154394).

4.4.2 Astrocyte differentiation

The hESC and hiPSC derived from healthy controls were differentiated into astrocytes based on neural induction protocol as described by Denham and Dottori (Denham & Dottori, 2011) with some modifications. Briefly, cells were plated onto a Poly-D-Lysine, Laminin (Life Technologies, USA) coated organ culture dish in neural induction media supplemented with LDN193189 (Sigma Aldrich, USA) [100nM] and SB431542 (Sigma Aldrich, USA) [10 μ M] for 7 days, then with fibroblast growth factor 2 (FGF2) [20ng/ml] (Lonza, USA) for an additional 7 days. Following neural induction, NPCs were plated onto Poly-D-Lysine, Fibronectin (Life Technologies, USA) coated dish, cultured in neurobasal medium with FGF2, EGF, and platelet-derived growth factor (PDGF)-AA [20ng/ml] (Lonza, USA) for 7 days, followed 7 days in various defined medium to promote final astrocyte differentiation, including (a) neurobasal medium, (b) neurobasal medium with FGF2, EGF, and PDGF-AA [20ng/ml] (Denham & Dottori, 2011), (c) DMEM/F-12 with astrocytes growth supplement (AGS) (Science Cell Research Laboratories, USA) (Juopperi et al., 2012), or (d) DMEM/F-12 with insulin growth factor (Shenandoah Biotechnology Inc, USA) [200ng/ml], activin-A (Miltenyl

Biotec, DEU)[10ng/ml], and heregulin β (Lonza, USA) [10ng/ml] (Shaltouki et al., 2013) (**Figure 4.1**). A chemical dissociation method (0.5mM EDTA) was also trialed instead of mechanical harvesting for collecting and re-plating cells during neural induction and differentiation stages in order to increase yield.

4.4.3 Primary human foetal and adult astrocytes

Primary human foetal astrocytes lines were established from brain tissue kindly donated by consenting patients undergoing pregnancy terminations, between gestational ages of 14-16 weeks, at Dr Marie Maroondah Reproductive Health Clinic, Victoria, Australia. Primary human adult astrocyte line was established from brain tissue donated by consenting patient undergoing temporal lobectomy as treatment for temporal lobe epilepsy at Royal Melbourne Hospital. Ethics approval was granted by the University of Melbourne (ID: 1135373) covering tissue collection for research use. Establishment of astrocytes was carried out as previously described (Trillo-Pazos et al., 2000). Briefly, tissue was mechanically disaggregated to single cell suspension, and seeded in T175 culture flask at 3×10^5 cells/ml in DMEM/F-12 containing FBS [10%], L-glutamine [1mM] and gentamicin sulphate [20 μ g/ml] (all reagents from Life Technologies, USA). Media change was performed weekly, and culture was passaged at least twice prior to experiments to remove any contaminating neurons.

4.4.4 Gene expression analysis

Total RNA was extracted using Purelink RNA Minikit (Life Technologies, USA) as per manufacturer's instruction. RNA quality was analyzed using TapeStation (Agilent Genomics, USA) before being converted to cDNA using SuperScript IV Reverse Transcriptase (Life Technologies, USA) according to manufacturer's instruction. All RNA samples used in this

study has RIN between 8.6 to 9.7. Quantitative real-time PCR was performed using Taqman gene expression mastermix and Taqman gene expression assays (Life Technologies, USA) including octamer-binding transcription factor 4 (*OCT4*) (Hs04260367_gH), *SOX2* (Hs01053049_s1), *PAX6* (Hs00240871_m1), *S100 β* (Hs00902901_m1), *GFAP* (Hs00909233_m1), *ALDH1L1* (Hs01003842_m1), *EAAT1* (Hs00904823_g1), *EAAT2* (Hs01102423_m1), *TUBB3* (Hs00801390_s1) as well as reference genes *GAPDH* (Hs02758991_g1), *PPIA* (Hs04194521_s1) and *HMBS* (Hs00609297_mq). qRT-PCR was performed with 20ng of cDNA template per reaction. Statistical analysis of gene expression levels in different samples was performed using student t-test in IBM SPSS Statistics (IBM, USA).

4.4.5 Immunocytochemistry

Cells were cultured on coverslips as per above differentiation protocol, fixed with 4% paraformaldehyde (PFA) (Sigma Aldrich, USA) for 20mins and blocked in 0.1% Triton-X PBS with 3% NGS for 1 hour at room temperature. Subsequently, samples were incubated with primary antibodies anti-GFAP [1:1000] (Dako, USA), anti-S100 β [1:500] (Sigma Aldrich, USA), anti-EAAT1 [1:250] (Abcam, USA), or anti-Beta III Tubulin [1:1000] (Millipore, USA) in 0.1% Triton-X PBS with 1.5% NGS overnight at 4°C, followed by secondary antibodies Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 594 goat anti-mouse [1:1000] (Life Technologies, USA) in 0.1% Triton-X PBS with 1.5% NGS for 1 hour at room temperature. These samples were then counterstained with DAPI [1 μ g/ml] (Sigma, USA) and mounted with ProLong Gold Antifade Mountant (Life Technologies, USA).

4.4.6 Flow cytometry analysis

6 weeks differentiated astrocytes from hESC and hiPSC were fixed with ice cold 2% PFA for 10 minutes, washed in cold PBS, then blocked with 0.1% Tween PBS with 3% NGS for 1 hour

at room temperature, followed by incubation with anti-GFAP [1:200] or anti-S100 β [1:100] in 0.1% Tween PBS with 1.5% NGS for 1 hour at room temperature. Following incubation with secondary Alexa Fluor 488 goat anti-rabbit or Alexa Fluor 488 goat anti-mouse [1:1000] in 0.1% Tween PBS with 1.5% NGS in the dark, cells were resuspended in cold PBS with 3% NGS and 3% FBS and analyzed using BD FACSVerse (BD Biosciences, USA).

4.4.7 Enzyme-linked immunosorbent assay (ELISA)

6 weeks differentiated astrocytes from hESC and hiPSC were exposed to Poly I:C [100ng/ml] or vehicle for 7 days. The culture media were then harvested and analyzed for pro-inflammatory markers IL-6 and Rantes as per manufacturer's instruction (ELISAKit.com, AUS). Standard curve dilution was used to determine the concentration of IL-6 ($r^2=0.996$) and Rantes ($r^2=0.999$) in our samples, and samples absorbance were analyzed at 450nm using SpectraMax M3 plate reader (Molecular Devices, USA).

4.4.8 Statistical analysis

The mRNA expressions were compared using a 2-way ANOVA with Dunnett's or Bonferonni's multiple comparisons test. For ELISA, the levels of IL-6 and Rantes between Poly I:C exposed cells and controls were compared using t-tests. All statistical tests were performed using Graphpad Prism 7 (GraphPad Software, USA).

4.5 Results

4.5.1 Defined media to promote expression of astrocyte markers

hESC and hiPSC were initially induced to neural progenitors using small molecule inhibitors of SMAD signaling, SB431542 and LDN193189 in neural induction media for 7 days. This was followed by expansion of progenitors, using FGF treatment in neural induction media for a further 7 days. Neural progenitors were mechanically or chemically harvested, and cultured on a fibronectin coated surface, with FGF, EGF and PDGF-AA in neurobasal media to promote differentiation towards glial progenitors. Further differentiation of glial progenitors to mature glia was assessed using four different media conditions including (a) neurobasal medium (NBM), (b) neurobasal medium with FGF, EGF, and PDGF-AA (NBM-FEP), (c) DMEM/F-12 with astrocytes growth supplement (DMEM-AGS), or (d) DMEM/F-12 with IGF, heregulin β , and activin-A (DMEM-IHA) (**Figure 4.1**).

RNA from 4 week differentiated astrocytes derived from H9 cells were harvested for qPCR. All RNA samples used in this study have RNA Integrity number (RIN) between 8.6 to 9.7, measured using TapeStation 2200 (Agilent Genomics, USA). Amongst the four different differentiation media compared, gene expression profiling revealed that DMEM-AGS and NBM-FEP produce cells expressing all astrocyte markers assayed, including *S100 β* , *ALDH1L1*, *EAAT1*, and *EAAT2*, except *GFAP* where the levels were not consistently detected in all replicates (**Figure 4.1**). Given the significantly higher expression of early astrocyte specific marker *S100 β* in DMEM-AGS and NBM-FEP, the 2 defined media were further considered. DMEM-AGS contained slightly higher expression levels of *EAAT1* and *EAAT2* compared to NBM-FEP (ns). Given its simplicity, common usage in the culture of primary astrocytes and our gene expression results, DMEM-AGS was selected as the astrocyte enriching media for ongoing culturing and further characterization of astrocyte differentiation.

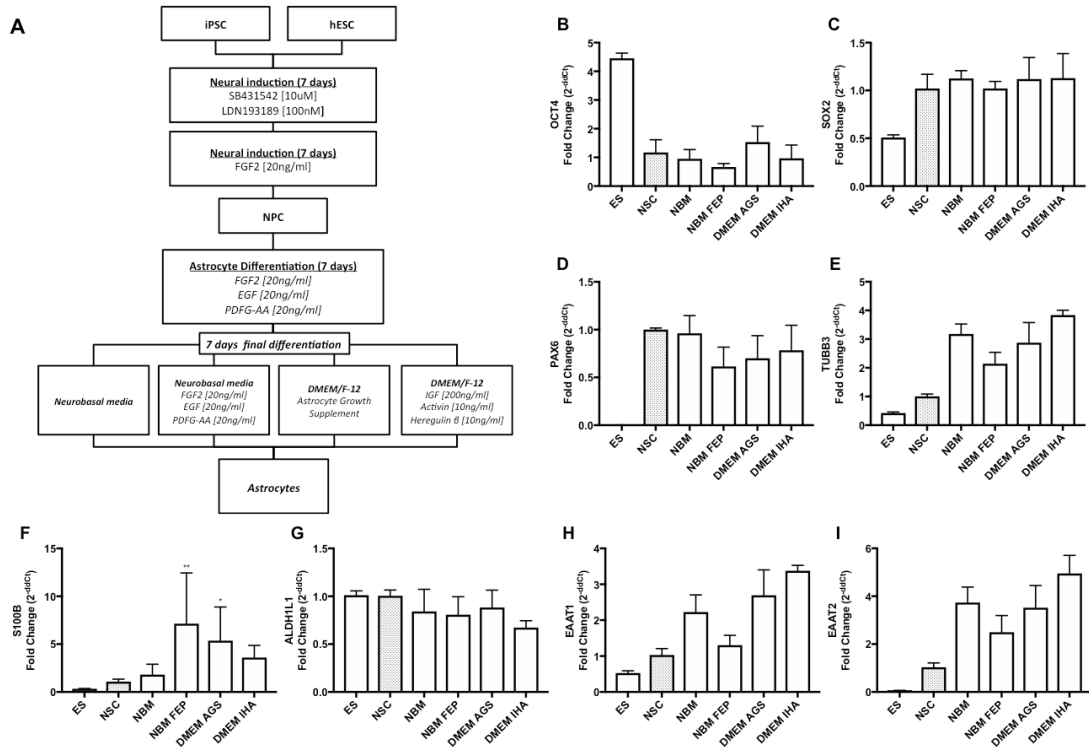


Figure 4.1 | Differentiation protocols and gene expression profile comparison of hPSC derived astrocytes using different defined media

(A) Astrocyte differentiation of hESC and iPSC. The cells were exposed to LDN193189 [100nM] and SB431542 [10μM] for 7 days, then FGF2 [20ng/ml] for 7 days on laminin coated dish. Neural progenitor cells were then harvested and plated on fibronectin coated dish and cultured with FGF2, EGF, and PDGF-AA [20ng/ml] for 7 days, followed by various defined media for 7 days to further promote differentiation into astrocytes. **(B-I)** Gene expression profiles of H9 (1) ESC, (2) NSC, and astrocytes differentiated from various defined media conditions including (3) NBM alone, (4) NBM with FGF2, EGF, and PDGF-AA (NBM FEP), (5) DMEM/F-12 with astrocytes growth supplement (DMEM AGS), as well as (6) DMEM/F-12 with IGF-1, Heregulin-B, and Activin-A (DMEM IHA). (* $p \leq 0.05$, ** $p \leq 0.01$)

4.5.2 Chemical dissociation promotes astrocyte morphology

To improve the efficiency and yield of the differentiation, we tested the protocol with chemical dissociation compared to mechanical dissociation to allow for a larger scale culture. Cells were incubated in EDTA [0.5 μ M] for 2 minutes, to dissociate off the culture surface for transfer and replating between hESC and hiPSC maintenance through to neural induction, as well as from NPCs to final astrocyte differentiation. qPCR data demonstrates that both mechanical and chemical dissociation using EDTA yield cells population with similar levels of astrocyte markers expression, suggesting that EDTA dissociation method is suitable for this differentiation protocol (**Figure 4.2**). Importantly, chemical dissociation with EDTA appeared to yield a more homogenous single cell population at the final differentiation stage, as opposed to a mixture of neurosphere-like colonies and single cells with astrocyte morphology around the periphery of these colonies when mechanical dissociation was used (**Figure 4.2**). We determined that chemical dissociation is the preferred method for differentiation as it promotes homogenous single cells population.

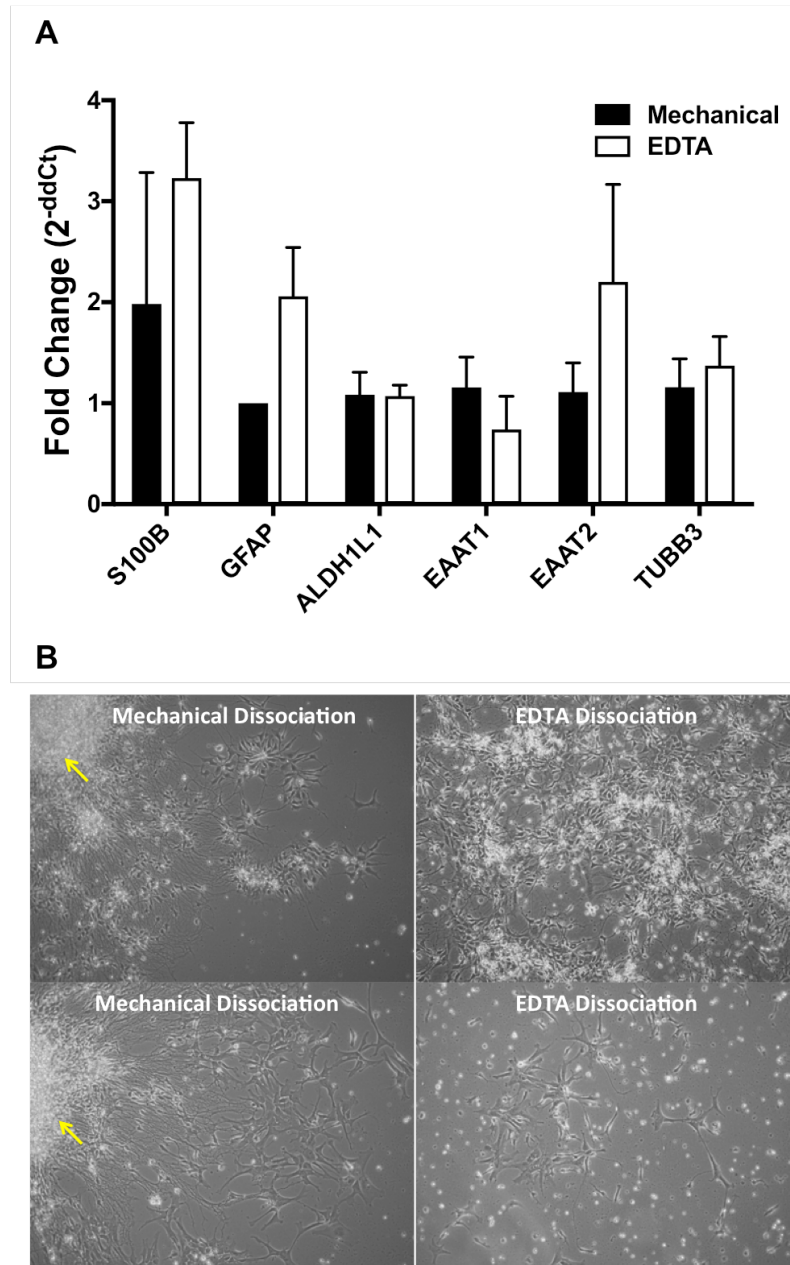


Figure 4.2 | Gene expression profile and morphological comparison of astrocytes derived using mechanical and chemical dissociation protocol.

(A) qRT-PCR results show that mechanically differentiated and enzymatically differentiated astrocytes have comparable gene expression profile. **(B)** When mechanically passage (left), cells form neurosphere-like colonies (yellow arrow) with small amount of single cells around the periphery of colonies. EDTA dissociation yields more homogenous single cells (right). Images shown were acquired at 10x magnification.

4.5.3 Comparison of hESC and hiPSC astrocytes with primary human astrocytes

We performed qPCR to compare gene expression profiles of 4 and 6 weeks unpassaged differentiated hESC (H9) and hiPSC (CERA007) astrocytes to that of human primary foetal and adult astrocytes (**Figure 4.3**). The differentiation protocol was extended from 4 to 6 weeks, with the aim of promoting astrocyte maturity as the key mature astrocyte marker GFAP was not consistently detected 4 weeks. Our results demonstrated hESC and hiPSC-derived astrocytes showed morphology similar to primary human foetal and adult astrocytes in culture (**Figure 4.3**). In addition, an increase in all astrocytes markers (*S100 β* , *GFAP*, *ALDH1L1*, *EAAT1*, and *EAAT2*) was observed in both cell lines when culture was extended from 4 weeks to 6 weeks. We also examined the level of *S100 β* and *GFAP* in culture extended to 7 weeks, showing further increase in mRNA expression.

4.5.3.1 Comparison with foetal astrocytes

When compared to primary human foetal astrocytes, 6 weeks old hESC derived astrocytes demonstrated comparable gene expression levels of *ALDH1L1* ($p=0.329$) (ie. 6w hESC derived astrocytes not significantly different to primary human foetal astrocytes), although the expression of *S100 β* (6w: $p<2.9\times 10^{-22}$, 7w: $p<3.0\times 10^{-20}$) and *GFAP* (6w: $p=1.4\times 10^{-5}$, 7w: $p=6.3\times 10^{-5}$) were significantly lower in 6 and 7 weeks hESC derived astrocytes respectively compared to human foetal astrocytes. While 6 and 7 weeks differentiated hiPSC derived astrocytes had significantly lower expression of *S100 β* (6w: $p=1.9\times 10^{-22}$, 7w: $p=8.9\times 10^{-21}$), *GFAP* ($p=6\times 10^{-5}$), *ALDH1L1* ($p=0.003$), *EAAT1* ($p=2.8\times 10^{-5}$) and *EAAT2* ($p=2\times 10^{-16}$) compared to human primary foetal astrocytes. Our findings indicate that hESC derived astrocytes using our defined protocol for 6 weeks express mature astrocyte markers including *S100 β* , *GFAP*, *ALDH1L1*, *EAAT1*, and *EAAT2*, with some markers at a comparable level to human primary foetal astrocytes. Although hiPSC derived astrocytes express all markers investigated, they

were significantly lower than that of human primary foetal astrocytes, suggesting the possible lack of maturation. Compared to human primary foetal astrocytes, our 6 weeks old hESC derived astrocytes expressed significantly higher neuronal marker *TUBB3* ($p=5.9 \times 10^{-5}$), while hiPSC derived astrocytes is not significantly different ($p=0.058$).

4.5.3.2 Comparison with adult astrocytes

When compared to primary human adult astrocytes, our hESC derived astrocytes had significantly higher expression of *ALDH1L1* ($p=0.019$), *EAAT1* ($p=0.026$), *EAAT2* ($p=2.3 \times 10^{-5}$), and *TUBB3* ($p=9.3 \times 10^{-10}$), while expression of *S100β* (6w: $p=0.573$, 7w: $p=0.252$) and *GFAP* (6w: $p=1.0$, 7w: $p=0.979$) were not significantly different. While the hiPSC derived astrocytes showed similar mRNA level of *S100β* (6w: $p=0.910$, 7w: $p=0.959$), *GFAP* (7w: $p=1.0$), *ALDH1L1* ($p=0.095$), *EAAT1* ($p=0.477$) and *EAAT2* ($p=0.493$) when compared to primary human adult astrocytes. Both hESC ($p=9.3 \times 10^{-10}$) and hiPSC ($p=0.009$) derived astrocytes had higher level of *TUBB3* compared to primary human adult astrocytes.

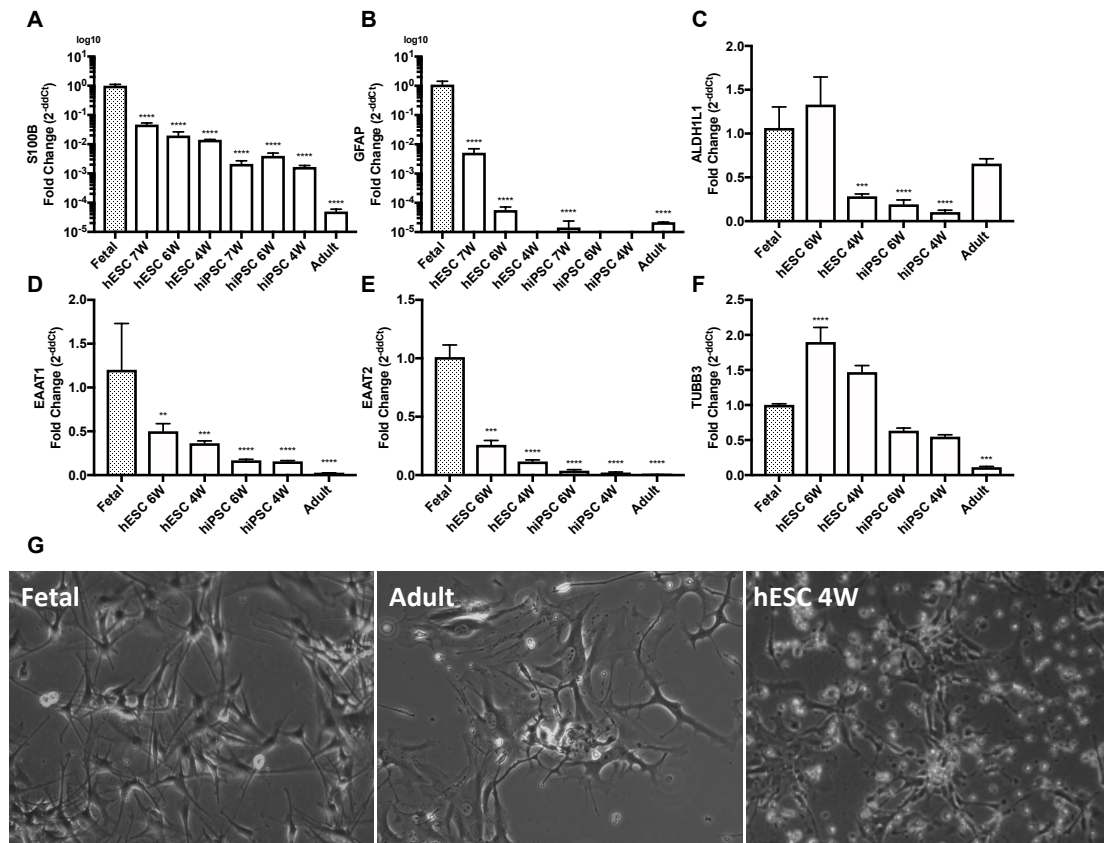


Figure 4.3 | Gene expression and morphological comparison of hPSC-derived astrocytes to primary human astrocytes

(A-F) Gene expression profile of hESC and hiPSC derived astrocytes as compared to primary human foetal astrocytes and human adult astrocytes in culture. **(G)** Bright field images of primary human foetal astrocytes, primary human adult astrocytes, and 4 week hESC-derived astrocytes in culture at 10x magnification. (** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$)

4.5.4 hESC and hiPSC derived astrocytes express mature astrocyte markers at the protein level

Both hESC and hiPSC lines were shown to express increased level of *GFAP* when culture differentiation time was extended from 4 weeks to 6 and 7 weeks (**Figure 4.3**). Due to low mRNA expression level of *GFAP*, we conducted an immunofluorescence staining at 4, 6 and 8 weeks' time point in order ascertain the protein expression level of astrocyte markers GFAP as well as S100 β and EAAT1 to assess if these values reflected our mRNA data. While optimizing our protocol for the length of differentiation to obtain a detectable level of GFAP, we also stained cells for β 3 Tubulin to visualize any neurons present within the cultures. GFAP was detectable at 4, 6 and 8 weeks time point (**Figure 4.4**), with a minority of neuronal cells (β 3 Tubulin positive cells) present within our cultures of hESC and hiPSC derived astrocytes. Additionally, differentiated astrocytes at 4,6 and 8 weeks also express S100 β and EAAT1 (**Figure 4.4**).

Utilizing the defined media DMEM-AGS differentiation protocol for 6 weeks, we quantified proportions of cells expressing astrocyte markers using fluorescence activated cell-sorting (FACS) analysis. Results based on 3 independent differentiation replicates on each cell lines revealed that among the hESC differentiated cells, 39.54% express S100 β and 83.87% express GFAP; while for hiPSC differentiated cells, 60.66% express S100 β and 78.50% express GFAP (**Figure 4.4**). Taken together, the immunofluorescence and FACS results confirm the expression of mature astrocyte markers in our cultures from week 6 onwards.

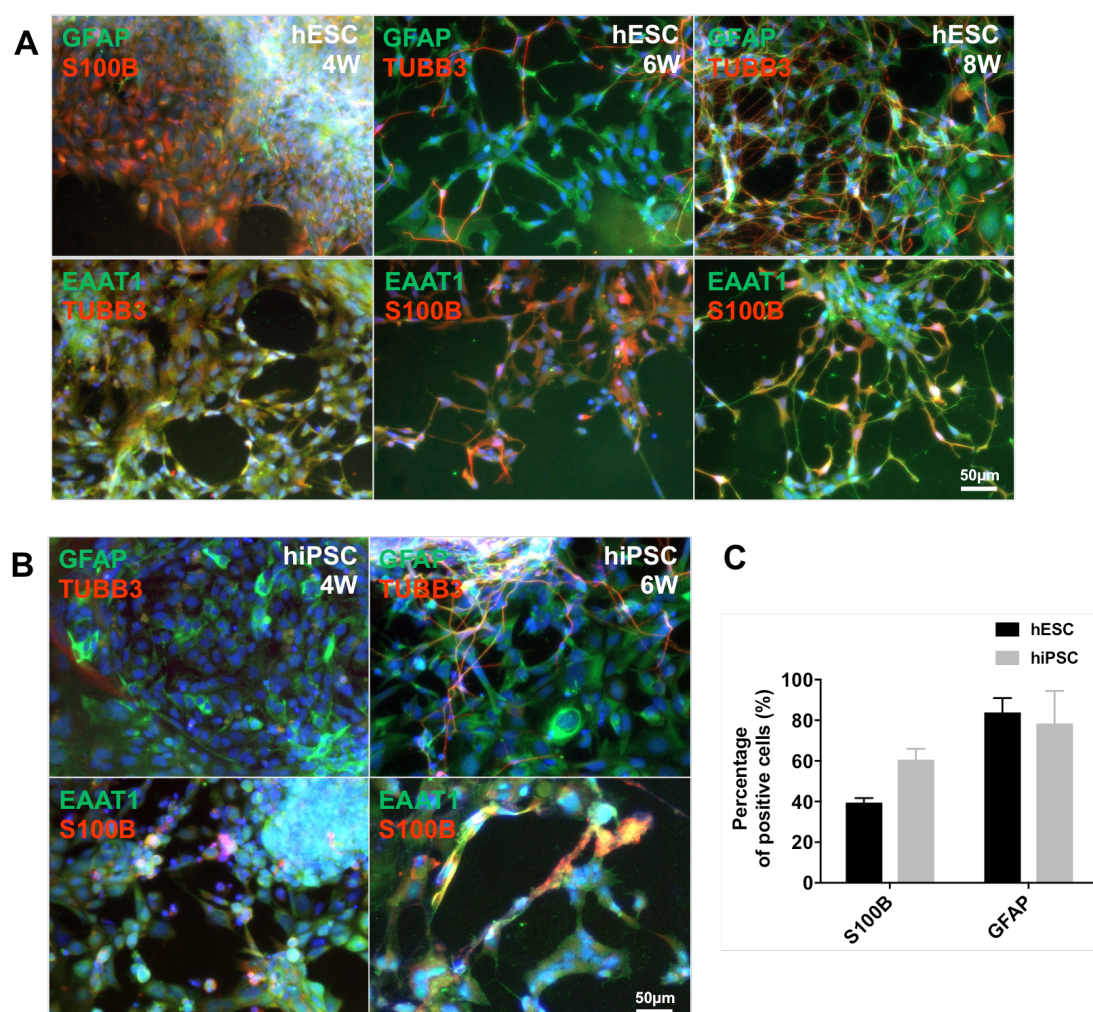


Figure 4.4 | Protein expression of astrocyte markers in hPSC derived astrocytes

Protein expression of astrocyte markers GFAP, EAAT1, and S100 β , along with neuronal marker TUBB3 for **(A)** hESC 4,6 and 8 weeks in culture, as well as **(B)** hiPSC 6 and 8 weeks in culture. **(C)** Percentage of 6 week differentiated hESC and hiPSC derived astrocytes expressing S100 β and GFAP analysed using FACS.

4.5.5 hESC and hiPSC derived astrocytes show typical functional activation and reactivity

To assess the functional maturation of our astrocytes, cells were exposed to Poly I:C, a TLR3 ligand, at concentrations known to stimulate astrocyte activation and reactivity. 6 weeks hESC differentiated astrocytes exposed to Poly I:C [100ng/ml] were found to have significantly higher levels of Rantes compared to controls ($p=0.034$), while no significant changes to the level of IL-6 ($p=0.983$) were observed. hiPSC derived astrocytes were shown to have significant higher level of Rantes compared to controls ($p=0.037$) and no significant changes in the level IL-6 ($p=0.327$) (**Figure 4.5**). This suggests that our 6 week hESC and hiPSC derived astrocytes demonstrate some activation properties as observed in human primary foetal astrocytes, secreting the pro-inflammatory factor Rantes following Poly I:C exposure.

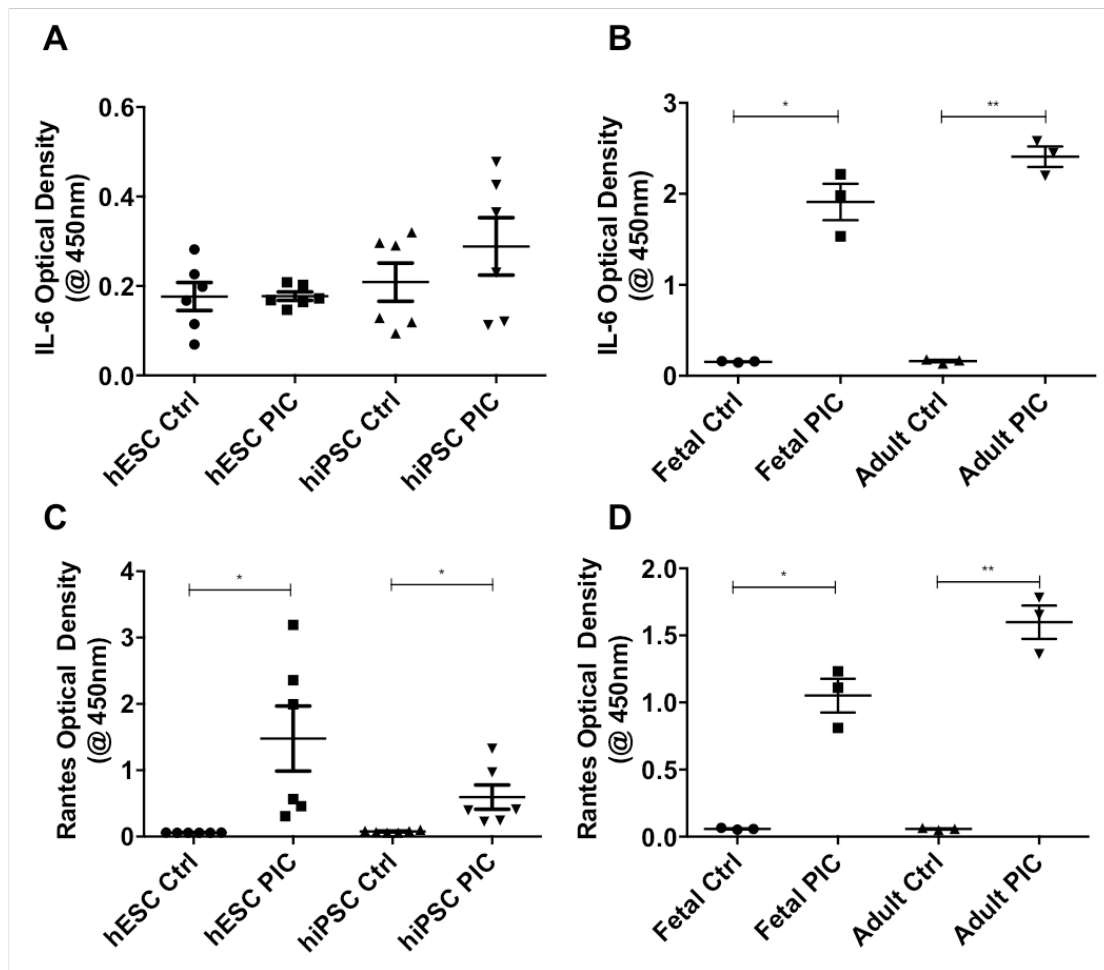


Figure 4.5 | ELISA of hPSC-derived astrocytes exposed to Poly I:C for 7 days

(A-D) hESC (H9) and hiPSC (007) exposed to poly I:C [100ng/ml] for 7 days, showed an increased level of Rantes in H9 and 007. (* $p \leq 0.05$, ** $p \leq 0.01$)

4.5.6 Long-term maintenance and passaging of hESC and hiPSC-derived astrocytes

Astrocytes derived from hESC and hiPSC could be maintained in primary human astrocyte culture media containing DMEM/F-12 with L-glutamine and gentamicin and 10% FBS for at least 7 passages while still maintaining astrocyte properties as shown by qPCR and immunostaining of astrocyte markers (**Figure 4.6**), with 1-2 weeks culture between each passage and weekly media change. Additionally, these cells can be passaged using TryPLE and cultured on non-substrate-coated tissue culture surface, as per maintenance protocol used for primary human astrocytes. Furthermore, hESC and hiPSC derived astrocytes can also be cryopreserved in 10% DMSO and subsequently thawed for culture resumption, while retaining the astrocyte morphology (**Figure 4.6**). These properties of the differentiated cells are significant, allowing for longer term, larger scale experiments while maintaining the consistency of cells.

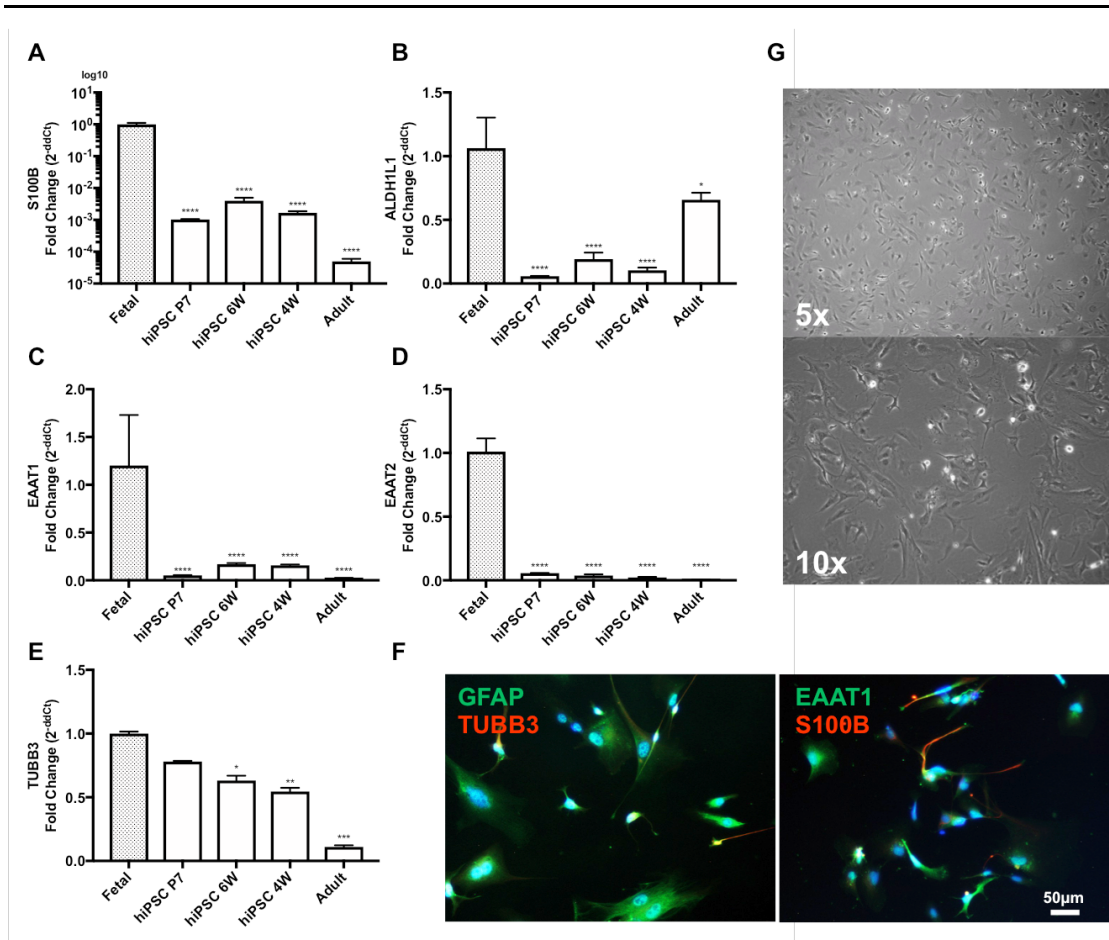


Figure 4.6 | Gene and protein expression of hPSC-derived astrocytes after long-term maintenance and passaging.

(A-E) qPCR showing gene expression profile of P7 hiPSC-derived astrocytes as compared to primary human foetal astrocytes and primary adult astrocytes in culture. **(F)** Immunocytochemistry of GFAP, S100β, EAAT1 and TUBB3 in hiPSC-derived astrocytes at P7. **(G)** Brightfield images of P6 hiPSC-derived astrocytes DIV3 following thawing from cryopreservation in liquid nitrogen. (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$)

4.6 Discussion

The current study reports a highly efficient protocol for differentiating astrocytes from hESC and hiPSCs using a monolayer induction method. Within 42 days of culture, we successfully derived homogenous astrocyte cultures from hESC and hiPSC, with the majority of cells expressing the astrocytic markers including S100 β , GFAP, EAAT1, EAAT2, and ALDH1L1. Our protocol is consistent with a recently published study, utilizing a commercially available media for final differentiation from NPCs generated using monolayer neural induction method, with further characterization against primary human foetal and adult astrocytes (Tcw et al., 2017). To our knowledge, this is the most efficient protocol to date for deriving astrocytes from hESC and hiPSC using monolayer induction, with the previous shortest protocol prior to this requiring 90 days (Roybon et al., 2013). We also present a side-by-side expression comparison of hESC and hiPSC derived astrocytes versus human primary foetal and adult astrocytes. hESC and hiPSC astrocytes derived using our protocol showed comparable gene expression levels of astrocytic markers to human primary foetal astrocytes in culture via qPCR data. In addition, our hESC derived astrocytes further showed inflammation properties similar to primary human foetal astrocytes when exposed to activation agent poly I:C. While we appreciate that differences between human foetal and adult astrocytes exist, our work nonetheless represents an important step forward in validating the use of stem cell derived astrocytes for *in-vitro* modelling of neuron-glia interactions during normal brain development as well as the potential breakdown of these relationships in neurological disorders.

The EB neural induction method is more commonly utilized to derive astrocytes, with several variations of protocols to generate astrocytes from NPCs ranging from 30 days to 6 months

(Jones et al., 2017; Juopperi et al., 2012; Krencik et al., 2011; Krencik & Zhang, 2011; Mormone et al., 2014; Shaltouki et al., 2013; Tcw et al., 2017). Among these, Shaltouki et al. (2013) successfully generated mature astrocyte populations within 35 days from NPCs using defined media containing heregulin-B (an EGF-like ligand), IGF-1 and activin-A. Jones et al. (2017) later adapted this protocol and generated astrocytes in 30 days from NPCs, utilizing a similar defined media including BMP2, CNTF, insulin, and EGF. We employed a similar approach to TCW et al. where the initial test of various defined-media was selected based on previously published protocols, however a much more comprehensive list of 11 media were tested in their study (Tcw et al., 2017). Within our investigation we tested differentiation of astrocyte using previously published protocols detailing 4 defined media, including NBM and NBM-FEP (Denham & Dottori, 2011), DMEM-AGS (Juopperi et al., 2012), as well as DMEM-IHA (Shaltouki et al., 2013). Our current protocol involved 14 days of neural induction from hESC and hiPSC, comprising double SMAD inhibition with SB431542 and LDN193189, followed by an FGF expansion phase and finally 28 days DMEM-AGS induced differentiation from NPCs to generate mature astrocytes. The superior performance of our selected media DMEM-AGS to differentiate NPCs was in line with that reported in TCW et al. where AGS was superior among 11 different growth factors defined media and commercially available media tested (Tcw et al., 2017). Therefore, our methodology and results provide support for AGS utilizing a monolayer neural induction approach, which can therefore be easily incorporated into any existing monolayer neuronal differentiation protocol. In addition, our findings verify to an extent the recent findings reported by TCW et al (Tcw et al., 2017).

Fibronectin was utilized in the current study for surface coating due to its astrocyte enrichment properties. For this investigation, we chose to avoid the use of laminin as

coating substrate given its tendency to favor neuronal over glial fates (Hall, Lathia, Caldwell, & French-Constant, 2008; Ma et al., 2008). Astrocytes in culture were shown to secrete high levels of fibronectin as opposed to laminin, which is believed to be involved with stimulating the growth and proliferation of astrocytes via $\alpha 5 \beta 1$ integrin receptor and P2Y1 receptor (Xia & Zhu, 2014; Yoshida & Takeuchi, 1991). Primary rat astrocytes have also showed tendency to adhere to and migrate towards the orientation of fibronectin-coated surfaces (van Strien et al., 2011; Zuidema et al., 2014). Within our differentiation protocol, we believe that substitution of fibronectin for laminin (which is more commonly used) acted as a more suitable ECM for astrocyte proliferation and maturation *in-vitro*, thereby providing an advantageous environment for promoting astrocyte differentiation from hESC and hiPSC.

Cellular interactions also appear to influence the final cell fate of NPCs. Solanki et al. (2010) manipulated cell-cell interaction via culturing NPCs on surface coated with different geometrical patterns and dimensions of ECM protein, where it was shown that a higher cell-cell interaction promotes neuronal fate while lower cell-cell interaction promotes glial fate. Dissociation related apoptosis due to single cell suspension or harsher choice of dissociation buffer also diminishes NPC and neuronal survival, improving the culture purity of glial fate committed cells (Jager et al., 2016; Rungsiwut et al., 2013; Wachs et al., 2003). Taken together, we believe that our choice of chemical dissociation for replating limits cell-cell interaction by separating NPCs into single cells and reseeding at low density, which favors glial cell fate while filtering out neuronal cells, thereby enriching for astrocyte populations. Moreover, chemical dissociation also allowed for more replicable results compared to mechanical dissociation where neural rosettes need to be dissected out manually. This simplifies the complexity of the differentiation protocol, and is more time efficient, which allows for scaling up of differentiating cultures for high throughput experiments.

Characterization of hESC and hiPSC derived astrocytes at 6 weeks revealed that they express mature astrocyte markers, including *S100 β* , *GFAP*, *ALDH1L1*, *EAAT1*, and *EAAT2*, and are similar in this regard to primary human foetal astrocytes. Although mRNA expression levels of *ALDH1L1* was comparable to those of human primary foetal astrocytes in culture, our hESC and hiPSC derived astrocytes showed lower levels of *EAAT1* and *EAAT2*, as well as very low levels of *S100 β* and almost undetectable levels of *GFAP*. Upon immunocytochemical investigation, we detected that most cells express S100 β and GFAP at the protein level despite the low mRNA levels for these two markers. This result may potentially represent the active translation of mRNA to protein products as part of the maturation process followed by reductions in production of mRNA once sufficient cellular levels of these proteins are achieved. Furthermore, upon exposure to the neuroimmune activator, poly I:C, our hESC derived astrocytes showed a similar pro-inflammatory response to primary foetal astrocytes, demonstrating increased release of Rantes upon treatment with poly I:C. This finding demonstrates the functional maturation of our astrocytes in the context of a key role of astrocytes in responding to neuroinflammation. However, the lack of increase in these factors in our hiPSC derived astrocytes may suggest that these cells were less mature compared to foetal or hESC derived astrocytes, despite expressing mature markers such as GFAP. This raises an important question with regard to developing suitable *in-vitro* models that not only demonstrate the appropriate morphology and expression profile but also demonstrate key processes and responses indicative of a mature adult phenotype. In this regard it is important to validate in vitro models against primary cells that closely reflect human cells. Utilizing human primary foetal astrocytes as well as adult astrocytes for this investigation provided us with a baseline for evaluating the validity of our hESC and hiPSC derived astrocytes, including functional validity.

Our results revealed hESC and hiPSC derived astrocytes more closely resembled primary foetal astrocytes in gene expression profiles. Taken together with recently published work of Sloan et al., as well as with astrogenesis being a prolonged postnatal development, *in-vitro* differentiation of mature astrocytes that closely resembles primary human astrocytes still demand an extended timeframe. These differences also highlight the need for comparing expression profiles for both hESC and hiPSC derived neuronal and glial populations to neurons and glia derived from adult human brain tissue, in order to aid the development of more appropriate and translational in vitro disease models for adult related neurological disorders. However, while biopsied tissue specimens used in the current study represent one such source, one must take into consideration that gene expression profiles for this tissue will to an extent be confounded by an underlying pathology, in our case epilepsy. Notwithstanding gene expression differences resulting from astrocytes being in a more reactive state for the adult brain tissue, our comparison of gene expression profiles across human foetal and adult astrocytes as well as our hESC and hiPSC derived astrocytes still offer a window of comparison for astrocytes in various maturational and developmental states and how they relate to our stem cell derived glial populations.

Yet another advantage of our 6 weeks differentiated astrocytes, is that they could maintain viability while being cultured in primary astrocyte culture medium without additional growth factors. Therefore, once final astrocytes differentiation is achieved, the use of expensive growth factors can be omitted, with as little as weekly media change required. This greatly increases the feasibility of conducting high throughput, cost-effective *in-vitro* modelling. In addition, we have also demonstrated that our hESC and hiPSC derived astrocytes can be passaged and replated on a surface without ECM substrate coating, as well as demonstrating

good cellular viability following cryopreservation and thawing from liquid nitrogen, while retaining morphology and gene expression properties of astrocyte. Taken together, these features greatly reduce the cost of cell maintenance and enable archiving of cell lines for continued experimental use. Unlike neuronal differentiation, which usually requires large-scale neural induction to produce large numbers of cells, our protocol allows for small-scale neural/glial induction with the ability to scale up at the final differentiation stage, including maintaining consistency by omitting batch-to-batch differences that may occur during neural induction.

For the current work, hiPSC astrocyte differentiation was limited to one cell line of hESC and control hiPSC each, mainly due to resources and time constraints, given the maintenance of hiPSC and NSC can be laborious and time consuming. Although the work was conducted with technical triplicates over at least three independent differentiation batches, having more than two biological replicates of hiPSC could strengthen the robustness of our current findings. This is especially important given the known variability between hiPSC from different donors and transformation batches. Furthermore, our characterisation of hPSC-derived astrocytes was limited to mRNA expression data, while verification of these markers using western blotting or quantitation of immunostaining in future work could verify changes at the protein level. In addition to the mGluR5 binding assay I employed, other functional studies such as glutamate uptake, calcium-signalling, multi-electrode array (MEA) as well as binding activity of other receptors including glutamate transporters (EAAT1 and EAAT2) and TLRs would further reveal a more detailed functional maturation profile of these cells.

In conclusion, the current study offers a simple and efficient method for astrocyte differentiation from hESC and hiPSC, using monolayer neural induction system, achieved within 42 days. Astrocytes derived from this protocol have also been shown to express mature markers as observed in primary human foetal astrocytes, as well as the ability to secrete pro-inflammatory factors IL-6 and Rantes upon activation with Poly I:C. However, given the differences between foetal and adult astrocytes, heterogeneity of astrocytes, as well as ongoing research investigating astrocytes subtypes and regional markers, future improvement of differentiation protocols and characterization is still required to generate region and subtype specific astrocyte population data for more translatable *in-vitro* models.

CHAPTER 5 General Discussion

5.1 Significance and outcomes

The first aim of my PhD research was based on evidence in the literature suggesting that potential astrocyte activation in autism brain may be restricted to the white matter and not present to the same extent in the grey matter. I conducted the first post-mortem stereological investigation of astrocyte density and activation morphology in the white matter of the DLPFC in autism. Here, our findings showed a lack of evidence for astrocyte activation in the DLPFC white matter, as well as no increase in astrocyte somal size. Furthermore, no alteration in overall astrocyte process length was seen between autism and controls. Taken together, these results suggest that astrocyte activation in the autism brain may not be as severe as previously reported or thought. Astrogliosis can occur at different severity levels, where mild to moderate reactivity is characterised by increased GFAP expression with limited proliferation as well as preservation of astrocytic domains. During severe astrogliosis, increased proliferation of astrocytes is generally observed, with disruption of astrocytic domains. In the case of prolonged and intensive reactivity, glial scar formation begins to take place, forming a protective barrier to areas undergoing severe neuroinflammation (Sofroniew & Vinters, 2010). As this intense reactivity of astrocytes is usually accompanied by neurodegeneration, which is absent in the autism brain, it is reasonable to assume that mild to moderate astrogliosis, if any, is likely to be present in the autism brain. Our findings of a lack of gross astrocytic pathology are in keeping with this perspective and perhaps require more functional and sensitive measures to detect mild or moderate astrocyte perturbation. However, our findings could also indicate that any associated astrocytic pathology may be present and restricted to early stages disease progression within the first 2 years of life, which may have resolved with age. Despite a lack of gross abnormalities in astrocyte morphology, disruption of astrocytic functions may still

be present in the autism brain. Therefore, in thinking about future neuropathological investigations, it will be important to extend on the current study by examining functional astrocytic markers as well as the contribution of other glia cells, especially microglia in facilitating astrocytic functioning and potentially causing astrocyte dysfunction.

Given the key roles that astrocytes play in neurodevelopment, including neurogenesis, synaptogenesis, and glutamatergic functioning, as well as the co-occurrence of both astrocyte activation and disruption to glutamatergic functioning in autism, we aimed to investigate the interrelationship between these two potential pathophysiological pathways. We conducted an *in-vitro* study utilising primary human foetal astrocytes to investigate the effect of astrocytic activation on glutamatergic functioning and how altering glutamatergic signalling via modulators of mGluR5 may have the capacity to ameliorate astrocyte activation. Following Poly I:C induced activation via TLR3, we observed downregulation of mGluR5 mRNA and binding activity. Interestingly, the reduction of mGluR5 expression and astrocyte activation have both been reported in the autism brain. However, it is unclear which one of these potential pathological events precedes the other, and whether there is an interaction between the two events. Our findings also demonstrate evidence for other glutamatergic signalling disruption following astrocyte activation and associated neuroinflammation, including decreased gene expression of GRIA3, as well as increased gene expression of SHANK3, both molecules that have been implicated in autism. This suggests that astrocyte activation may be a potential mechanism that leads to disruption in glutamatergic signalling in autism. However, there may be many other factors at play, and it is unclear if this is a direct effect of interrupted astrocyte physiological functions, or an indirect effect of astrocytic pro-inflammatory cytokines release. Nevertheless, our results suggest that astrocyte activation can lead to disturbance of mGluR5 signalling, together with

dysregulation of other components of the glutamatergic pathway. Therefore, it is important for future investigations to extend on this knowledge and assess if modulation of mGluR5 could be a potential target to alleviate astrocyte activation with possibly reduced neuropathological consequences. This could be achieved by exposing astrocyte cultures concurrently to Poly I:C and mGluR5 modulators to determine if Poly I:C related changes, such as cytokines levels, as well as astrocytic mRNA markers, can be reversed. Then, a co-culture of astrocytes treated with mGluR6 modulators and untreated controls with neurons could reveal the effect of these astrocytic treatment on potentially alleviating astrocyte activation related neuropathology.

Many studies have employed hiPSC from autism patients to model developmental neurogenesis, using protocols for neuronal differentiation. However, there is still a lack of a reliable and efficient glial differentiation protocol from human stem cells, including astrocyte differentiation. During my PhD, I have successfully improved upon an existing laboratory protocol for glial differentiation by deriving astrocytes displaying mature characteristics from hPSC within 42 days using a monolayer neural induction system. To our knowledge, this is the most efficient differentiation protocol to date utilising monolayer neural induction system, with the previously reported protocol requiring at least 90 days to generate mature astrocytes (Roybon et al., 2013). A recently published study has also reported a similar finding, where the same defined media as used in our study was shown to have similar efficiency of 30 days differentiation, compared to our protocol of 28 days differentiation from NPCs to mature astrocytes (Tcw et al., 2017). The development of this method provides a useful tool for astrocyte *in-vitro* disease modelling not only in autism, but also for other neurological disorders. I demonstrated that these astrocytes resemble primary human foetal astrocytes morphologically, express gene and protein of astrocyte markers, as well as

being capable of releasing pro-inflammatory cytokines IL-6 and Rantes when exposed to Poly I:C. I have also observed an increased resemblance towards primary human foetal astrocytes as the culture time is extended. Furthermore, I have shown that these cells could be maintained in long term culture, thereby minimising batch-to-batch variation due to neural induction and differentiation as seen in stem cells derived neuronal cultures. Another advantage of cryopreserving our stem cells derived astrocytes is the convenience of cultures being able to be suspended and resumed at a later time without the need to recommence the time and resource consuming differentiation protocol from hPSC.

5.2 Neuroinflammation in autism brain

Recent findings of astrocyte and microglia reactivity in the autism brain, has shifted our neurocentric view of autism pathophysiology towards inflammation related to the activation of these glia cells (Chana et al., 2015; Crawford et al., 2015b; Edmonson et al., 2014; Laurence & Fatemi, 2005; John T. Morgan et al., 2010). However, the precise mechanisms of how glial activation could contribute to abnormal neuronal functioning and how these alterations relate to autism core symptomatology still remain largely unclear. Within the body of work in this thesis, we focused on astrocytes due to their essential involvement in neurogenesis and synaptogenesis, as well as glutamatergic signalling, all processes that have been demonstrated to be disrupted in the autism brain (Petrelli, Pucci, & Bezzi, 2016). However, our post-mortem stereological study failed to show evidence of astrocyte activation. Not only does this point to the need for investigation with a more functional approach and at an earlier time point of the disorder, it emphasises the need to consider the reciprocal interaction between neurons and glia, as well as between different glia cell types.

While astrocytes are known to be involved in regulating neurogenesis, neurotransmission and metabolic homeostasis, it is unclear how these functions may be interfered when they are activated. For instance, in the autism brain, activation of astrocytes could lead to secretion of cytokines and possibly morphological changes that may bias astrocyte functions towards pro-inflammatory, deleterious tendencies, instead of maintaining their normal physiological roles that are essential for supporting neuronal health and connectivity. These could be clarified by mechanistic studies looking at specific measures of astrocytic physiological functions such as examining intracellular level of glutamate, glutamate synthase, and glutamine to determine the glutamate reuptake/clearing functioning. Of interest to work conducted in aim 2, measuring levels of extracellular glutamate could be a

useful indicator for potential glutamate excitotoxicity, and whether the capacity of EAAT1 and EAAT2 in synaptic glutamate clearing is disrupted. This could clarify whether neuronal abnormalities seen in the autism brain are related to glutamate toxicity as a result of reduced functioning of these transporters following astrocyte activation. In addition, the elevated level of cytokines such as IL-6 at a pathological level could also be detrimental to normal neuronal functioning despite findings suggesting low level during early developmental timeframe could be beneficial for neurogenesis.

Within the CNS, microglia also play a major role in the innate immune system and are responsible for assessing the brain microenvironment for signs of pathogenic activity around neuronal processes and synapses, as well as astrocyte processes (Tremblay, Lowery, & Majewska, 2010). Just like astrocytes, these microglia are involved in the physiological functioning of the brain, including synaptic communication, as part of the learning and memory process (Parkhurst et al., 2013). Microglia also play critical roles in synaptic pruning via a phagocytic mechanism facilitated by the CX3C chemokine receptor 1 (CX3CR1), targeting smaller and weaker synapses tagged by the complement cascade initializing protein (C1q) and complement 3 (C3) for removal (Schafer et al., 2012; Sominsky et al., 2017; Stevens et al., 2007). Furthermore, the presence of microglia appears to be crucial for certain astrocytic functions, including TLR4 response where the presence of microglia are essential, as well as enhancing astrocytic TLR2 and TLR3 response (Holm et al., 2012). Activated microglia secrete factors including TNF α that have also been shown to induce astrocyte reactivity and trigger associated mGluR5 dysregulation. Given the significant interrelationship that exist between astrocytes and microglia, studies investigating the role of neuroinflammation in disorders such as autism would benefit from taking a more

interactive approach to assessing the contribution of different glial populations and their activation to autism pathogenesis.

The maternal inflammation theory has also received a significant amount of attention in recent years, with supporting evidence including rodent offspring exposed to Poly I:C and LPS expressing autistic-like behaviours and phenotypes. With the current understanding that these compounds bind to and activate TLR3 and TLR4 respectively, the mechanism very likely involves both astrocyte and microglia activation driven neuroinflammation, and potentially disruption to mGluR5 glutamatergic signalling. It is also possible that acute and chronic pathogen exposure may lead to different severity of glial activation. In astrocytes, acute pathogen exposure may only lead to mild/moderate astrogliosis with minimal physiological disruptions, whereas chronic exposure may lead to severe astrogliosis with glial scar formation (Sofroniew & Vinters, 2010). In microglia, acute pathogen exposure can lead to a pro-inflammatory M1 activation, which initiates the first line of defence. This is followed by M2 anti-inflammatory state, which involves immunoregulation and repair once pathogenic threats are removed. A prolonged pathogen exposure may cause a bias towards M1 as oppose to anti-inflammatory M2 state, which can lead to further microglial related damage (Tang & Le, 2016). Given the early postnatal occurrence of autism symptoms and related early neuropathological events, such as brain overgrowth, it is more than likely that even an acute inflammatory response could lead to detrimental impairment due to the vulnerability of developing brain to any insult. This highlights the importance of early treatment regime, which relies heavily on the availability of an early detection biomarker. Furthermore, it is also crucial that potential pharmaceutical targets or receptors are expressed in the early developing brain, and that drugs can cross the BBB to enter the brain. Despite a large body of work suggests that activation of astrocytes and microglia occur in the

autism brain, individuals with autism are not generally assessed for or given the medical diagnosis of encephalitis, therefore treatment targeting neuroinflammation is also not usually considered.

Although many potential biomarkers including neuroinflammatory factors can be detected in the CSF and blood, a specific biomarker for autism has not been established due to the heterogeneity of the disorder. While some preliminary data suggests that markers of oxidative stress and DNA methylation may be predictive of autism-like behaviour, the clinical utility is lacking as the model requires in depth nonlinear regression analysis of multiple metabolites, and is constructed based on a very small cohort of 83 individuals with autism and 76 controls (Howsmon, Kruger, Melnyk, James, & Hahn, 2017). In addition, the common presentation of neuroinflammation in other psychiatric and medical conditions such as schizophrenia, ADHD and epilepsy make characterising a specific neuroinflammation signature for autism a difficult proposition. Many of these conditions are also common comorbid conditions of autism and/or have overlapping symptoms, which make the discovery of an autism exclusive biomarker even more challenging. Therefore, identification of the underlying aetiology of autism and elucidation of an autism specific biomarker still represent a big gap in our current knowledge and understanding on autism.

5.3 Human pluripotent stem cell models of autism

Modelling autism can be challenging due to the symptoms heterogeneity of the disorder, as well as the involvement of higher cognitive functions that are difficult to model *in-vitro*, and in non-human *in-vivo* models. Furthermore, autism has a polygenic genetic composition, where multiple common variants are found to contribute to the genetic risk, while some other may contribute to resilience. These combinations are also more than likely highly different between individuals, which make single gene mutation modelling inappropriate. Furthermore, it is difficult to obtain brain tissues corresponding to the critical pathophysiological state of autism at early postnatal and possibly prenatal periods.

When utilising the hiPSC system, there are several considerations to take into account. The pluripotency of reprogrammed cells are usually confirmed using markers including SOX2 and OCT4 at both the mRNA and protein levels, as well as karyotyped to rule out any genetic abnormalities introduced by the reprogramming process (Kathuria, Sala, Verpelli, & Price, 2017). These quality control precautions ensure the integrity of hiPSCs, which is especially critical when modeling conditions such as autism where genetic variations play a key part in the pathophysiology. However, batch-to-batch variations still exist even between hiPSCs transformed independently but derived from the same source.

The recent success in three-dimensional culturing of brain organoids has begun to bridge the distance between *in-vitro* and *in vivo* modelling, providing the capability of mimicking brain development *in-vitro*. These organoids recapitulate features of human cortical development, showing distinct organisation of progenitor zones and neuronal zones including transient corticogenesis structures (Kadoshima et al., 2013; Lancaster et al., 2013). Furthermore,

neurons within the organoids are shown to be mature and have spontaneous activity, as well as forming functional synapses (Pasca & Sloan, 2015). These characteristics allow for the investigation of early neurodevelopmental abnormalities associated with autism within transient developmental structures such as the preplate and subplate via the use of iPSC organoids. Organoids are also advantageous compared to animal models, with the minimal ethical concerns, low maintenance costs and shorter timeframe (Jorfi, D'Avanzo, Kim, & Irimia). However, compared to non-human in-vivo models, the organoids still lacks peripheral sensory inputs to the brain from various organs such as the cardiovascular, respiratory and digestive systems.

While autism hiPSC-derived neurons are considerably well characterised, there are limited studies reporting astrocytic differences in hiPSC models of autism relative to controls. This could mainly be due to the lack of an efficient protocol to differentiate mature and functional astrocytes, up until recently. Williams and colleagues showed that wild type neurons cultured with RTT hiPSC-derived astrocytes display deficits in morphology, including reduced somal size, neurite length and terminal ends when compared to wild type astrocytes, and these were rescued by IGF-1 (Williams et al., 2014). Another study reported increased microtubule dependent vesicular transport in RTT hiPSC derived astrocytes compared to controls, as measured via live cell imaging (Delépine et al., 2016).

In terms of treatment development, hiPSCs can serve as a highly informative mean of testing different potential therapeutic agents. In response to drugs exposure in hiPSC, outcome and responses at the cellular and molecular levels could be used to predict response of patients to specific drugs including potential side effects, prior to investing in clinical trials. One such example is present in syndromic autism, where IGF-1, gentamicin and MECP2 expression

were found to rescue neuronal growth and synaptogenesis in hiPSCs generated from RTT patients (Marchetto et al., 2010). Recently, the same group has also reported similar therapeutic effects of IGF-1 on rescuing neuronal networks in idiopathic autism hiPSCs (Marchetto et al., 2017). Among a library of 43 compounds tested, Nageshappa and colleagues identified a novel therapeutic candidate NCH-51 in alleviating synaptic activity of RTT MECP2 hiPSC derived neurons to similar level seen in controls (Nageshappa et al., 2016).

When combined with recent technologies in biomedical research, hiPSCs provide a model for various disease specific mechanistic studies. Genetic editing with CRISPR/cas9 is a powerful tool that can be applied on patient derived hiPSCs in order to tease out the specific effect of deletion or modification of a particular candidate gene at the single nucleotide level. A CRISPR mediated gene correction will also reveal the potential benefit of correcting pathological gene mutation. Within a FXS hiPSC model, Park and colleagues reported reversion of FMR1 methylation and expression following correction of CGG repeats with CRISPR/Cas9 (Park et al., 2015). Amongst the recent success of CRISPR/Cas9 technology has been the ability to correct genes in human embryos with no evidence of detrimental off-target effect (Ma et al., 2017). Many research groups around the world are working on the clinical feasibility of CRISPR as a gene therapy. Once the major concern of off-target effects are minimised to a clinically safe level, CRISPR combined with hiPSC technology could initiate a new era of patient specific genetic therapy where patient derived stem cells can be corrected for mutations, and then transplanted back to the host. Very recently, a research group from Sun Yat-sen University in China has commenced the world's first clinical trial of CRISPR, for the treatment of human papillomavirus (HPV). Based on the NIH clinical trials database, there are at least 20 other CRISPR trials announced for 2017/2018, most are which are to be carried out in China. While this could potentially be achieved for syndromic

forms of autism with single genes mutation, gene therapy for idiopathic autism certainly presents a more significant challenge, due to its polygenic nature and varying genetic architecture between individuals. However, many other considerations remain, including ethical concerns, resource availability and other potential unwanted side effects.

Although hiPSC disease modelling has contributed to significant advancement in our understanding of brain physiology, there are still shortfalls that make the use of human samples and animal models inevitable. Despite the differences between mouse and human brain cells, including a lack of complexity and size of mouse astrocytes, animal models still provide a greater understanding of neurobiology mechanisms within a complete and mature brain microenvironment, as well as sensory inputs from peripheral organ systems (Zhang et al., 2016). Despite being able to differentiate stem cells into different neuronal and glial subtypes, iPSC-derived cells still do not display the same morphology, expression patterns or functionality compared to adult brain cells. Furthermore, many differentiation protocols rely on the use of small molecules and proteins to enrich for certain cell types where cells are not proliferative after terminal differentiation. The need to conduct a new batch of neural induction and differentiation for each experiment contributes to batch-to-batch variability between experiments, and is very time consuming. hiPSCs models including the 3D organoids are still lacking the mature and complex neuronal networks resembling the mature brain, along with the lack of glial populations (Jorfi et al.). Furthermore, these models also do not display cortical subregion segregation as the mature brains, where clear lobules structure could be seen. Fundamentally, hiPSC models of autism do not provide behavioural phenotype data, despite many benefits in recapitulating a patients underlying genetic architecture. Therefore, until further improvement of organoids to better

recapitulate brain connectivity, architecture and microenvironment, animal models remain crucial for understanding brain physiology and modelling disease.

5.4 Current intervention and therapies for autism

Early diagnosis and intervention in autism is the key to maximising therapeutic outcomes. Individuals who received intervention as early as 18 months have been shown to display improvement in core autism symptoms (Dawson et al., 2010; Estes et al., 2015). Findings have also demonstrated that treatment effectiveness is greatly enhanced by the high plasticity of the developing brain where learning or experience-dependent processes contribute to synaptic formation and pruning (Sullivan, Stone, & Dawson, 2014). To date, behavioural interventions such as the Early Start Denver Model (ESDM) and Applied Behavioral Analysis (ABA) remain the main therapeutic approaches for treating autism core symptoms (Bhat, Acharya, Adeli, Bairy, & Adeli, 2014; Dawson et al., 2010; Matson et al., 2012). While pharmaceutical approaches have been used in some individuals to target comorbid behavioural issues, there is still no FDA approved drug to target autism core symptoms. Atypical antipsychotics are used for aggression and self-injury, with selective serotonin reuptake inhibitors (SSRIs) used for anxiety and mood symptoms, while psychostimulants are also used for hyperactivity (Ji & Findling, 2015; Lacivita, Perrone, Margari, & Leopoldo, 2017). Among atypical antipsychotics used, risperidone and aripipazole are the only compounds that are currently FDA approved to alleviate aggression and irritation associated with autism, with minor side effects including mild sedation, fatigue, drowsiness and increased appetite (Lacivita et al., 2017).

The imbalance of E/I signalling and synaptic dysfunction have been implicated significantly in autism, along with the mGluR5 theory of FXS based on animal studies showing enhanced mGluR5 dependent LTD. As such, mGluR5 has been a popular drug target for many autism pharmacological studies, as well as many other psychiatric disorders including schizophrenia and anxiety disorder (Huber, Gallagher, Warren, & Bear, 2002; Niswender & Conn, 2010).

Mavoglurant (AFQ056), an mGluR5 antagonist, was shown to rescue molecular and behavioural phenotypes in FMR1 KO mice, with a phase 2 randomised controlled trial showed significant treatment efficacy in autism subgroup with complete DNA methylation. However, further phase 2b clinical trials failed to show efficacy of mavoglurant compared to placebo (Berry-Kravis et al., 2016; Gantois et al., 2013; Jacquemont et al., 2011; Levenga et al., 2011).

Despite the previous unsuccessful clinical trials with mGluR5 antagonists, recent improvement in the understanding of GPCRs and the discovery of their allosteric modulation with non-competitive molecules opened up new possibilities of pharmacological designs. Although it is challenging to design competitive ligands capable of crossing the blood brain barrier, allosteric modulators are generally non-competitive small molecules that bind to alternative sites, and are able to cross of the BBB due to their smaller structure (Lacivita et al., 2017). These compounds include Fenobam, a clinically validated NAM that has anxiolytic properties, as well as the structurally similar MPEP, MTEP and GRN-529, which have been found to alleviate autism behaviour in BTBR mice, including repetitive self-grooming. (Lacivita et al., 2017; Pecknold, McClure, Appeltauer, Wrzesinski, & Allan, 1982; Swanson et al., 2005). Alternatively, PAMs such as CDPPB, NCFP and VU0409551 were found to display antipsychotic and cognitive enhancing effects with minimal neurotoxicity (Lacivita et al., 2017).

Alleviation of neuroinflammation by targeting glia activation has also been suggested as a potential therapeutic approach to improve autism outcomes (Juan I. Rodriguez & Janet K. Kern, 2011). Our current findings showed a decrease in mGluR5 activity and reduction in mRNA expression, along with dysregulation of genes in the downstream glutamatergic

pathway following astrocyte activation. Based on this, modulation of mGluR5 with PAM may alleviate some of the downstream glutamatergic disruption, and potentially other astrocyte activation related neuroinflammatory response such as cytokines release. Further work to elucidate the role of astrocytes and neuroinflammation in autism pathophysiology could be investigated with autism hiPSC derived astrocytes. When compared to control hiPSC derived astrocytes, autism hiPSC derived astrocytes may have an elevated activation profile with increased cytokines levels, and glutamatergic mRNA expression dysregulation. If so, it would be important to investigate if modulation of mGluR5 in these autism hiPSC derived astrocytes could lead to any potential therapeutic effect in alleviating glutamatergic dysregulation, and potentially some of the autism related phenotypes. However, any potential improvement may only be limited to restoration of cellular abnormalities such as neuronal morphology, synaptic functioning, and the rescue may not be sufficient alter behavioural abnormalities. While it is important to consider astrocyte activation and astrocytic mGluR5 as a potential target to relieve autism neuropathology, evidence also points to microglial activation in the autism brain, along with microglial mGluR5 dysregulation.

5.5 Limitations & future directions

While the findings within the current body of work have contributed to understanding the involvement of astrocytes in the pathophysiology of autism, several limitations should be taken into consideration while interpreting the results.

Access to human brain tissue enabled us to establish primary human astrocyte cultures from foetal and adult brain tissues. While the use of primary human astrocytes in our in-vitro studies provided insightful data from human cells, there is nonetheless variation between cultures established from different donors when compared to immortalised cell lines. Due to the sparse availability of tissues and time constraint, as well as the difficulties associated with successfully setting up cultures from adult brain tissue, work within this thesis was conducted based on foetal astrocytes cultures from 2 different donors and a single adult astrocyte culture. Therefore, interpretation of data based on our adult astrocytes cultures should be done with caution given the low number of cell lines. In addition, the adult astrocytes came from temporal lobectomy of epilepsy patients and hence the cells could have increased activation due to the underlying neurological disorder as well as potential genetic mutations. Nevertheless, for my studies they provided a useful phenotypic comparison as mature adult astrocytes when compared to early life foetal astrocytes and stem cell derived astrocytes. However, not only did our findings reveal differences between foetal and adult astrocytes in cultures, there were also differences between the two foetal astrocyte cultures. Therefore, future work would benefit from having primary astrocyte cultures established from an increased number of donors.

Extending from the current body of work, many questions are yet to be answered or clarified within the field of research into the aetiology and pathology of autism. Currently, neuropathological studies of autism focusing on glial cells remain very limited despite a significant amount of work on neuronal abnormalities. Future work including post-mortem and neuroimaging studies should further explore any potential astrocyte, microglia and oligodendrocyte abnormalities, especially in regions involved in cognitive function and/or have been implicated in autism such as the DLPFC, ACC, amygdala and hippocampus. While further *in-vivo* and *in-vitro* studies could further investigate the involvement of mGluR5 glutamatergic signalling and how it could contribute to pathologies related to autism. This can be achieved by extending on our mGluR5 modulation work, using agonists, antagonists, PAMs and NAMs in activated and resting state astrocytes and microglia together with neuronal co-culturing. Furthermore, genetic manipulation of mGluR5 expression using CRISPR/Cas9 to model knockout, overexpression, single nucleotide polymorphisms (SNPs) could also provide valuable understanding of the potential role of genetic abnormalities in mGluR5 and how they play a role in regulating neuroinflammatory responses by glia. While we have established a protocol to image astrocytes cultured on glass cover slips under the helium ion microscope to visualise the ultrastructure, no characterisation or measurement was conducted due to time constraint (**Figure 5.1**). The high power and resolution of the HIM allows visualisation of ultrastructure such as receptors and proteins not usually visible using other imaging methods. Unlike the scanning electron microscope (SEM), the HIM is also able to image fresh cells without gold coating, which enable the visualisation of the true surface structure of the cells. Future work could also utilise this technique and characterise the potential ultra-structural differences between activated versus resting astrocyte and microglia.

Another major challenge for our stem cell differentiation of astrocytes would be to improve our current protocol to enable differentiation into different subtypes of astrocytes, especially protoplasmic and fibrous astrocytes. The low mRNA levels of GFAP in the stem cell derived astrocytes cultures suggest that our protocol may be biased towards derivation of protoplasmic astrocytes, which express very low amount of GFAP, with levels in these astrocytes sometimes undetectable. Furthermore, given the aberrant levels of GFAP observed mainly in the white matter of post-mortem autism brains, a protocol specific for WM specific fibrous astrocytes may be more beneficial for neuropathological studies related to autism. At the same time, characterisation of hiPSC derived astrocytes at different time points corresponding to their neurodevelopment timepoint would also significantly improve our understanding of astrogenesis and enable generation of age-specific cultures for future experiments.

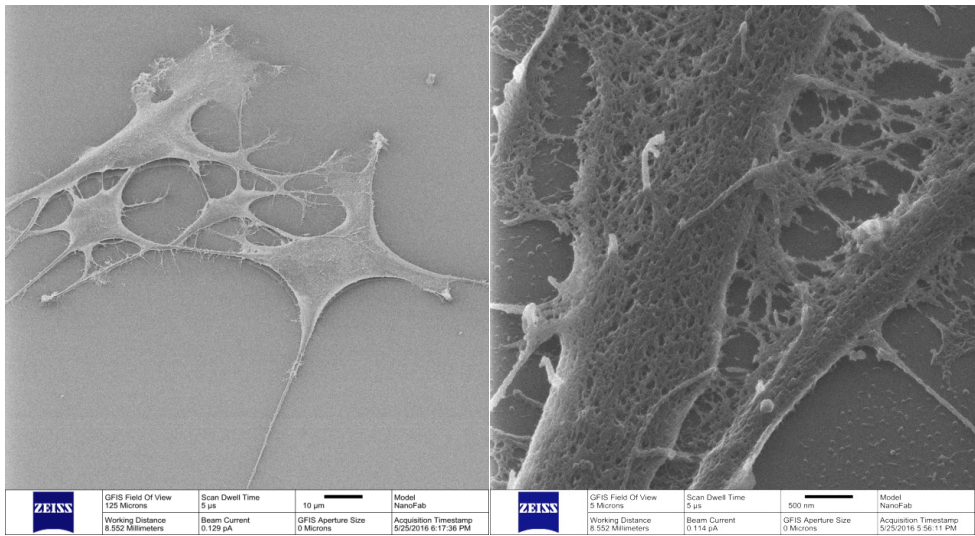


Figure 5.1 | Preliminary ultra-structural images of primary foetal astrocytes generated using Helium ion microscope.

5.6 Conclusion

The body of work carried out for my PhD combined findings from post-mortem autism brain, primary foetal astrocyte and hPSC-derived astrocyte cultures in an attempt to tease out the involvement of astrocyte activation and glutamatergic signalling in the aetiology of autism. In doing so, I conducted the first comprehensive stereological investigation of astrocyte density and activation morphology within the DLPFC white matter of autism brain. My findings suggest that astrocyte activation within the autism brain may not be as severe as previously appreciated, in spite of limitations with the age range for the cohort investigated. Using primary foetal astrocytes, I have shown that activation of astrocytes via a TLR3 driven mechanism can lead to dysregulation of genes involved in the glutamatergic pathway, including reduced mGluR5 signalling and expression. Finally, I have established a simple and efficient protocol to derive astrocytes from hiPSC within 42 days, the most efficient protocol to date using a monolayer neural induction system. I have also shown that these hiPSC derived astrocytes display a similar morphology to primary human astrocytes in culture, as well as expressing structural and functional astrocytes markers at the mRNA and protein level. This provides a simple and efficient protocol to derive mature and functional astrocytes as an *in-vitro* model not only for autism but also other neurological disorders.

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