Human Astrocytes in Down Syndrome

by

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July 2021

A thesis submitted in partial fulfillment of the requirements of the degree of Doctorate of Philosophy.

ABSTRACT

Down syndrome (DS) is caused by the triplication of human chromosome 21 and leads to significant alterations in brain development and function. Importantly, it is the most common genetic cause of intellectual disability. While changes to neurons in DS have been reported, molecular and physiological disruptions to other brain cells are less understood. Among the cell types whose alteration may affect multiple aspects of brain development, function, and response to injury are astrocytes, as they regulate many processes including synapse formation and plasticity, neurovascular coupling and extracellular ion and neurotransmitter homeostasis. Emerging evidence implicates astrocyte dysfunction in a variety of neurodevelopmental and neurodegenerative disorders, raising the possibility of a direct contribution of astrocytes to DS pathophysiology.

In this PhD thesis, I present two investigations which aim to better understand the impact of trisomy 21 on astrocytes in the human brain. Firstly, I characterized the epigenetic and transcriptional profile of human DS astrocytes derived from newly produced DS human induced pluripotent stem cells (hiPSCs). My research identified whole genome alterations of the transcriptome and epigenome, with a strong dysregulation of neurodevelopmental, cell adhesion, and extracellular matrix molecules. Along with these transcriptomic and epigenomic changes, DS astrocytes displayed functional perturbations in cell adhesion and motility. Importantly, this study demonstrates that DS is associated with genome-wide transcriptional and epigenomic changes that go beyond chromosome 21 and leads to functional alterations in astrocytes that may contribute to altered brain development and function in DS.

Secondly, I performed a study using a collection of post-mortem brain samples of DS individuals which identified specific perturbations to glial cells over time. In particular, I found that

glial cells form specialized structures termed Reactive Glial Nets (RGNs) around β-Amyloid (Aβ) plaques as found in sporadic Alzheimer's disease (AD). RGNs possess an inner ring of dysmorphic and activated microglia and an outer perimeter of dysmorphic pathological astrocytes. Having obtained post-mortem samples of DS individuals ranging from ages 33 to 67, I tracked the development of such structures and found that the density of astrocytes and microglia increases as DS individuals age. Interestingly, this study showed an uncoupling in the progression of two glial cell pathologies. While activated microglial cells progressively increased their density around Aβ plaques, reactive astrocytes exhibited a dramatic increase in density around Aβ plaques around 50 years of age. Importantly, this intensification of reactive astrocytic density correlates with the known increase in the severity of cognitive symptomology in DS individuals. This study shows the development and progression of glial cell alterations, and provides an important chronology for the sequence of events of AD neuropathology in DS.

This thesis provides a foundation for further studies to be performed on DS astrocytes and reinforces the importance of research on astrocytes in DS, a historically neurocentric field.

RESUME

La triplication du chromosome 21 qui engendre des altérations dans le fonctionnement du cerveau est la cause génétique la plus courante de déficience intellectuelle. L'impact de la trisomie 21 sur les neurones a été longuement étudié, en revanche les altérations des autres cellules du cerveau sont peu connues. Les astrocytes font partis de ces cellules chez qui des altérations pourraient affecter le développement du cerveau. Les astrocytes notamment, régulent de nombreux processus tels que la formation de synapses, la plasticité neuronale et le couplage neurovasculaire. Ainsi les altérations de leurs fonctions causées par la trisomie 21 pourraient engendrer de nombreuses conséquences sur le développement du cerveau. Récemment la recherche a démontré que le dysfonctionnement astrocytaire est directement impliqué dans de nombreuses maladies neurodéveloppementales et neurodégénératives. Etant donné que les personnes trisomiques présentent des retards neurodéveloppementaux importants et développent la maladie d'Alzheimer à l'âge adulte, il est encore plus important de comprendre comment les astrocytes sont affectés par la triplication du chromosome 21, et les conséquences potentielles que cela a sur le développement du cerveau et plus tard sur son vieillissement.

Dans cette thèse je présente deux études qui ont pour objectif de mieux comprendre l'impact de la trisomie 21 sur les astrocytes. Premièrement j'ai caractérisé le profil épigénétique et transcriptionnel d'astrocytes humains trisomiques (issus de cellules souches pluripotentes induites), et j'ai identifié des altérations sur l'ensemble de l'épigénome et du transcriptome avec une forte dérégulation de molécules neurodéveloppementales, de l'adhésion cellulaire, et de la matrice extracellulaire. De plus les astrocytes trisomiques ont des altérations fonctionnelles de l'adhésion et de la motilité cellulaire. En conclusion, cette étude démontre que la triplication du chromosome 21 est associée à des altérations transcriptionnelles, épigénétiques et fonctionnelles qui sont susceptibles de

contribuer au dysfonctionnement cérébral et au retard de développement chez les personnes trisomiques.

En second lieu j'ai effectué une étude sur des échantillons de cerveaux humains d'individus trisomiques qui a permis d'identifier la présence de structures spécialisées appelées agglomérations gliales réactives autour des plaques d'amyloïde. Celles-ci possèdent un anneau interne de microglies activées et une sphère externe d'astrocytes réactifs et dysmorphiques. Ayant obtenu des échantillons d'individus trisomiques allant de trente-trois à soixante-sept ans j'ai ensuite suivi le développement de ces structures et ai découvert que les densités astrocytaires et microgliales augmentent avec l'âge. Etonnamment cette étude a révélé un découplage temporel entre les deux pathologies gliales avec une augmentation progressive de la densité microgliale à partir de quarante ans et une augmentation secondaire drastique de la densité astrocytaire autour de cinquante ans. L'augmentation de la densité astrocytaire se produit au même moment que l'arrivée des symptômes cliniques de la maladie d'Alzheimer. Cette étude permet donc d'ajouter la chronologie de la formation d'agglomérations gliales réactives aux autres neuropathologies de la maladie, afin de compléter la séquence des évènements de la pathologie d'Alzheimer chez les personnes trisomiques.

Les études présentées dans cette thèse identifient des altérations chez les astrocytes trisomiques allant d'altérations épigénétiques et transcriptomiques à des changements fonctionnels dans l'adhésion et la motilité cellulaire, jusqu'à des altérations de l'organisation spatiale des astrocytes dans le cerveau de personnes trisomiques. Cette thèse apporte un travail de fondation pour que de futures études soient réalisées sur les astrocytes trisomiques et démontre l'importance d'étudier les astrocytes trisomiques dans un domaine historiquement neurocentré.

DEDICATION

To Guillaume for his unconditional love and support,

To Gabrielle and Raphael for making my life wonderfully messy and beautiful,

And to Abigaelle, for whom I became a researcher in the first place, and for whom I hope that this contribution to research will one day make a difference.

ACKNOWLEDGMENTS

First of all, I would like to thank my PI and mentor Dr Keith Murai for giving me the opportunity to join his laboratory and perform research that I am deeply passionate about. His constant enthusiasm and passion for science led to many discussions and fruitful projects. Thank you for your support, guidance and encouragement and for helping me grow as a researcher and as a person.

I would also like to thank my committee members and collaborators Dr Naguib Mechawar and Dr Carl Ernst for their support and helpful discussions. Thank you Dr Naguib Mechawar and Dr MA Davoli for your help and support in processing the post-mortem tissue samples. Thank you Dr Carl Ernst and Dr Huashan Peng for teaching me how to culture and reprogram iPSCs.

My sincere thanks to our collaborators, for their support and great ideas. Thank you Dr Arnold Hayer for helping me perform the astrocyte motility experiments. Thank you to Dr Mayran and Dr Drouin for your help and expertise in the analysis of our epigenetic data.

Thank you to the members of the Murai lab who have been incredible mentors, collaborators and friends. Thank you Emma, Todd, Erin, Gael, Sabrina, Alex, Abby, Chris, Benny, Yorka and David for the stimulating discussions, for sharing with me your expertise and your passion and, most of all, for your support and for being my friends and lab family for the last 7 years!

Thank you to my CRN buddies Marie and Sejal, for making me laugh, for helping me when I needed it, for our endless discussions in the hallway, and for being there for me at the most important times of my life. Thanks for being my Canadian family.

Thank you to my family and my parents for always pushing me to aim for the stars and supporting me every step of the way. Thank you to my siblings for being amazing people, for being interested in what I do and always supportive. And thank you Abi for being you, and for giving me a drive and reason to do better research every day.

Thank you, Gabrielle and Raphael, for being incredible little humans who motivate me to be better every day. And finally thank you Guillaume for your love, for being my best supporter and the one I can always count on, and for having followed me across the world and always believing in me.

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ABBREVIATIONS

AD	Alzheimer's Disease
Aβ	Beta Amyloid
ALS	Amyotrophic Lateral Sclerosis
APP	Amyloid Precursor Protein
AQP4	Aquaporin 4
ASD	Autism Spectrum Disorder
ATAC-seq	Assay for Transposase Accessible Chromatin sequencing
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
BBB	Blood Brain Barrier
bFGF	Basic Fibroblast Growth Factor
BMP	Bone Morphogenic Protein
CNS	Central Nervous System
DS	Down syndrome
DSCR	Down Syndrome Critical Region
EAAT1	Excitatory amino acid transporter 1
EAAT2	Excitatory amino acid transporter 2
ECM	Extracellular Matrix
FXS	Fragile X Syndrome
GABA	γ-amino butyric acid
GDNF	Glial Derived Neurotrophic Factor

GECIs	Genetically-encoded Calcium Indicators
GFAP	Glial Fibrillary Acidic Protein
GLAST	Glutamate Aspartate Transporter 1
GLT1	Glutamate Transporter 1
GO	Gene ontology
GS	Glutamine Synthetase
H_2O_2	Hydrogen Peroxide
HD	Huntington's Disease
HSACh21	*Homo sapiens* chromosome 21
iNOS	Nitric Oxide Synthetase
IPSC	Induced Pluripotent Stem Cell
hiPSC	Human Induced Pluripotent Stem Cell
KCC2	Type 2 K^+/Cl^- cotransporter
KLF4	Krüppel-like factor 4
KO	Knock Out
LTD	Long-Term Depression
LTP	Long-Term Potentiation
MS	Multiple Sclerosis
mTOR	mammalian target of rapamycin
NKCC1	Na-K-2Cl cotransporter-1
NFTs	Neurofibrillary tangles
NO	Nitric Oxide
NPCs	Neural Precursor Cells

Oct4	Octamer-binding transcription factor 4
PCDH	Protocadherin
PD	Parkinson's Disease
RGNs	Reactive Glial Nets
RNA-seq	RNA sequencing
RTT	Rett Syndrome
SCNT	Somatic cell nuclear transfer
Sox2	SRY-Box Transcription Factor 2
SOD1	Superoxide Dismutase 1
TBI	Traumatic Brain Injury
TGF-β	Transforming Growth Factor Beta
TNF-α	Tumor Necrosis Factor Alpha
TSP-1	Thrombospondin 1

PREFACE

This thesis is manuscript-based in accordance with the guidelines from Graduate and Postdoctoral Studies at McGill University and includes three original manuscripts, one of which has been published. The contributions of the authors included on these manuscripts are highlighted here.

Manuscript 1: Astrocytes in Down Syndrome Across the Lifespan.

Blandine Ponroy Bally and Keith K. Murai

Under Revision at Frontiers in Cellular Neuroscience

Author Contributions:

BPB and KKM together wrote and revised the manuscript

Manuscript 2: Human iPSC-Derived Down Syndrome Astrocytes Display Genome-Wide Perturbations in Gene Expression, an Altered Adhesion Profile, and Increased Cellular Dynamics.

Blandine Ponroy Bally, W. Todd Farmer, Emma V. Jones, Selin Jessa, J. Benjamin Kacerovsky, Alexandre Mayran, Huashan Peng, Julie L. Lefebvre, Jacques Drouin, Arnold Hayer, Carl Ernst, and Keith K. Murai.

Hum Mol Genet 2020 Mar 27;29(5):785-802. doi: 10.1093/hmg/ddaa003.

Author Contributions:

BPB and KKM conceived the project and wrote the manuscript. BPB contributed to all experiments. WTF and SJ contributed to RNA-seq and ATAC-seq analysis and manuscript editing. EVJ contributed

to biochemistry and immunolabeling experiments, preparation of cells for ATAC-seq, and helped with hiPSC-derived astrocytes, as well as manuscript editing. AM helped with preparation and planning of the ATAC-seq experiment. AM and JD edited the manuscript. JL provided PCDH antibodies and edited the manuscript. BK performed Ca^{2+} imaging experiments and analysis. AH helped with the cell motility assays, performed related analysis, and edited the manuscript. HP and CE provided support with generating hiPSCs and CE edited the manuscript.

Manuscript 3: Progressive Glial Cell Pathology and Age-Related Acceleration as Astrocyte Reactivity in Down Syndrome-Associated AD.

Blandine Ponroy Bally, Maria Antonietta Davoli, Naguib Mechawar and Keith K. Murai

Manuscript In Preparation

Author Contributions:

BPB and KKM conceived the project and designed the experiments with contributions by NM. MAD prepared and sliced the human post-mortem human tissue. BPB performed all of the experiments and all of the analysis. BPB and KKM wrote the manuscript.

CONTRIBUTION TO ORIGINAL KNOWLEDGE

Author of a literature review covering the role of astrocytes throughout the lifespan of Down syndrome individuals

- Under Revision as Ponroy Bally et al, Frontiers in Cellular Neuroscience

Characterisation of the profile of hiPSC-derived astrocytes in Down syndrome

- Published as Ponroy Bally et al 2020 Human Molecular Genetics, Volume 29, Pages 785-802
- I reprogrammed 6 new patient-derived fibroblast cell lines: 3 DS individuals and 3 age-matched controls into iPSCs and performed validation of all lines.
- I developed a new astrocyte differentiation protocol.
- I differentiated 6 cell lines into astrocytes and performed quality control experiments which demonstrate that they are functional astrocytes.
- I performed the first published RNA-seq experiment on hiPSC-derived DS astrocytes which demonstrate whole-transcriptome alterations in DS astrocytes with especially strong dysregulation in the expression of ECM and cell adhesion molecule genes.
- I performed the first published ATAC-seq experiment on hiPSC-derived DS astrocytes which demonstrate genome-wide alterations in the chromatin structure of DS astrocytes with especially strong alteration of the chromatin structure of ECM and cell adhesion genes.
- I performed cell adhesion assays on DS astrocytes which demonstrated their alteration in cellular adhesion properties and motility.

Analysis of Glial Pathology in Ageing Down Syndrome Brain

- In Preparation as Ponroy Bally et al.
- I obtained a collection of 69 post-mortem brain tissue samples (39 DS and 30 aged matched controls) ranging from the ages of 22 to 67 years of age.
- I described profound AD neuropathology in DS individuals over the age of 50 that includes Aβ plaques and tau phosphorylation.
- I identified the presence of RGNs around Aβ plaques in DS individuals over the age of 50.
- I tracked the development of RGNs in DS individuals and found a progressive increase in the density of activated microglia around plaques which correlates with age.
- I demonstrated that increases in the density of reactive astrocytes also occur with age, however, a major increase in cell density occurs at age 50 and correlates with plaque load rather than with age.

INTRODUCTION

Down syndrome (DS) is a major genetic cause of intellectual disability and affects ~1 in 700 births worldwide (Parker et al., 2010). In DS, triplication of HSA21 (*Homo sapiens* chromosome 21) causes DS individuals to have pronounced intellectual disability that often coincides with brain disorders/diseases including epilepsy, autism, mental disability, and Alzheimer's disease (AD) (Antonarakis, 2017; Antonarakis, Lyle, Dermitzakis, Reymond, & Deutsch, 2004; Arya, Kabra, & Gulati, 2011; Asim, Kumar, Muthuswamy, Jain, & Agarwal, 2015; Glasson et al., 2002; Lott, 2012; Lott & Dierssen, 2010; Roizen & Patterson, 2003). Traditionally, a neurocentric approach, focusing on neurons and their connectivity, has been applied to understanding the mechanisms involved in DS brain pathophysiology with an emphasis on how the triplication of chromosome 21 leads to alterations in neuronal survival, homeostasis, synaptogenesis, brain circuit development, and neurodegeneration. However, recent studies have drawn attention to the role of non-neuronal cells, especially astrocytes, in DS.

Astrocytes were initially perceived as passive connective elements of the brain (Molenaar, 2003). However, they are now known to comprise a highly complex and dynamic cell population which is critical for brain development, homeostasis, and function. Importantly astrocytes have been implicated in virtually all brain diseases and conditions, and recent studies have shown that astrocytes represent an attractive therapeutic target in many CNS disorders and diseases. In Chapter 1, I review the critical functions of astrocytes in the healthy brain and the contributions of astrocytes in brain disorders, injuries, and diseases.

A shift in focus is also happening in the DS field as recent studies have drawn attention to the role of non-neuronal cells, especially astrocytes, in the DS brain. In Chapter 2, I review the alterations caused by the extra chromosome 21 in astrocytes and the repercussions which these have on the DS

brain during development, adulthood and in ageing. Importantly this literature review highlights the need for further research on the altered properties of DS astrocytes and the implications for CNS health and function.

In this PhD thesis, I aimed to characterize human astrocytes in order to identify and describe alterations caused by the triplication of chromosome 21 in astrocytes. In Chapter 3, I harnessed the potential of human induced pluripotent stem cells (hiPSC) technology to understand the properties of DS human astrocytes. For this, I created six new patient-derived hiPSC lines which I differentiated into astrocytes. I identified whole-transcriptome alterations in gene expression and whole-genome alterations in chromatin organization of these cells. Importantly both approaches showed a strong dysregulation of neurodevelopmental, cell adhesion, and extracellular matrix (ECM) molecules. Finally, I performed cell adhesion assays and live cell tracking experiments which showed that DS astrocytes have altered cellular adhesion and motility properties. Overall, this study provides the scientific community with six hiPSC cell lines to analyze, and identifies global changes in gene expression of DS astrocytes which can be used for further investigations.

One of the main challenges for ageing DS individuals is the fact that they are at high risk for developing AD pathology and dementia. Astrocytes and microglia are known to play important roles in AD by being drivers and regulators of neuroinflammation and other CNS pathologies. These cells are also known to aggregate around Aβ plaques in sporadic AD where they form structures called reactive glial nets (RGNs). In DS-associated AD, studies have reported the accumulation of glial cells near Aβ plaques, however, no comprehensive studies have been performed on the spatial rearrangement of activated and reactive glial cells as DS individuals age and develop AD pathology. Therefore, in Chapter 4, I studied whether RGNs form in the ageing DS brain. Considering the near universal development of AD pathology in DS individuals beyond forty years of age, I monitored the

development of glial pathology in DS individuals across four decades of time using post-mortem brain samples, and found that RGNs indeed form in the DS brain and increase their complexity over time. Interestingly, while RGN development showed a strong correlation with Aβ plaque load, it was not correlated with plaque type. Furthermore, while microglial cells progressively increased their association with Aβ plaques with age in DS, changes in reactive astrocytes significantly increased beyond fifty years of age in DS individuals. This timing coincides with the known decrease in cognitive abilities in the DS population.

The findings presented in this PhD thesis represent significant advances in our understanding of the gene expression and functional alterations present in DS astrocytes, as well as, their ability to respond to the accumulation of Aβ plaques in the ageing DS brain. This thesis also provides the scientific community with newly reprogrammed patient-derived hiPSC lines along with several unique datasets which will enable future investigations. I hope the discoveries made in this thesis will motivate others to study astrocytes and make important progress in the development of strategies for treating DS.

CHAPTER 1

Literature Review of Astrocytes in the Healthy and Diseased Brain

The concept of "neuroglia" was first introduced in 1858 by physician and scientist Rudolf Virchow, referring to a substance that connects elements of the brain (Molenaar, 2003). This was followed by the visualization of different glial cell types, including astrocytes, by early anatomists including Camillo Golgi who referred to astrocytes as passive elements which acted as "brain glue" (Golgi, 1873) to embed neurons (Oberheim, Goldman, & Nedergaard, 2012). However, it was not until detailed descriptions of astrocytes by Santiago Ramon y Cajal and others, that the complex and heterogeneous nature of astrocytes begin to be fully appreciated. At the time, Ramon y Cajal and colleagues postulated that astrocytes may be important regulators of brain homeostasis, brain architecture and nutrition (Cajal, 1895). Surprisingly, it took almost a century for studies to begin to prove that astrocytes are a physiologically dynamic and heterogeneous cell population with key roles throughout the brain.

1.1 ASTROCYTE FUNCTION IN THE HEALTHY BRAIN

1.1.1 ASTROCYTE MORPHOLOGY AND HETEROGENEITY

Astrocyte heterogeneity was initially described by Santiago Ramon y Cajal and William Lloyd Andriezen who in 1893 described two different types of glial cells in the white and grey matter, the fibrous and protoplasmic astrocytes, respectively (Andriezen, 1893). These two astrocyte populations show substantial morphological differences. Protoplasmic astrocytes in the grey matter display a sponge-like appearance with numerous fine processes. Today, it is known that these processes contact synapses and play an important neuromodulatory role (Bushong, Martone, Jones, & Ellisman, 2002; Keyser & Pellmar, 1994; Oberheim et al., 2012). In comparison, fibrous astrocytes are less complex

and contain fewer branching processes. They are organized along white matter tracts and contact the nodes of Ranvier and blood vessels (Lundgaard, Osorio, Kress, Sanggaard, & Nedergaard, 2014). The functions of fibrous astrocytes are less clear. Since they associate with blood vessels with their processes (Marin-Padilla, 1995), they are thought to communicate with the vasculature to regulate ionic and metabolic homeostasis in the CNS (Vasile, Dossi, & Rouach, 2017). In addition to these two main astrocyte subtypes, other specialized astrocytes have been identified. For example, Bergmann glia in the cerebellum form a specialized, polarized architecture adapted to the molecular layer of the cerebellar cortex. Bergmann glia play important roles in regulating the migration of granule cells during early postnatal development (Grosche, Kettenmann, & Reichenbach, 2002), give rise to fine processes that enwrap Purkinje cell synapses in the cerebellar cortex, participate in ion and glutamate homeostasis, and participate in motor skill learning (Saab et al., 2012). Müller glia are another specialized astrocyte type found in the retina with a highly polarized morphology that is fine-tuned to the surrounding retinal connectivity (Bringmann et al., 2006). Müller glia play important roles in the structural stabilization, regulation of ion homeostasis, neurotransmitter recycling, and neuronal survival in the retina (Newman & Reichenbach, 1996). Interestingly, there are additional types of astrocytes that appear to be unique to primates, especially humans (Oberheim et al., 2012; Oberheim et al., 2009). For example, specialized interlaminar astrocytes with cell bodies positioned in Layer I of cerebral cortex extend long interlaminar processes through several deeper cortical layers (Colombo, Yanez, Puissant, & Lipina, 1995). Varicose astrocytes which reside in layer V and VI of cortex, also extend long processes characterized by evenly spaced swellings or varicosities (Oberheim et al., 2009). Thus, astrocytes represent a heterogenous population of cells that can have specialized morphologies and functions in the brain.

Several levels of astrocyte heterogeneity are now appreciated by neuroscientists. Inter-regional astrocyte diversity refers to brain region-specific astrocyte types such as those mentioned above

including Bergmann and Müller glia which have specialized anatomies, gene expression profiles and functions tailored to the cerebellum and retina, respectively. Inter-regional astrocyte heterogeneity is largely related to the fact that these cells originate from distinct neuroprogenitors that give rise to astrocytes with different properties (Hochstim, Deneen, Lukaszewicz, Zhou, & Anderson, 2008). This allows for astrocytes to acquire major brain-region specific properties tailored to the neuronal populations within their immediate environment (Farmer & Murai, 2017). Interestingly, astrocytes also display heterogeneity within the same brain region, a concept referred to as intra-regional heterogeneity. Remarkably, neighboring astrocytes within the same brain region can have distinct gene expression profiles and functions. An important study performed in our lab showed that neurons generate intra-regional astrocyte diversity by secreting the molecule Sonic hedgehog that modifies astrocyte gene expression and function (Farmer et al., 2016). Both inter- and intra-regional astrocyte heterogeneity allow astrocytes to be become specialized and functionally adapted to the specific brain microenvironments in which they reside.

1.1.2 ASTROCYTES AS REGULATORS OF ION AND NEUROTRANSMITTER HOMEOSTASIS

Astrocytes are among the most numerous cell types in the brain and cover and tile most of the CNS. Remarkably, astrocytes interact with synapses to bring about changes both locally and globally in the modulation of neuronal activity. The maintenance of ion and neurotransmitter homeostasis is one of the central mechanisms by which astrocytes control neuronal activity (Sofroniew & Vinters, 2010). Astrocytes express molecules such as K^+ channels, Na+/K+ ATPases, and Na+/H+ exchangers which control ionic balance (Obara, Szeliga, & Albrecht, 2008). Astrocytes also harbor water channels such as aquaporin 4 (AQP4) to modulate fluid balance in the brain. AQP4 is densely clustered along specialized processes of astrocytes called endfeet that contact blood vessels (Nagelhus & Ottersen, 2013; Papadopoulos & Verkman, 2013). Endfeet are sites where astrocytes

have been shown to regulate blood flow through the release of modulators such as nitric oxide (NO) and prostaglandins that increase or decrease blood flow, respectively (Gordon, Mulligan, & MacVicar, 2007; Iadecola & Nedergaard, 2007). Collectively, astrocytes employ a number of mechanisms to modulate ionic balance, and water and blood flow in response to neuronal activity (Koehler, Roman, & Harder, 2009).

Astrocytes also play a key role in neurotransmitter homeostasis which is among their most critical functions in the brain (Sattler & Rothstein, 2006). Astrocytes are the principle cell type that recover synaptically-released neurotransmitters such as glutamate and GABA, recycling and shuttling precursors back to neurons (Mahmoud, Gharagozloo, Simard, & Gris, 2019). Glutamate is the primary excitatory transmitter in the brain and effective glutamate reuptake occurs through dedicated Excitatory Amino Acid Transporters (EAATs) such as EAAT1/GLAST and EAAT2/GLT1 (Danbolt, 2001; Schousboe, Scafidi, Bak, Waagepetersen, & McKenna, 2014). Importantly, loss of glutamate uptake by astrocytes leads to neuronal hyperexcitation and excitotoxicity which impacts neuronal health and survival (Mahmoud et al., 2019; Rothstein et al., 1996).

1.1.3 ASTROCYTE CALCIUM SIGNALLING AND GLIOTRANSMISSION

Unlike neurons, astrocytes are not electrically excitable and do not propagate action potentials. However, this does not mean that astrocytes are physiologically silent and do not have dynamic cellular changes that can be monitored *in vitro* and *in vivo*. Measuring intracellular calcium fluctuations in astrocytes is a predominant method used to study astrocyte signaling and dynamics. In the 1990s, cultured astrocytes were shown to have calcium oscillations in response to glutamate that propagated to neighboring cells (Cornell-Bell, Finkbeiner, Cooper, & Smith, 1990; Di Castro et al., 2011). Since these initial studies, the neuroscientist's toolkit to measure calcium activity has significantly improved in specificity and kinetics. For example, new versions of genetically-encoded calcium indicators

(GECIs) are now widely used in addition to traditional calcium sensitive dyes such as Fluo4-AM in measuring minute calcium fluctuations with high spatial and temporal precision (Oh, Lee, & Kaang, 2019). Through the use of calcium sensitive dyes and GECIs, it is clear that astrocytes display regulated increases in intracellular calcium both *in vitro* and *in vivo* (Gordon et al., 2009; Shigetomi et al., 2013). Notably, studies have shown that the majority of astrocyte calcium transients are small, localized events in the processes of astrocytes termed as microdomains (Shigetomi et al., 2013). However, the functional implications and physiological outcomes of these calcium events in microdomains still remain to be fully understood.

Many intrinsic and extrinsic factors are believed to contribute to calcium microdomain activity in astrocytes. *In vitro* studies have shown that calcium transients can be induced by several neurotransmitters such as adenosine triphosphate (ATP), GABA, glutamate, endocannabinoids, prostaglandins, adenosine and norepinephrine (Bezzi et al., 1998; Cotrina, Lin, Lopez-Garcia, Naus, & Nedergaard, 2000; Duffy & MacVicar, 1995; Kang, Jiang, Goldman, & Nedergaard, 1998; Navarrete & Araque, 2008; Porter & McCarthy, 1995). These neurotransmitter-induced calcium events have been demonstrated *in vitro* to induce calcium-dependent exocytosis of neurotransmitters (Araque, Parpura, Sanzgiri, & Haydon, 1998; Cotrina, Lin, & Nedergaard, 1998; Parpura et al., 1994; Schell, Molliver, & Snyder, 1995). This phenomenon termed gliotransmission was later validated in acute brain slices and shown to enable astrocytes to respond to neuronal activity through the release of neuroactive molecules such as ATP and D-serine, which in turn modulate neuronal activity (Araque et al., 2014; Pasti, Volterra, Pozzan, & Carmignoto, 1997; Porter & McCarthy, 1996). While bidirectional communication between astrocytes and neurons is well-known to occur in various experimental contexts (Ashhad & Narayanan, 2016; Fossat et al., 2012; Le Meur, Mendizabal-Zubiaga, Grandes, & Audinat, 2012; Wang et al., 2006), the extent by which gliotransmission regulates neuronal activity *in vivo* to control behaviour requires further investigation.

1.1.4 ENERGY AND METABOLISM

The brain is a highly metabolic tissue that consumes about 20% of the body's energy despite representing only 2% of total body weight (Erbsloh, Bernsmeier, & Hillesheim, 1958). The high metabolic nature of the brain is related to the large number of energy consuming cells (approximately 10^{10} in mammals) including neurons, each of which must maintain strict ion gradients and support thousands of synapses (Deitmer, Theparambil, Ruminot, Noor, & Becker, 2019). In addition to this high energy demand, a major challenge for the brain also lies in the proper distribution of energy substrates. In fact, there can be 3-6 layers of cells between two capillaries, resulting in many brain cells only in indirect contact with the blood supply (Mathiisen, Lehre, Danbolt, & Ottersen, 2010). This is where astrocytes are able to bridge the gap between blood vessels and neurons and their synapses in order to transfer energy substrates like glucose from blood. By contacting blood vessels through their endfeet and neuronal cell bodies and synapses, astrocytes possess an anatomical organization which makes them well-positioned to maintain energy supply to neurons in the brain (Deitmer et al., 2019; Prebil, Jensen, Zorec, & Kreft, 2011; Sofroniew & Vinters, 2010).

In addition to transferring glucose from the bloodstream to neurons, astrocytes are also able to store energy in the form of glycogen in the CNS (Phelps, 1972). Glycogen is located in small granules in astrocytes and found in larger quantities in areas of increased synaptic density where the energy demand is higher (Phelps, 1972). These glycogen stores can be utilized to maintain neuronal activity during hypoglycemia and periods of high neuronal activity (Brown & Ransom, 2007; Duran, Gruart, Lopez-Ramos, Delgado-Garcia, & Guinovart, 2019; Suh et al., 2007). With increased energy demand, astrocytic glycogen is metabolised to lactate which is then transported to neurons through the astrocyte-neuron lactate shuttle, thereby allowing for rapid transfer of energy substrates to neurons (Brown, Baltan Tekkok, & Ransom, 2004; Cali, Tauffenberger, & Magistretti, 2019). Thus, the

specialized structure and biochemical properties of astrocytes allow them to maintain the brain's energy supply, as well as, mobilize additional energy supplies when demand is increased.

1.1.5 ASTROCYTES AND THE BLOOD BRAIN BARRIER

The blood brain barrier (BBB) is a diffusion barrier which impedes the exchange of certain molecules on the basis of size and polarity between blood vessels and the brain parenchyma (Abbott, Ronnback, & Hansson, 2006). It is made up of endothelial cells, perivascular pericytes and astrocyte endfeet (Abbott et al., 2006). Astrocytes have been shown *in vitro* to induce barrier properties in endothelial cells through the release of Transforming Growth Factor Beta (TGF-β), Glial Derived Neurotrophic Factor (GDNF), Basic Fibroblast Growth Factor (bFGF) and angiopoetin 1 (Abbott et al., 2006; Beck, Vinters, Hart, & Cancilla, 1984; Igarashi et al., 1999; S. W. Lee et al., 2003), suggesting a role for astrocytes in the induction of the BBB. However, their exact role in the induction of the BBB remains unclear and controversial since *in vivo* studies have revealed that certain aspects of the BBB become functional in the brain before the appearance of astrocytes (Haseloff, Blasig, Bauer, & Bauer, 2005; Saunders, Ek, Habgood, & Dziegielewska, 2008). It is more clear in the mature CNS where astrocytes are needed for the maintenance of the BBB in the adult brain through specific signalling pathways (Araya et al., 2008; Heithoff et al., 2021). Astrocyte reactivity and dysfunction are tied to disruptions to the BBB (Alvarez, Katayama, & Prat, 2013). Notably, BBB impairments are associated with several brain disorders/diseases such as AD, Parkinson's Disease (PD), and epilepsy and also occur during brain ischemia and trauma (Daneman, 2012). However, whether astrocytes play a primary role in causing BBB impairments in these disorders/diseases remains to be fully understood.

1.1.6 ROLE OF ASTROCYTES IN SYNAPTOGENESIS AND SYNAPTIC PRUNING

Studies over the last two decades have shown that astrocytes are major contributors to synaptogenesis and synaptic pruning and maintenance (Baldwin & Eroglu, 2017; Christopherson et

al., 2005; Chung et al., 2013; Molofsky et al., 2014). Initial discoveries by the laboratory of Ben Barres demonstrated that adding astrocytes to neuronal cultures was sufficient to promote synaptogenesis and increase spontaneous activity of neurons (Meyer-Franke, Kaplan, Pfrieger, & Barres, 1995; Pfrieger & Barres, 1997). Further studies showed that astrocyte conditioned medium itself was sufficient to induce synaptogenesis in neuronal cultures, and that astrocytes can promote synaptogenesis through the release of soluble, synaptogenic molecules (Ullian, Sapperstein, Christopherson, & Barres, 2001). Many astrocyte-derived synaptogenic factors have now been identified including thrombospondins, which are multi-domain glycoproteins that increase synaptogenesis by their interaction with their neuronal receptor, calcium channel subunit α2δ-1 (Christopherson et al., 2005; Eroglu et al., 2009). Other astrocyte-secreted molecules such as SPARC and Hevin are also essential for the generation of functional synapses in the mammalian nervous system. Hevin is secreted by astrocytes localized to excitatory synapses and is required for the formation and maturation of glutamatergic synapses (Kucukdereli et al., 2011). SPARC on the other hand does not act as a synaptogenic molecule but instead inhibits Hevin induced synaptogenesis (E. V. Jones et al., 2011; Kucukdereli et al., 2011). Thus, astrocytes are equipped with specific molecular factors that promote and fine-tune synapse formation in the CNS.

Neurons are known to produce an excess of number of synapses during development. This is followed by a period of experience-dependent synaptic elimination which optimizes neuronal connectivity. Synaptic elimination, also called pruning, occurs during development during which weak and unnecessary synapses are believed to be eliminated from the neuronal circuit (Bosworth & Allen, 2017; E. Lee & Chung, 2019). The physical act of removing synapses was originally thought to be performed solely by microglial cells. However, accumulating evidence has shown that astrocytes also participate in synaptic pruning during development (Stevens et al., 2007). Astrocytes can directly regulate synaptic pruning through astrocyte-mediated phagocytosis using the astrocytic phagocytosis

receptors MERTK and MEGF10 (Chung et al., 2013). They also indirectly control pruning by promoting the neuronal expression of phagocytic markers which are then targeted by microglia. This process occurs through the astrocytic release of TGF-β which induces the expression of complement C1q in synapses and causes complement-dependent synaptic refinement by microglia (Bialas & Stevens, 2013). Therefore, astrocytes utilize discrete signaling molecules and receptors to communicate with neurons and microglia in order to eliminate unwanted synapses during development.

1.2 ROLES OF ASTROCYTES IN BRAIN INJURY AND DISEASE

1.2.1 REACTIVE ASTROCYTES

The studies described above highlight the complex nature of astrocytes and their many roles in the brain. As astrocytes are important for brain development and function, it is not surprising that astrocyte dysfunction occurs in nearly all CNS disorders and diseases. A common feature of most injuries and pathologies of the CNS is astrocyte reactivity. Astrocyte reactivity involves changes in the molecular, cellular and functional state of astrocytes (M. A. Anderson, Ao, & Sofroniew, 2014; Eddleston & Mucke, 1993; Escartin et al., 2021). The severity of astrocyte reactivity varies according to the extensiveness and duration of the disease or insult, and is regulated by inter- and intracellular molecules in a context-dependent manner (Sofroniew, 2009). Mild astrocyte reactivity occurs in situations of mild perturbation to the CNS (Mena et al., 2011) or in contexts where astrocytes are located distal to a more severe injury site. In these situations, astrocytes become hypertrophic and upregulate the intermediate filament protein glial fibrillary acidic protein (GFAP) and other genes (Sofroniew, 2009; Wilhelmsson et al., 2006). With mild astrocyte reactivity, there is often little or no reorganization of tissue architecture and astrocytic proliferation is minimal or absent (Sofroniew,

2020). After the injury, astrocyte reactivity can be resolved, allowing astrocytes to return to a normal state (Wilhelmsson et al., 2006).

On the contrary, more significant astrocyte reactivity occurs with moderate and severe injuries, neurodegeneration and infection (Sofroniew, 2009; Sofroniew & Vinters, 2010). This astrocyte reactivity is characterized by proliferation of astrocytes at the site of injury and an enhanced hypertrophy of their cell bodies and processes. These alterations to astrocytes lead to substantial reorganization of tissue architecture including the intermingling and overlap of astrocytic processes (Sofroniew & Vinters, 2010). Severe injuries can lead to the formation of glial scars formed on the border of the damaged tissue, for example, following a contusion injury, brain tumour or an Aβ plaque (Bush et al., 1999; Faulkner et al., 2004; Sofroniew, 2009; Voskuhl et al., 2009). Astrocytic scars are long-lasting and persist after the time of injury. Several studies have shown that astrocytic scars can serve a neuroprotective purpose by creating a barrier to isolate the site of injury from healthy CNS tissue. Astrocytic scars can block mobility of inflammatory cells which propagate inflammatory responses (Bush et al., 1999; Drogemuller et al., 2008; L. Li et al., 2008; Myer, Gurkoff, Lee, Hovda, & Sofroniew, 2006). Reactive astrocytes may also protect CNS cells and tissues by removing excess glutamate (Bush et al., 1999; Rothstein et al., 1996), degrading Aβ, facilitating BBB repair, supressing neuroinflammation or producing glutathione in order to combat oxidative stress (Koistinaho et al., 2004; Shih et al., 2003). However, reactive astrocytes may also be a major source of CNS pathology (Dossi, Vasile, & Rouach, 2018). For example, reactive astrocytes can block CNS repair through the synthesis of extracellular matrix (ECM) proteins such as collagen and proteoglycans which prevent axonal regeneration (Y. Chen & Swanson, 2003; Dossi et al., 2018). In addition, chronic astrocyte reactivity, as seen in neurodegenerative and autoimmune disorders, can promote neuroinflammation that exacerbates the disease state (Hsiao, Chen, Chen, Tu, & Chern, 2013; Liddelow et al., 2017; Perriard et al., 2015). Whether astrocyte reactivity is ultimately beneficial or detrimental in CNS

diseases remains a complicated and controversial topic (Ding et al., 2021). Studies have been performed in animal models in which certain aspects of astrocyte reactivity were supressed. However, these studies have shown mixed results with some studies reporting an improvement in the outcome following suppression of astrocyte reactivity (Argaw et al., 2012; Brambilla et al., 2009; R. Y. Kim et al., 2014), and other studies showing worsening of the outcome. The worsening of the outcome is believed to be attributed to a failure of glutamate uptake or increased inflammation due to loss of astrocytic scar formation (Drogemuller et al., 2008; Faulkner et al., 2004; Rothstein et al., 1996).

1.2.2 ASTROCYTE REACTIVITY IN CNS TRAUMA: TBI AND STROKE

Traumatic brain injury (TBI) is a major cause of premature death or life-long disability and is caused by various types of insults to that brain that lead to different levels of injury (Graham, McIntosh, Maxwell, & Nicoll, 2000). TBI can have direct and immediately recognizable consequences, but can also have the potential for long-term impairments through the development of epilepsy, behavioural disturbances or neurodegeneration. Astrocyte reactivity and scar formation are key components of CNS trauma and are implicated in the clinical outcome of TBI. Astrocytes are early responders to TBI and stimulate the response of other cells through molecules such as ATP (Burda, Bernstein, & Sofroniew, 2016; J. V. Kim & Dustin, 2006). Cellular and tissue damage and the extent of astrocyte reactivity varies with the severity of TBI. Astrocyte reactivity can range from a reversible reactivity in cases of mild TBI, to glial scar formation in cases of moderate to severe TBI. This is where irreversible changes to tissue architecture can occur along with astrocyte proliferation and the release of proinflammatory molecules. As discussed earlier, the formation of a glial scar can be protective by isolating the injury site to limit the spread of the inflammatory response to healthy, uninjured tissue (Bush et al., 1999; Sofroniew & Vinters, 2010). However, severe astrocyte reactivity and scar formation can inhibit neurogenesis and axonal regeneration post-TBI. Interestingly, knocking down GFAP expression improves neurogenesis and axonal regeneration (Wang, Messing, & David,

1997; Wilhelmsson et al., 2004). Further research is required to better understand how the timing and extent of astrocyte reactivity in TBI has both positive and negative effects on CNS regeneration and, ultimately, recovery.

Ischemic stroke is a type of brain injury which can lead to severe disability or death. Ischemic stroke is caused by the occlusion of blood vessels either by embolus and thrombus, and induces a reduced or blocked blood flow which leads to deficits in oxygenation, glucose delivery and ATP production. Combined, these conditions cause increased extracellular glutamate release and excitotoxicity of neurons (Sofroniew, 2000). Although astrocytes are less susceptible than neurons to excitotoxicity, stroke causes extensive astrocyte reactivity. Following a stroke, reactive astrocytes become proliferative and form an astrocytic scar around the ischemic infarct typically 7 to 10 days after the event. This scar can persist throughout the life of the patient (Sofroniew & Vinters, 2010). As with TBI, the role of the astrocytic scar may be neuroprotective by isolating injured tissue away from healthy tissue. However, transcriptomic studies have shown that reactive astrocytes in the penumbra close to the ischemic infarct express high levels of cytokines such as IL-6, IL-10, IL-1 and IL-1β. These cytokines are known to induce high levels of reactive oxygen species which can cause neuronal death (Rostworowski, Balasingam, Chabot, Owens, & Yong, 1997; Suzumura, Takeuchi, Zhang, Kuno, & Mizuno, 2006). In addition, reactive astrocytes express chemokines that can attract immune cells that, in turn, can propagate the neuroinflammatory response (Glabinski et al., 1996; Strack, Asensio, Campbell, Schluter, & Deckert, 2002).

1.2.3 ASTROCYTE REACTIVITY IN EPILEPSY

Epilepsy is one of the most common neurological conditions affecting 1% of the world's population (Dossi et al., 2018). It is characterized by aberrant neuronal activity that causes repetitive and recurrent seizures. Seizures disrupt normal brain function and can lead to tissue damage and a

worsening of the condition over time (Dossi et al., 2018). Most cases of epilepsy are idiopathic with unknown cause. However, epilepsy can accompany acquired brain injuries, or be caused by infections, birth defects, or rare genetic mutations (Goldberg & Coulter, 2013; Pandolfo, 2011). Astrocyte reactivity occurs in most forms of epilepsy, although the extent of the reactivity is variable. It is particularly apparent with hippocampal sclerosis, a product of epilepsy associated with hippocampal neuronal loss (Thom, 2014). In hippocampal sclerosis, reactive astrocytes proliferate in areas of pyramidal cell loss. In cases of severe epilepsy, reactive astrocytes have been demonstrated to lose their non-overlapping territories. These astrocytes exhibit an increase in the overlap of their processes of up to tenfold, and this process is associated with an increase in synaptogenesis (Oberheim et al., 2008).

In addition to simply responding to epileptic foci, astrocytes can be direct players in the mechanisms involved in chronic forms of epilepsy. Mutations in Kir4.1, an inwardly rectifying potassium channel which mediates spatial potassium buffering and neuronal excitability, have been shown to cause epileptic seizures (Buono et al., 2004; Heuser et al., 2010). Likewise, reductions in the expression, trafficking and membrane anchoring of the water channel AQP4 can induce seizures (Nagelhus, Mathiisen, & Ottersen, 2004) (Eid et al., 2005; T. S. Lee et al., 2004). As mentioned earlier, astrocytes play important roles in neurotransmitter homeostasis and are responsible for the uptake of glutamate released at synapses. This is done by the glutamate transporters EAAT2/GLT1 and EAAT1/GLAST (Danbolt, 2001; Marcaggi & Attwell, 2004). Once inside the astrocyte, recovered glutamate is converted into glutamine by the enzyme glutamine synthetase (GS) before being shuttled back to neurons (Rose, Verkhratsky, & Parpura, 2013). Increases in extracellular glutamate can cause neuronal excitotoxicity and neuronal cell death in epilepsy. Interestingly, several reports have demonstrated decreases in the expression of EAAT2/GLT1, EAAT1/GLAST and GS in epilepsy, opening the possibility that molecular changes in astrocytes can drive physiological alterations that

result in epilepsy (Eid, Behar, Dhaher, Bumanglag, & Lee, 2012; Mathern et al., 1999; Proper et al., 2002).

1.2.4 ASTROCYTE REACTIVITY IN NEURODEGENERATIVE DISEASES: PD, ALS, AND AD

A variety of factors are believed to contribute to neurodegenerative diseases including genetic, environmental, and other unknown causes. Remarkably, astrocyte reactivity has been described in most neurodegenerative diseases. Here, I provide an overview of neurodegenerative diseases where astrocyte reactivity has been observed and may participate in disease onset or progression.

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disease where there is a progressive degeneration of motor neurons in the spinal cord, brain stem and motor cortex. The mechanisms by which this occurs remain uncertain as 90% of ALS cases are sporadic, while only 10% of cases are familial with autosomal dominant transmission (Ghasemi & Brown, 2018). In both sporadic and familial ALS, the disease includes a non-cell autonomous component. In ALS, reactive astrocytes surround both upper and lower degenerating motor neurons (Kushner, Stephenson, & Wright, 1991). Notably, reactive astrocytes in ALS have a selective or total loss of glutamate transporters which can lead to excitotoxicity and motor neuron cell death (Fray et al., 1998; Maragakis & Rothstein, 2006; Rattray & Bendotti, 2006). Furthermore, reductions in Kir4.1 and perturbations in potassium homeostasis are believed to contribute to neuronal hyperexcitability and neuronal cell death (Bataveljic, Nikolic, Milosevic, Todorovic, & Andjus, 2012; Kaiser et al., 2006). Reactive astrocytes in ALS also upregulate the expression of inducible Nitric Oxide Synthetase (iNOS), a soluble enzyme which produces NO. NO inhibits cellular respiration and leads to neuronal depolarization and glutamate release, which can also lead to neuronal death. (Almer, Vukosavic, Romero, & Przedborski, 1999; Sasaki, Warita, Abe, & Iwata, 2001; Stewart, Land, Clark, & Heales, 1998; Stewart, Sharpe, Clark,

& Heales, 2000). On a similar theme, 20% of familial ALS and 5% of sporadic ALS cases have mutations in the gene encoding for Superoxide Dismutase 1 (SOD1) (Rowland & Shneider, 2001). SOD1's main function is to dismutate free superoxide radicals ($O_2^{\bullet -}$) into molecular oxygen (O_2) along with the less reactive hydrogen peroxide (H_2O_2), thus eliminating free radicals that cause oxidative stress (McCord & Fridovich, 1969). Mutations in the SOD1 gene lead to the malfunction of this protein and accumulation of free radicals. Importantly, studies have shown that mutations of SOD1 in astrocytes (but not in other cell types) leads to the production and secretion of molecules which are selectively toxic to motor neurons but not spinal cord interneurons (Di Giorgio, Carrasco, Siao, Maniatis, & Eggan, 2007; Nagai et al., 2007). Therefore, astrocytes are important and direct contributors to motor neuron cell death in ALS.

Astrocytes also participate in PD, which is the second most common type of neurodegenerative disease affecting 1-2% of the elderly population. Clinically, PD is characterized by motor abnormalities such as tremor at rest, rigidity and bradykinesia (Poewe et al., 2017). The specific role of astrocytes in PD remains poorly understood. Mild reactivity of astrocytes is observed in the brains of PD patients with some rare cases of severe astrocyte reactivity (Forno, DeLanney, Irwin, Di Monte, & Langston, 1992). Astrocytes may have both neuroprotective, as well as, neurotoxic properties in PD. Indeed, astrocytes are able to take up α-synuclein (the synaptic protein which aggregates to form Lewy bodies in PD), and post-mortem studies show that astrocytes accumulate aggregates of α-synuclein at levels which are correlated with neuronal loss and are mostly found in brain regions which possess large amounts of Lewy bodies (Wakabayashi, Hayashi, Yoshimoto, Kudo, & Takahashi, 2000). Interestingly, the astrocytic α-synuclein aggregation has also been found in the striatum and dorsal thalamus, two brain regions in which Lewy bodies do not develop, therefore suggesting that astrocytes can take up α-synuclein and potentially prevent it from forming Lewy bodies (H. J. Lee et al., 2010). On the other hand, α-synuclein causes astrocyte reactivity which, in turn,

produces an inflammatory response and the production of reactive oxygen species in PD (Matias, Morgado, & Gomes, 2019). The precise roles and extent of astrocytic involvement in PD remains to be better understood.

Among neurodegenerative diseases, reactive astrocytes are likely best characterized in AD. AD is the most common cause of dementia, accounting for 60-80% of dementia in the elderly population (Wortmann, 2012). AD is characterized by a subtle decline in episodic memory, followed by a broader decline in cognitive abilities including long-term memory, language skills, attention as well as personality changes (Kelley & Petersen, 2007). Histopathologically, there are two major hallmarks of AD, the accumulation of extracellular Aβ plaques, and the formation of intracellular neurofibrillary tangles composed of hyperphosphorylated tau (Ballard et al., 2011). However, over the last 15 years, neuroinflammation has emerged as a third major feature of AD, and may provide a link between the other two hallmark pathologies. Indeed, studies have shown the presence of a inflammatory response in post-mortem brains of AD patients (Verkhratsky, Olabarria, Noristani, Yeh, & Rodriguez, 2010). While neuroinflammation is a self-defence mechanism that occurs in response to a pathogen or injury, it appears to be a chronic condition in the case of AD (Kinney et al., 2018). This chronic inflammation is largely attributed to microglia and astrocytes which release molecules such as proinflammatory cytokines. Indeed, Aβ has been shown to trigger production and secretion of pro-inflammatory molecules such as IL-6, IL-1 and Tumor Necrosis Factor Alpha (TNF-α) in astrocytes, which may contribute to neurodegeneration (Fakhoury, 2018). Concomitant with Aβ deposition and neuroinflammation, the glutamate transporters EAAT2/GLT1 and EAAT1/GLAST have been reported to be reduced in the brains of AD patients. Decreases in glutamate transporter expression correlate with higher Braak stages of the disease (Jacob et al., 2007). Likewise, alterations to the GABA transporter GAT3 and GABA producing enzyme GAD67 have also been reported in post-mortem brain tissue of AD patients (Mitew, Kirkcaldie, Dickson, & Vickers, 2013; Wu, Guo, Gearing, & Chen,

2014) further suggesting a generalized breakdown in glutamate and GABA homeostasis that accompanies AD neuropathology and neuroinflammation.

Alterations in astrocyte morphology and organization in AD were first suggested in 1910 by Alois Alzheimer who identified glial cells in close association with damaged neurons and in close proximity to Aβ plaques (Verkhratsky et al., 2010). Today, we know that increased GFAP expression and hypertrophy of astrocytes occurs in several regions of the AD brain (temporal, occipital, parietal and frontal lobes) and that GFAP expression is prevalent in close proximity to plaques (Kashon et al., 2004). Studies have shown that Aβ isolated from plaques of AD patients can trigger astrocyte reactivity *in vitro* (DeWitt, Perry, Cohen, Doller, & Silver, 1998), suggesting that Aβ, at least in part, is responsible for astrocyte reactivity. Interestingly, astrocytes have been observed to accumulate Aβ in their cytoplasm and processes, in a possible attempt to remove Aβ and prevent plaque formation (Pike, Cummings, & Cotman, 1995). The amount of Aβ accumulation in astrocytes appears to be proportional to the amount of surrounding neuropathology (Nagele, D'Andrea, Lee, Venkataraman, & Wang, 2003). Importantly, the accumulated astrocytic Aβ is of neuronal origin, likely derived from phagocytosis of degenerating synapses and dendrites (Nagele et al., 2004). Astrocytes surrounding plaques form structures that resemble glial scars (Kato et al., 1998; Sofroniew & Vinters, 2010). A recent study performed in our lab demonstrated that astrocytes interact with microglia to form complex three-dimensional glial cell structures in sporadic AD (Bouvier et al., 2016). A more complete description of these structures will be presented in Chapter 4.

CHAPTER 2

Astrocytes in Down Syndrome Across the Lifespan

Blandine Ponroy Bally[1] and Keith K. Murai[1]

2.1 ABSTRACT

Down syndrome (DS) is the most common genetic cause of intellectual disability in which delays and impairments in brain development and function lead to neurological and cognitive phenotypes. Traditionally, a neurocentric approach, focusing on neurons and their connectivity, has been applied to understanding the mechanisms involved in DS brain pathophysiology with an emphasis on how triplication of chromosome 21 leads to alterations in neuronal survival and homeostasis, synaptogenesis, brain circuit development, and neurodegeneration. However, recent studies have drawn attention to the role of non-neuronal cells, especially astrocytes, in DS. Astrocytes comprise a large proportion of cells in the central nervous system and are critical for brain development, homeostasis, and function. As triplication of chromosome 21 occurs in all cells in DS (with the exception of mosaic DS), a deeper understanding of the impact of trisomy 21 on astrocytes in DS pathophysiology is warranted and will likely be necessary for determining how specific brain alterations and neurological phenotypes emerge and progress in DS. Here, we review the current understanding of the role of astrocytes in DS, and discuss how specific perturbations in this cell type can impact the brain across the lifespan from early brain development to adult stages. Finally, we highlight how targeting, modifying, and/or correcting specific molecular pathways and properties of astrocytes in DS may provide an effective therapeutic direction given the important role of astrocytes in regulating brain development and function.

2.2 GENERAL FEATURES OF THE DS BRAIN

DS is a genetic condition found in approximately 1 in 400 births and results from the presence of an extra copy of human chromosome 21 (Hattori, Fujiyama, & Sakaki, 2001; J LEJEUNE, 1959). Trisomy 21 alters gene expression in all cells of the body and results in characteristic facial features, hypothyroidism, hearing and vision abnormalities, cardiac and gastric malformations, and importantly, delayed brain and cognitive development (Baburamani, Patkee, Arichi, & Rutherford, 2019; Vicente, Bravo-Gonzalez, Lopez-Romero, Munoz, & Sanchez-Meca, 2020). Neurodevelopment is atypical and extremely variable in DS. Notably DS individuals present intellectual disability ranging from mild to severe (30 to 70 of Intellectual Quotient (IQ)) (Maatta, Tervo-Maatta, Taanila, Kaski, & Iivanainen, 2006). Such intellectual disability manifests itself by disrupting working memory and verbal short-term memory (Chapman & Hesketh, 2000; Lanfranchi, Baddeley, Gathercole, & Vianello, 2012). Analyses of the DS brain and animal models have shown reduced brain volume, as well as, simplified gyral appearance in human samples (Pinter, Eliez, Schmitt, Capone, & Reiss, 2001). Furthermore, in children with DS, the brain has reduced cortical area but an increased cortical thickness (N. R. Lee et al., 2016). Later, during early to middle adulthood, the DS brain shows signs of premature aging and shrinkage of crucial brain regions needed for learning and memory and executive function, such as the hippocampus and prefrontal cortex (Koran et al., 2014). During middle to late adulthood, essentially all DS individuals develop AD neuropathology, including ß-amyloid (Aß) plaques, tauopathy, neurodegeneration and neuroinflammation (Mann & Esiri, 1989; K. Wisniewski, Howe, Williams, & Wisniewski, 1978; K. E. Wisniewski, Wisniewski, & Wen, 1985).

The neurodevelopmental and intellectual deficits observed in DS are strongly linked to alterations in brain connectivity. Indeed, connectivity in the brain of DS individuals is reported to be perturbed at multiple levels. MRI studies have shown that DS individuals have altered functional connectivity and synchrony (J. S. Anderson et al., 2013; Figueroa-Jimenez et al., 2021; Pujol et al., 2015). However, disruptions in connectivity are not uniform across the brain, but rather occur in areas

where anatomical alterations have been reported such as the hippocampus, anterior cingulate cortex (ACC), and the frontal lobe (Aylward et al., 1999; Carducci et al., 2013), and are consistent with the cognitive deficits observed with DS. Excessive connectivity along with increased inter-brain regional connectivity are thought to contribute to poor adaptative behaviours and lower IQ (J. S. Anderson et al., 2013; Pujol et al., 2015). At the cellular level, a reduction in neuronal production and premature neuronal death are observed in DS and implicated in the brain size reduction (Contestabile et al., 2007; Guidi et al., 2008; Guidi, Ciani, Bonasoni, Santini, & Bartesaghi, 2011). Abnormal dendritic arborization, dendritic spine density and morphology are also reported in DS (L. E. Becker, Armstrong, & Chan, 1986; Takashima, Becker, Armstrong, & Chan, 1981; K. E. Wisniewski, Laure-Kamionowska, & Wisniewski, 1984), indicating disrupted formation and maintenance of cellular connectivity.

While neuronal changes have been widely described in DS, it is far less clear how trisomy 21 impacts non-neuronal cells which are essential for brain development, function, and homeostasis. Interestingly, recent studies analysing the transcriptome of human DS brains have revealed a dysregulation of genes involved in oligodendrocyte differentiation, these genes include *TMEM63A*, *MYRF*, *PLD1*, *RTKN*, *ASPA*, *OPALIN*, *ERBB3*, and *EVI2A* (Olmos-Serrano et al., 2016). This is consistent with defects in axonal myelination and altered psychomotor development in DS individuals (K. E. Wisniewski & Schmidt-Sidor, 1989). Alterations of oligodendrocytes are also present in the Ts65dn mouse model (the most studied DS mouse model which consists of a partial trisomy made up of a distal portion of mouse chromosome 16 and a centromeric portion of mouse chromosome 17 (Davisson et al., 1993)), where defects in myelin are attributed to impairments in oligodendrocyte maturation and an overall reduction in the number of mature myelinating oligodendrocytes (Olmos-Serrano et al., 2016). Thus, trisomy 21 appears to impact non-neuronal cells including oligodendrocytes, an important brain cell type that ensures the fidelity of axon potential conduction.

2.3 ASTROCYTES IN NEURODEVELOPMENTAL DISORDERS AND NEURODEGENERATIVE DISEASES

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2.4 DS ASTROCYTES AND BRAIN SIZE

As astrocytes play important roles in brain disorders and diseases from early developmental to adult stages, it is important to consider how astrocytes are affected by triplication of chromosome 21. Although studies of astrocytes in DS are few in number when compared to studies of neurons, recent studies are providing insights into how trisomy 21 can directly impact astrocytes to affect brain development and function throughout the lifespan. A characteristic feature of the DS brain is reduced brain volume which likely contributes to the intellectual disability of DS individuals. This reduced brain volume has been reported as early as in the second trimester of pregnancy (Patkee et al., 2020) and is caused by a significant reduction in neuronal number (Schmidt-Sidor, Wisniewski, Shepard, & Sersen, 1990). It should be noted that cell counting in the brain is not an easy task and can be associated with significant analytical artefact which is described in this review (von Bartheld, Bahney, & Herculano-Houzel, 2016). Therefore, additional and up to date studies performed in humans would be useful in order to calculate more precise numbers and ratios of astrocytes and neurons in DS. However considering the large body of evidence reporting smaller brain volumes, decreased neuronal numbers in humans, animal models, from *in vivo* and *in vitro* analysis, it is largely accepted that this phenomenon occurs in the DS brain. New studies using contemporary techniques would nevertheless allow for clarity and preciseness in the exact numbers and ratios. It is suspected that there are multiple causes of the reduction in neuronal number, including a decrease in neuronal differentiation during development and increased neuronal cell death throughout the life of DS individuals (Contestabile et al., 2007; Guidi et al., 2008; Guidi et al., 2011). Notably, the reduction in neuronal differentiation is believed to be caused by a gliogenic shift, meaning that neuroprogenitor cells alter their differentiation ability in favor of astrocytes rather than neurons (C. Chen et al., 2014; Guidi et al., 2008; Zdaniuk, Wierzba-Bobrowicz, Szpak, & Stepien, 2011). Several mechanisms can cause the gliogenic shift in DS, among which is a decrease in progenitor cell proliferation (Contestabile et al., 2007; Roper et al., 2006;

Trazzi et al., 2013) and deficits in the Sonic hedgehog signaling pathway which have been directly shown to cause a reduction in the production of neurons (Currier, Polk, & Reeves, 2012; Das et al., 2013; Roper et al., 2006; Trazzi et al., 2013). Remarkably, studies have shown that correcting these deficits can rescue neuronal number in a DS animal model (Das et al., 2013). Indeed, with a single injection of a Sonic hedgehog agonist in newborn mice, the Reeves' group restored neuronal number as well as behavioural deficits in the DS model. Furthermore, studies performed *in vitro* demonstrated that excessive levels of AICD (amyloid intracellular domain), which results from the cleavage of APP by γ-secretase were responsible for the increase in expression of Ptch1 and therefore for the malfunctioning of the Sonic hedgehog pathway. Importantly this study also showed that the treatment of neuronal precursor cells with a γ-secretase inhibitor normalized AICD and restored neurogenesis and gliogenesis levels to normal levels *in vitro* (Figure 1) (Giacomini et al., 2015). Additional pathways have also been suggested to induce a gliogenic shift in DS, such as an increase in progenitor cell oxidative stress and apoptosis caused by the simultaneous overexpression of S100ß and amyloid precursor protein (APP) (both of which are genes located on chromosome 21) (Lu et al., 2011). Finally, overactivation of the JAK-Stat pathway in progenitor cells due to Dyrk1a overexpression (also a chromosome 21 gene) has been suggested to drive aberrant gliogenesis in DS (Kurabayashi, Nguyen, & Sanada, 2015; H. C. Lee et al., 2019). Overall, the gliogenic shift in DS and the pathways described could potentially be targeted to rescue neuronal number deficits and restore cell populations that are competent to form normal brain cell number and connectivity.

2.5 DS ASTROCYTES AND THE DEVELOPMENT OF BRAIN CONNECTIVITY

In DS, neuronal connectivity is believed to be disrupted at several levels from individual synapses to whole circuits. Alterations in synapse density and shape have been reported in the brains of DS individuals, along with defects in dendritic outgrowth and arborization (Benavides-Piccione et

al., 2004; Coyle, Oster-Granite, & Gearhart, 1986; Ferrer & Gullotta, 1990; Golden & Hyman, 1994; Marin-Padilla, 1976). The extensiveness of such morphological abnormalities are correlated with the severity of intellectual disability (Zdaniuk et al., 2011). Interestingly, dendritic arborization and synaptogenesis are processes which are regulated by astrocytes in the developing brain through the expression and/or release of various neuroactive factors (Christopherson et al., 2005; Mauch et al., 2001; Verkhratskiĭ & Butt, 2007). Interestingly, thrombospondin 1 (TSP-1), a known astrocyte-secreted synaptogenic factor (Christopherson et al., 2005), is expressed at lower levels in cultured DS astrocytes (Garcia, Torres, Helguera, Coskun, & Busciglio, 2010). Its lowered expression is responsible for perturbations in dendritic spine morphology and decreases in synapse number in co-cultures of human DS astrocytes with rodent neurons (Figure 2). This deficit can be mitigated by supplementation with recombinant TSP-1 (Garcia et al., 2010). Future experiments, such as those investigating TSP-1 in DS animal models, will be important in assessing how lower TSP-1 levels contribute to DS synaptic changes that impact synapse and circuit formation, and cognitive processes such as learning and memory formation.

In addition to defects in synapse development, perturbations in the effectiveness of GABA synaptic transmission are also implicated in DS (Contestabile, Magara, & Cancedda, 2017). GABA is the main inhibitory neurotransmitter in the mature brain. However, during development, GABA transmission is known to be excitatory (Ben-Ari, 2002). During the postnatal period, GABAergic responses in neurons switch from being excitatory to inhibitory due to decreases and increases in the expression of the chloride transporters NKCC1 and KCC2, respectively, which regulate intracellular chloride concentration (Ben-Ari, 2002). In DS, inhibitory GABA transmission in the adult brain is altered and rendered excitatory. This is supported by studies in the Ts65dn mouse model that have shown that synaptic plasticity and memory deficits can be corrected when inhibitory GABA transmission is restored (Deidda et al., 2015). Intriguingly, astrocytes can regulate intracellular GABA

concentrations and the GABA excitatory-inhibitory switch *in vitro* (Y. X. Li, Schaffner, Walton, & Barker, 1998). This is mainly through the secretion of BDNF, which downregulates NKCC1 levels (Eftekhari et al., 2014). Studies have demonstrated that both BDNF and NKCC1 levels are altered in DS (Deidda et al., 2015). Indeed the reduction in BDNF levels (Tlili et al., 2012) and upregulation of NKCC1 cause excitatory GABAergic transmission in the adult brain (Figure 3)(Deidda et al., 2015). Thus, astrocytes may alter the GABA switch in the DS brain through their altered BDNF secretion and cause cellular and circuit-level deficits in excitation/inhibition in the developing brain.

Unlike neurons, astrocytes do not have action potentials. Instead, they exhibit dynamic physiological changes visualized through intracellular calcium elevations. Such communication is coordinated by intracellular calcium transients which can be driven by neuronal activity (Khakh & McCarthy, 2015). These calcium events are thought to induce release of neuroactive molecules including gliotransmitters which can alter neuronal activity and the activity of neighboring astrocytes (Angulo, Kozlov, Charpak, & Audinat, 2004; S. Lee et al., 2010). In DS, aberrant calcium dynamics have been reported both in rodent models (Muller, Heinemann, & Schuchmann, 1997) and in one study using induced pluripotent stem cell (iPSC)-derived human astrocytes in which spontaneous calcium fluctuations were increased (Mizuno et al., 2018). These aberrant calcium dynamics are believed to cause a reduction in neuronal excitability when co-cultured with neurons. Remarkably, S100ß overexpression causes aberrant calcium signaling, and pharmacological intervention on this pathway restores calcium dynamics along with neuronal excitability (Mizuno et al., 2018). Thus, targeting calcium dynamics in DS astrocytes may improve aberrant neuronal activity patterns in DS. However, a recent study performed by our group did not detect similar alterations in spontaneous or evoked calcium fluctuations in three different iPSC-derived DS astrocyte lines (Ponroy Bally et al., 2020). The reason for this discrepancy is unclear. However, it should be noted that the two studies were performed using different IPSC cell lines and that in Mizuno et al., only one isogenic line was

used. Therefore, additional studies are required using a larger number of iPSC lines in order to better understand the impact of trisomy 21 on astrocyte calcium dynamics.

2.6 GENOME-WIDE TRANSCRIPTIONAL ALTERATIONS IN DS ASTROCYTES

Until recently, it was believed that the DS phenotype was largely caused by the altered gene dosage of a small number of genes located in the DS Critical Region (DSCR) of chromosome 21 (Delabar et al., 1993; Korenberg, 1990). The DSCR extends for approximately 5.4 Mb on HSA21q22 and was shown to be necessary and sufficient to induce a DS phenotype. Many key genes are in that region such as DYRK1A, APP, S100β and SOD1 (N. P. Belichenko et al., 2009). However, studies now show that trisomy 21 has broader and more complex effects well beyond those directly associated with the DSCR (Korbel et al., 2009; Lyle et al., 2009). A recent study reported changes to the global chromatin architecture in DS, with potential genome-wide effects on the transcriptome (Letourneau et al., 2014). Other studies have reported global transcriptional alterations in DS from analysis of a range of tissues including brain (Lockstone et al., 2007; Mao et al., 2005; Olmos-Serrano et al., 2016; Saran, Pletcher, Natale, Cheng, & Reeves, 2003), heart (Mao et al., 2005), blood (Pelleri et al., 2018) and thymus(Pelleri et al., 2018), as well as, in individual cell types including fibroblasts (Pelleri et al., 2018), fetal cells (FitzPatrick et al., 2002), lymphoblastoid cell lines (Sullivan et al., 2016), induced pluripotent stem cells (iPSCs) (Briggs et al., 2013; Gonzales et al., 2018; Pelleri et al., 2018; Weick et al., 2013), and neurons (Briggs et al., 2013; Gonzales et al., 2018; Huo et al., 2018; Weick et al., 2013). With respect to astrocytes, microarray analysis has detected dysregulation of many mRNAs in these cells (C. Chen et al., 2014). A more recent study from our group using an Assay for Transposase Accessible Chromatin sequencing (ATAC-seq) on control and DS iPSC-derived astrocytes uncovered thousands of differently accessible chromatin sites across the genome in DS astrocytes, with an even split of increased and decreased accessibility (Ponroy Bally et al., 2020). Concomitantly, RNA

sequencing (RNA-seq) revealed a global dysregulation of the transcriptome of DS astrocytes that differed significantly from DS neuroprogenitors (Ponroy Bally et al., 2020). As expected, DS astrocytes showed an upregulation of genes on chromosome 21 such as DYRK1A, S100β, APP, SOD1 and SUMO3. However, 93% of dysregulated genes were found outside of chromosome 21 and were distributed across the genome. Interestingly, mRNAs encoding cell adhesion and extracellular matrix (ECM) related genes were especially altered and led to impaired adhesive properties of these cells. This is particularly interesting as alterations in cell adhesion and the ECM have also been reported in various cell types and tissues in DS (Conti et al., 2007; Gonzales et al., 2018; Huo et al., 2018). Further investigation into cell adhesion changes of astrocytes is needed to better understand their relationship to neurodevelopmental and age-related changes observed in the DS brain.

2.7 DS ASTROCYTES AND NEURONAL INJURY

Trisomy 21 is expected to impact astrocyte physiology throughout the lifespan of DS individuals. Mitochondrial dysfunction and oxidative stress may be particularly relevant in this context given that these processes are associated with DS and are a common feature of all DS cells and tissues including astrocytes (Izzo et al., 2018). Consistent with this, DS astrocytes contain a fragmented mitochondrial network that is composed of mostly shorter mitochondria and few elongated mitochondria (Helguera et al., 2013). This type of mitochondrial network is correlated with reduced ATP production and increased ROS production (T. Yu, Robotham, & Yoon, 2006). This lowered mitochondrial activity in DS astrocytes may be an adaptative and protective mechanism. Indeed, DS astrocytes are able to increase their mitochondrial activity if stimulated, but this exacerbates free radical formation, lipid peroxidation, and cell death (Helguera et al., 2013). Consistent with this, increased oxidative stress has been reported in various DS cell types, including in astrocytes and increases in iNOS and nitrite/nitrate concentrations have been reported in the conditioned medium of iPSC-

derived astrocytes. Importantly, an increase in astrocytic oxidative stress can cause an increase in neuronal cell death (C. Chen et al., 2014; J. Hu, Ferreira, & Van Eldik, 1997). Thus, mitochondrial dysfunction and oxidative stress in DS astrocytes may impact the health of neurons and contribute to neuronal cell death observed within the DS brain (Figure 4).

2.8 ASTROCYTES AND ALZHEIMER'S DISEASE PATHOLOGY IN DS

Improvements in health care systems and management of co-morbidities in DS have led to a dramatic increase in life expectancy for DS individuals from 12 years of age in 1949 to 60 years of age in 2004 (Bittles & Glasson, 2004). This increase in life expectancy has also led to the discovery of age-related conditions in DS, the main one being AD neuropathology. Indeed, by age 40, most (if not all) DS individuals present AD neuropathology including Aß plaques, neurofibrillary tangles, neurodegeneration, and neuroinflammation (Mann, 1988; Mann & Esiri, 1989; Motte & Williams, 1989; Zigman & Lott, 2007). The prevalence of dementia in DS-associated AD is similar to sporadic AD (Oliver, Crayton, Holland, Hall, & Bradbury, 1998), although evaluating the cognitive decline in DS is challenging due to the pre-existing intellectual impairment. Novel cognitive tests are currently being developed and deployed to better assess the abilities of DS individuals (Dekker et al., 2018; Sinai, Hassiotis, Rantell, & Strydom, 2016). Importantly, dementia leads to global deterioration and is associated with the mortality of over 70% of DS individuals making it the main cause of death in DS (Hithersay et al., 2018). The exact causes of death are commonly caused by swallowing difficulties which are common in late-stage dementia and which increase susceptibility to aspiration pneumonia or pneumonia caused by the accidental inhalation of food or fluids into the lungs (Degerskar & Englund, 2020). In familial AD, ~10% of cases are caused by mutations in the amyloid precursor protein (APP) protein, which is a precursor to toxic Aβ in plaques. The APP gene is found on

chromosome 21 and its overexpression is thought to be the primary cause of AD in DS as it causes rapid accumulation of Aβ with age (Head, Lott, Wilcock, & Lemere, 2016; Margallo-Lana et al., 2004).

In AD, chronic neuroinflammation is believed to exacerbate Aβ burden and possibly exacerbate neurofibrillary tangle formation related to tau hyperphosphorylation, thus potentially linking two hallmarks of AD pathology (Kinney et al., 2018). In DS, astrocyte reactivity may be a major contributor to AD pathology given that glial reactivity has been reported to occur as early as 2 days postnatally (Griffin et al., 1989). This early astrocytic reactivity occurs prior to wide-spread Aß plaque formation and neuronal degeneration, and hence is in a position to play a primary role in AD pathology. Consistent with this, early overexpression of S100ß in astrocytes and neuronal APP overexpression have been shown to activate microglia and increase IL-1β expression, which in turn exacerbates APP production in neurons and glial cells (Barger & Harmon, 1997; Goldgaber et al., 1989; L. Liu, Li, Van Eldik, Griffin, & Barger, 2005). These events appear to be self-propagating, as IL-1β and S100ß have both been reported to induce microglial cell activation and astrocyte reactivity with overexpression of themselves, as well as, neuronal APP (Goldgaber et al., 1989; Sheng et al., 1996). Notably, glial activation and cytokine production occur during childhood in DS, many years before the accumulation of Aβ plaques (Griffin et al., 1989). Taken together, upregulation of neuronal APP and astrocytic S100ß, and cytokines such as IL-1ß, may drive neuronal stress, glial activation, and DS-related neuropathological changes characteristic of AD.

A noticeable cellular feature accompanying AD pathology is oxidative stress. Increased oxidative stress and ROS production occurs in various cell types in DS throughout the entire lifespan including in astrocytes. Increased ROS production in the ageing DS brain is known to damage proteins, lipids, and DNA which alters neuronal function and ultimately aggravates neurodegeneration in DS (Busciglio & Yankner, 1995; F. Di Domenico et al., 2013; Perluigi & Butterfield, 2012; Perluigi, Di Domenico, & Buttterfield, 2014). Studies have demonstrated that increased ROS levels in neurons

leads to altered processing of APP and accumulation of Aβ (Busciglio et al., 2002; Cenini et al., 2012; Coskun & Busciglio, 2012; Perluigi & Butterfield, 2012). Interestingly, the spread and extent of oxidative stress increases with age and correlates with Aβ levels (Lott, Head, Doran, & Busciglio, 2006). The progressive and chronically high level of oxidative stress is therefore implicated in neuronal death and believed to contribute to neurodegenerative processes and cognitive dysfunction in the DS brain (Perluigi & Butterfield, 2012). Correcting oxidative stress early in DS may help ameliorate premature ageing and slow the progression of AD neuropathology with which there are no therapies. Consumption of vitamin-rich diets and vitamin supplementation may also combat neurodegeneration, since vitamins are known antioxidants and reduce oxidative stress (Bhatti, Usman, Ali, & Satti, 2016). Thus, targeting oxidative stress pathways may hold promise for the future treatment of DS-associated AD.

Excitotoxicity is another major event which lead to neuronal death and neurodegeneration and may be related to AD-related neuropathology in DS. Astrocyte reactivity has been shown to exacerbate excitotoxicity and neurodegeneration through the overexpression of the metabotropic receptor mGluR5. mGluR5 is expressed in both astrocytes and neurons and is important for neuron-glial cell communication in both the healthy and injured brain (Aronica et al., 2003; Bradley & Challiss, 2012; D'Antoni et al., 2008). mGluR5 is prevalent in the developing brain and is involved in processes such as proliferation, differentiation, and survival of neuronal progenitors (Di Giorgi Gerevini et al., 2004). mGluR5 expression is lower in the adult brain, except in areas with active neurogenesis (Catania et al., 1994; Romano, van den Pol, & O'Malley, 1996). Importantly, mGluR5 upregulation has been reported in brains of AD and DS individuals (Dolen & Bear, 2008; Kumar, Dhull, & Mishra, 2015; Oka & Takashima, 1999). This mGluR5 upregulation is specific to astrocytes in DS and occurs as early as mid-gestation and persists postnatally (Iyer, van Scheppingen, Milenkovic, Anink, Lim, et al., 2014). Aged DS individuals with AD pathology also present even higher levels of mGluR5 in

astrocytes, especially in astrocytes in close vicinity to Aβ plaques. This suggests that Aβ may stimulate upregulation of mGluR5 expression in astrocytes (Iyer, van Scheppingen, Milenkovic, Anink, Lim, et al., 2014). Interestingly, astrocytic mGluR5 is activated by soluble Aβ in sporadic AD which has been found to generate calcium oscillations and the release of glutamate, thus enhancing the neuronal excitotoxicity (Kumar et al., 2015; Shrivastava et al., 2013). Targeting glutamate excitotoxicity pharmacologically could therefore be beneficial in DS associated AD and several treatment options are currently available. These include therapy for hypoactivity of glutamate receptors by inducing the receptors with glycine and cycloserine; blocking the NMDA receptor with the antagonist memantine, and targeting oxidative stress with estrogen and antioxidant supplementation (Butterfield & Pocernich, 2003). Interestingly clinical trials have been performed testing the efficacy of memantine in the DS population, these revealed a complete absence of effect in the DS population which is surprising considering the presence of glutamate excitotoxicity in the DS brain (Hanney et al., 2012). Further research is required in this area to develop appropriate therapies for DS associated AD.The mammalian target of rapamycin (mTOR) pathway is also known to be altered in DS astrocytes. The mTOR pathway is an important signaling pathway which responds to a large variety of environmental stimuli and regulates essential processes such as cell growth and proliferation, metabolism, protein synthesis, synaptogenesis and apoptosis (Dazert & Hall, 2011; Laplante & Sabatini, 2012; Wong, 2013). Dysregulation of this pathway has a major impact on the nervous system and has been reported to occur in various neurological diseases such as tuberous sclerosis (Orlova & Crino, 2010), ASD (Tsai et al., 2012), and DS. This pathway has also been identified as a molecular link between Aβ accumulation and cognitive dysfunction in sporadic AD. Intriguingly, mTOR inhibitors can reverse cognitive dysfunction and reduce Aβ load in a mouse model of AD (Caccamo, Majumder, Richardson, Strong, & Oddo, 2010; Ma et al., 2010; Spilman et al., 2010) and hyperactivation of the mTOR pathway has been identified both in the developing and aged DS brain (Iyer, van Scheppingen, Milenkovic,

Anink, Adle-Biassette, et al., 2014; Perluigi, Pupo, et al., 2014). A recent study showed that iPSC-derived DS astrocytes cause mTOR hyperactivation in control neurons and exacerbate the hyperactivation in DS neurons (Araujo et al., 2018). Targeting mTOR hyperactivation in astrocytes and neurons may therefore be a plausible target for mitigating some aspects of AD pathology in DS.

2.9 FINAL PERSPECTIVE ABOUT ASTROCYTES AND DS

New discoveries are challenging the neurocentric view of DS and leading to a more complete understanding of the contributions of other brain cell types including astrocytes to DS pathophysiology. Recent studies have revealed myriad ways astrocytes can participate in DS across the lifespan. Although these studies still remain relatively few in number, they provide an important launching point for investigating how trisomy 21 alters their properties which may have profound effects on the developing and aging DS brain. Since astrocyte development largely occurs postnatally, there may be an attractive therapeutic window for correcting genetic or molecular alterations in DS to improve brain function and prevent cellular changes including AD-related neuropathology. Harnessing new technologies such as single cell RNA-seq to investigate transcriptional profiles and cellular heterogeneity in DS will allow additional detailed characterization of astrocytes in the DS brain. This technology has been used in other diseases such as in Huntington's disease where several transcriptional states of astrocytes were identified (Al-Dalahmah et al., 2020). Use of patient-derived iPSCs is also a relatively new technology that allows the study of human DS cells. This approach is compatible with high-throughput screening methods that can be used to identify new compounds that correct aberrant cellular pathways caused by trisomy 21. However, a current limitation of iPSC research is the limited availability of independent DS cell lines. Many studies have used the same iPSC lines, and it is clear that genetic background can have an important impact on cellular phenotypes observed, especially in DS. New patient-derived iPSC lines need to be created and shared among the

scientific community in order to take advantage of this powerful approach. Finally, establishing new animal models of DS is an important future direction for the field to help validate findings *in vivo* and test new hypotheses. DS is a particularly challenging to model in mice as it requires the triplication of genes of a whole human chromosome which are spread out in several chromosomes in mice. There are many different animal models of DS which all have their strengths and weaknesses and summarized in this review (Herault et al., 2017). Access to new models and application to innovative technologies such as single-cell RNA-seq and iPSCs will help build a more complete picture of the cellular changes occurring in DS and provide further optimism that effective therapies for DS can be found.

2.10 FIGURES

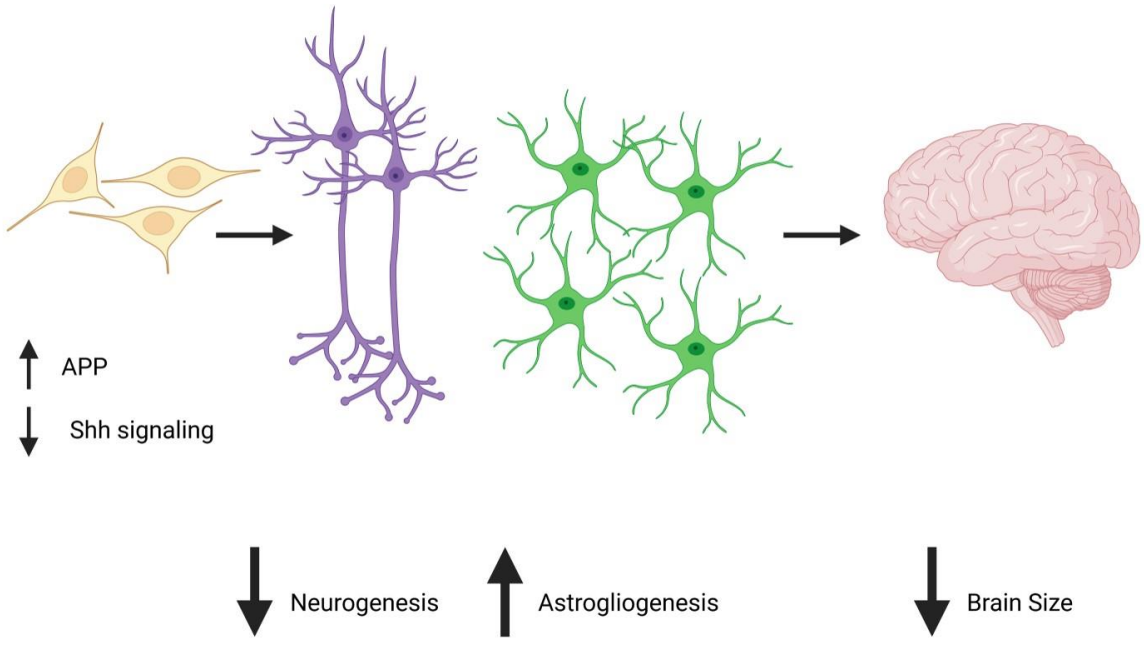

Figure 1: APP overexpression in neuroprogenitor cells decreases Shh signaling and is believed to be responsible for an increase in astrogliogenesis and a decrease in neurogenesis. (Created in Biorender)

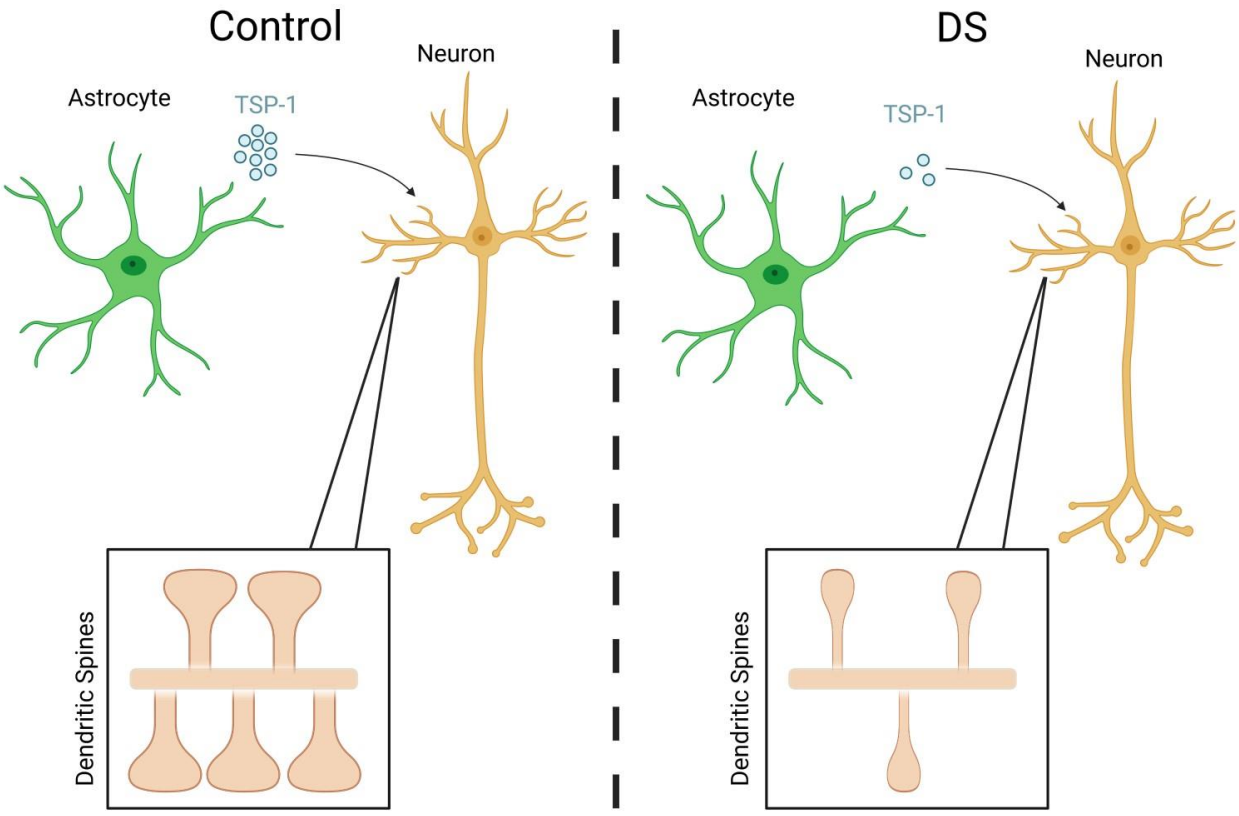

Figure 2: Reductions in the astrocytic production and secretion of TSP-1 from DS cells causes abnormal spine shape and number. (Created in Biorender)

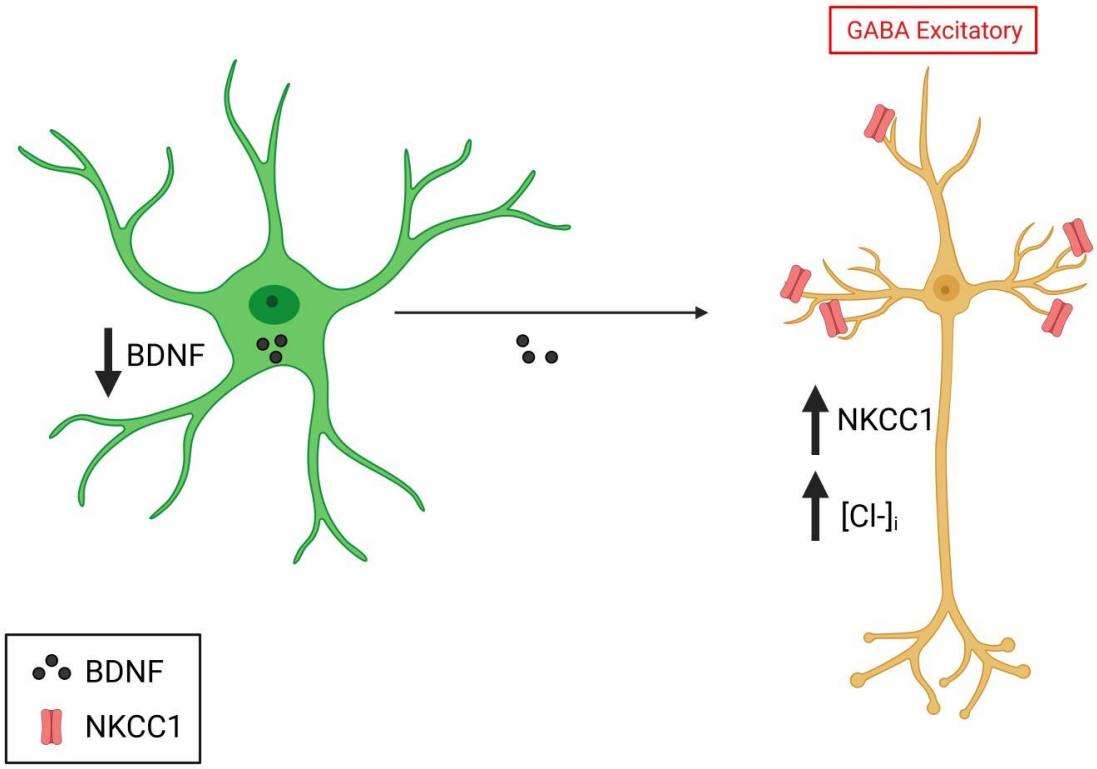

Figure 3: Implication of astrocytes in the GABA switch in DS. (Created in Biorender)

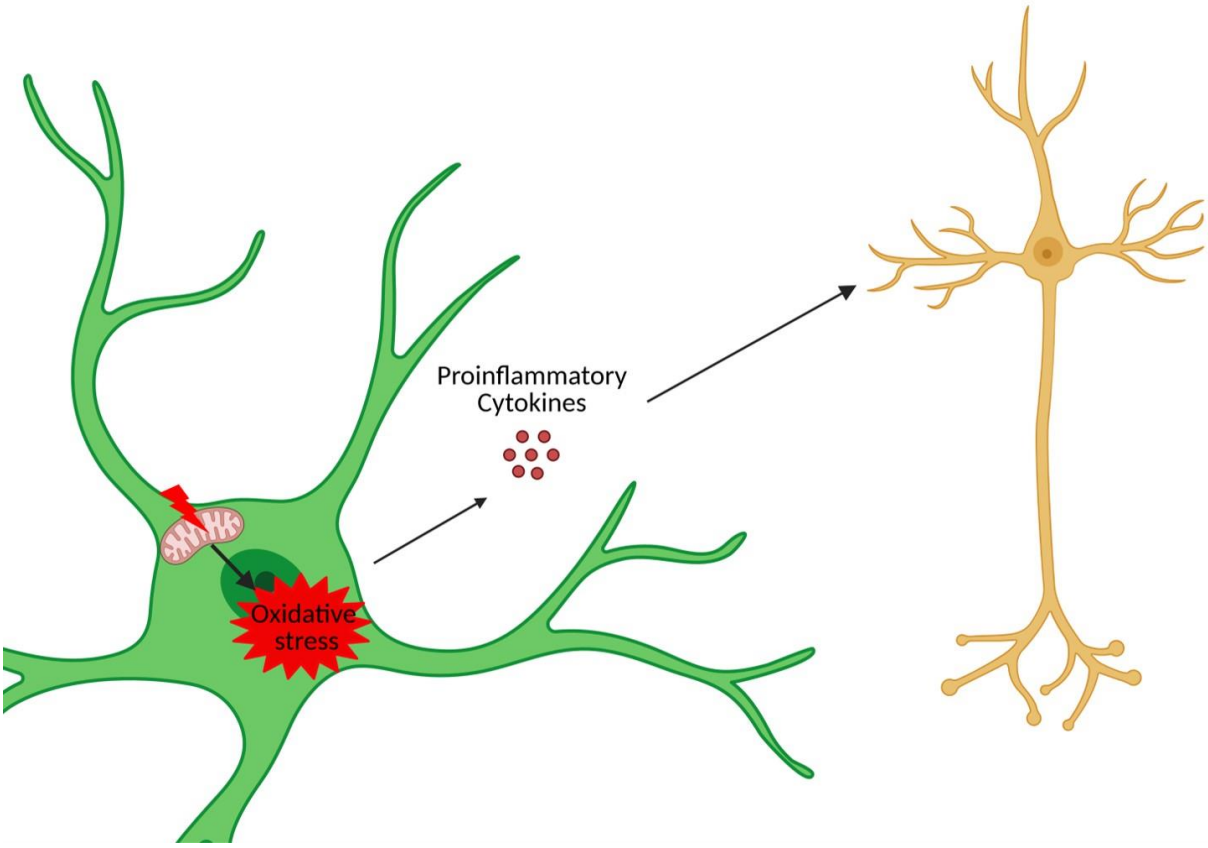

Figure 4: Astrocytic oxidative stress and mitochondrial dysfunction in neuronal death. (Created in Biorender)

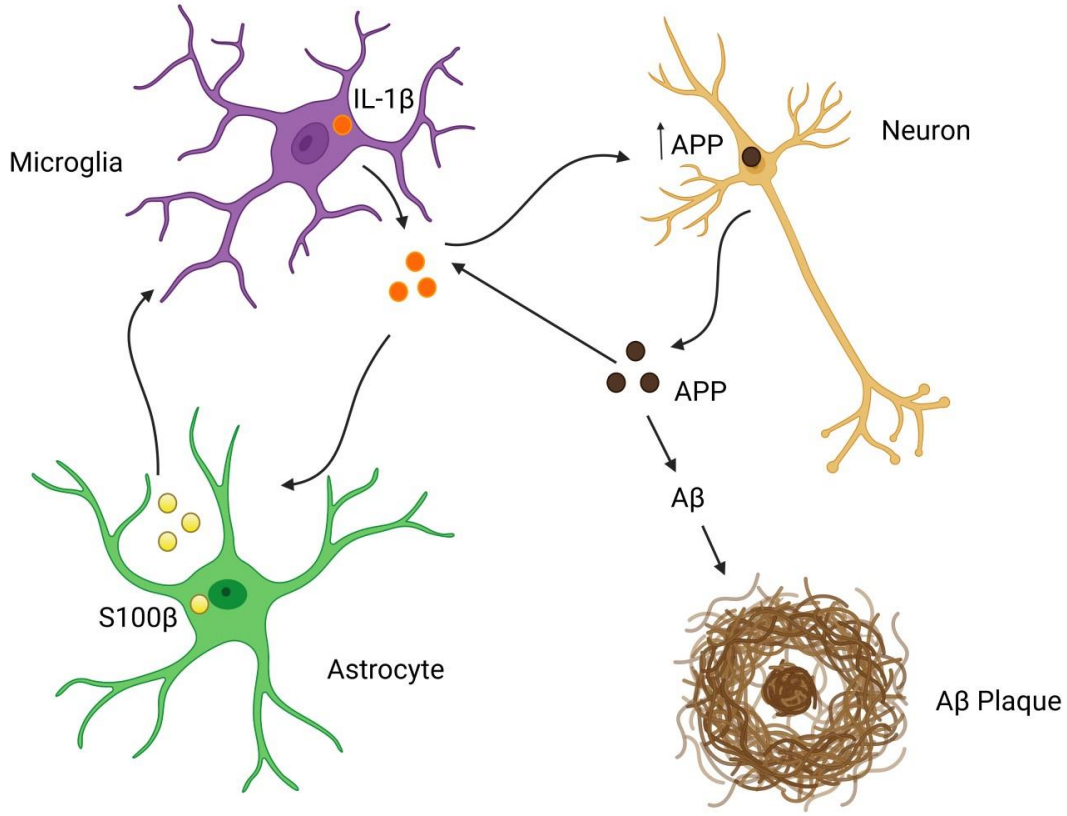

Figure 5: Astrocytes as drivers of Aβ pathology in the DS brain? (Created in Biorender)

PREFACE TO CHAPTER 3

MODELING DISEASE WITH HUMAN IPSCS

In 1962, Dr. John Gurdon showed that the nucleus of a terminally differentiated cell was able to be restored to an early embryonic state by transferring it into a depleted fertilized egg cell. Furthermore, he showed that this transplanted terminally differentiated cell could then go on to become a tadpole and ultimately a frog (Gurdon, 1962; Gurdon, Laskey, & Reeves, 1975). Through these experiments he demonstrated two major findings: 1) that all nuclei contain the genes required to be reprogrammed into a stem cell, and 2) that the factors expressed in the cytoplasm of an oocyte are able to reprogram a terminally differentiated cell into a stem cell. These experiments, called somatic cell nuclear transfer (SCNT) are the foundation for what later led to the discovery of iPSCs.

Thirty years later, Dr Shinya Yamanaka and Dr Kazutoshi Takahashi performed studies in the mouse embryo in which they searched for factors able to induce the reprogramming of somatic cells to pluripotent cells. They identified four transcription factors: octamer-binding transcription factor 4 (Oct4), SRY-box transcription factor (Sox2), c-Myc, and Krüppel-like factor 4 (Klf4) which they called the Yamanaka factors. These factors are sufficient for the reprogramming of terminally differentiated cells into iPSCs (Takahashi & Yamanaka, 2006). A year later they showed that a similar approach could be used for humans, and they created the first human iPSCs (hiPSCs) (Takahashi & Yamanaka, 2006).

This novel technology completely revolutionized research and medicine, and in 2012 Dr Gurdon and Dr Yamanaka received the Nobel Prize in Physiology or Medicine for their outstanding and innovative contributions. Since 2007, the field of iPSC research has grown exponentially, with the development of numerous reprogramming protocols which allow for the reprogramming of several cell types such as fibroblasts, CD34+ peripheral blood cells, renal epithelial cells isolated from urine

and keratinocytes (Loh et al., 2009; Xue et al., 2013). Many protocols have been established to differentiate these iPSCs into various cell lineages.

In the last 13 years there has been an explosion in iPSC research. Many studies have used hiPSCs to model human diseases and for early stages of drug discovery. Indeed, these cells are especially useful to model rare diseases for which there are no existing models, as well as, for studying human cells which are difficult to obtain. iPSCs have been especially useful in neuroscience, a discipline in which cells are hard to access and in which complex and human specific neurological diseases occur which cannot always be well-represented in animal models. Protocols allowing the differentiation of iPSCs into neurons were quickly established two years following the original research of Dr Yamanaka (Wernig et al., 2008), and protocols for glial cell differentiation including astrocytes emerged a few years later (Emdad, D'Souza, Kothari, Qadeer, & Germano, 2012). Since 2012, many protocols have been developed to differentiate astrocytes from iPSCs (Chandrasekaran, Avci, Leist, Kobolak, & Dinnyes, 2016) and these have been used successfully to study the role of astrocytes in disease conditions such as PD (A. di Domenico et al., 2019; Rhee et al., 2011), AD (V. C. Jones, Atkinson-Dell, Verkhratsky, & Mohamet, 2017), neuroinflammation (Zhou et al., 2019), ALS (Birger et al., 2019) and others (Juopperi et al., 2012; Serio et al., 2013).

CHAPTER 3

Human iPSC-Derived Down Syndrome Astrocytes Display Genome-Wide Perturbations in Gene Expression, an Altered Adhesion Profile, and Increased Cellular Dynamics.

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3.1 ABSTRACT:

Down syndrome (DS), caused by the triplication of human chromosome 21, leads to significant alterations in brain development and is a major genetic cause of intellectual disability. While much is known about changes to neurons in DS, the effects of trisomy 21 on non-neuronal cells such as astrocytes are poorly understood. Astrocytes are critical for brain development and function, and their alteration may contribute to DS pathophysiology. To better understand the impact of trisomy 21 on astrocytes, we performed RNA-sequencing on astrocytes from newly produced DS human induced pluripotent stem cells (hiPSCs). While chromosome 21 genes were upregulated in DS astrocytes, we found consistent up- and down-regulation of genes across the genome with a strong dysregulation of neurodevelopmental, cell adhesion, and extracellular matrix molecules. ATAC (Assay for Transposase-Accessible Chromatin)-seq also revealed a global alteration in chromatin state in DS astrocytes, showing modified chromatin accessibility at promoters of cell adhesion and extracellular matrix genes. Along with these transcriptomic and epigenomic changes, DS astrocytes displayed perturbations in cell size and cell spreading as well as modifications to cell-cell and cell-substrate recognition/adhesion, and increases in cellular motility and dynamics. Thus, triplication of

chromosome 21 is associated with genome-wide transcriptional, epigenomic, and functional alterations in astrocytes that may contribute to altered brain development and function in DS.

3.2 INTRODUCTION:

Down syndrome (DS) is a major genetic cause of disability and affects ~1 in 700 births worldwide (Parker et al., 2010). In DS, triplication of HSA21 (*Homo sapiens* chromosome 21) causes DS individuals to have pronounced intellectual disability that often coincides with brain disorders/diseases including epilepsy, autism, mental disability, and Alzheimer's disease (AD) (Antonarakis, 2017; Antonarakis et al., 2004; Arya et al., 2011; Asim et al., 2015; Glasson et al., 2002; Lott, 2012; Lott & Dierssen, 2010; Roizen & Patterson, 2003). Analysis of DS brain and animal modeling studies have shown reduced brain volume and neuronal density (Pinter, Brown, et al., 2001; Teipel et al., 2003). This is attributed to reduced neurogenesis, increased neuronal apoptosis, and over-representation of glial lineages (Busciglio & Yankner, 1995; Guidi et al., 2008; Lu et al., 2011; K. E. Wisniewski, 1990; Zdaniuk et al., 2011). Abnormal dendritic arborization, spine morphology, and spine density have also been reported, indicating altered formation and maintenance of neuronal circuits in DS (L. E. Becker et al., 1986; P. V. Belichenko, Kleschevnikov, Salehi, Epstein, & Mobley, 2007; Garner & Wetmore, 2012; Takashima, Iida, Mito, & Arima, 1994).

While changes to neurons in DS have been reported, molecular and physiological disruptions to other brain cells are less understood. Among the cell types whose alteration may affect multiple aspects of brain development, function, and response to injury are astrocytes, as they regulate many processes including synapse formation and plasticity, extracellular ion and neurotransmitter homeostasis, and neurovascular coupling (Denis-Donini, Glowinski, & Prochiantz, 1984; Drejer, Larsson, & Schousboe, 1982; Farmer & Murai, 2017; Parpura et al., 1994; Zhang & Barres, 2010). Emerging evidence implicates astrocyte dysfunction in a variety of neurodevelopmental disorders and

neurodegenerative diseases (Phatnani & Maniatis, 2015; Sloan & Barres, 2014; Y. Yang, Higashimori, & Morel, 2013; Yates, 2015), raising the possibility of a direct contribution of astrocytes to DS pathophysiology. Consistent with this, astrocytes are more abundant in DS fetal cortex, and appear more morphologically mature (Zdaniuk et al., 2011). Elevated levels of glial fibrillary acidic protein (GFAP) and S100ß, as well as altered cellular morphology also indicate increased astrocyte reactivity in the adult DS brain (Jorgensen, Brooksbank, & Balazs, 1990; Mito & Becker, 1993). In DS mouse models, astrocytes decrease neuronal activity of cholinergic neurons (Nelson et al., 1997) while fetal tissue-derived DS astrocytes have reduced secretion of the synaptogenic factor thrombospondin-1 and are believed to contribute to the alteration of dendritic spine structure and reduced dendrite arborization (Garcia et al., 2010). DS astrocytes have also been shown to have disrupted mitochondrial morphology and function (Helguera et al., 2013) but with potentially greater anti-oxidant capacity versus euploid astrocytes (Sebastia et al., 2004). Recent advances with human induced pluripotent stem cell (hiPSC) technologies have shown increased oxidative stress in iPSC-derived DS astrocytes which results in the release of factors that promote neuronal apoptosis, implicating astrocytes in neuronal death and overall reduction in neuronal number (C. Chen et al., 2014). DS astrocytes also contribute to elevations in mTOR signaling and synaptic marker expression in neurons (Araujo et al., 2018). Thus, astrocytes may be primary effectors in DS pathophysiology. However, a detailed analysis of how trisomy 21 impacts the overall molecular profile and functional properties of astrocytes is still required.

To understand how trisomy 21 affects the molecular properties and function of astrocytes, we created astrocytes from newly produced DS hiPSCs. RNA-sequencing (RNA-seq) analysis showed genome-wide perturbations in gene expression in DS astrocytes with a strong dysregulation of molecules that function during nervous system development including those involved in axon guidance, cell adhesion/recognition, and extracellular matrix (ECM) organization. Interestingly, the same gene sets were not disrupted in isogenic DS neural precursor cells (NPCs), demonstrating that

the genome-wide changes in astrocytes are not transposed from an NPC state. Using ATAC-seq, we also observed global changes to the chromatin landscape in DS astrocytes and differential accessibility to the promoters of genes involved in cell adhesion and ECM organization. Consistent with these transcriptomic and epigenomic modifications, DS astrocytes have altered cell spreading, cell-cell and cell-substrate adhesion, motility and specific changes to Protocadherin (PCDH) mediated cellular adhesion. Overall, this study reveals that hiPSC-derived DS astrocytes have wide-spread transcriptomic and epigenomic changes that disrupt the adhesion profile of DS astrocytes, offering insight into non-neuronal changes that may occur in the developing and mature DS brain.

3.3 RESULTS:

GENERATION OF DS AND CONTROL HIPSCS, NPCS, AND ASTROCYTES

We created six new hiPSC lines from three DS and three control (CTL) fibroblast cell lines using episomal reprogramming techniques (Fig 1A). All DS and CTL fibroblast lines were obtained from the Coriell Institute for Medical Research. An additional DS hiPSC line was acquired from the American Type Culture Collection (ATCC). Fibroblast samples were matched for sex, age and ethnicity (S1 Table). All generated hiPSC lines expressed the pluripotency markers OCT4, TRA-1-60, and SSEA4 (Fig 1C and S2A Fig). All three CTL hiPSC lines showed a normal karyotype and three DS hiPSC lines were trisomic for chromosome 21 (Fig 1B). One DS line (AG04823) showed a disomic karyotype and an insertion in the X chromosome, and hence was not used for subsequent studies. CTL and DS lines will be made available to the scientific community.

DS and CTL hiPSC lines were differentiated into NPCs using a monolayer, double-Smad inhibition protocol (Chambers et al., 2009; Scott Bell, 2019) (Fig 1A), after which all lines expressed NPC markers (Nestin, SOX1, and PAX6; Fig 1C and S2B Fig). A monolayer protocol was then used to differentiate NPCs into astrocytes using differentiation protocols adapted from Shaltouki et al and

Roybon et al (Roybon et al., 2013; Shaltouki, Peng, Liu, Rao, & Zeng, 2013). Approximately 90 days after initiating astrocyte differentiation, the majority of cells expressed the astrocytic markers GFAP, S100ß and SOX9, and downregulated the NPC marker SOX1 (Fig 1D-E, S4A Fig and S5A Fig). Cells also displayed more complex morphologies typical of astrocytes *in vitro* (Fig 1D). Quantitative reverse transcriptase polymerase chain reaction (RT-PCR; now referred to as qPCR) analysis showed that CTL and DS astrocytes showed similar levels of astrocytic mRNAs including GFAP, EAAT1/GLAST, GS, SOX9, and ALDH1L1 (Fig 1F). This was consistent with immunofluorescence labeling which showed that DS and CTL astrocytes expressed similar amounts of ALDH1L1 and EAAT1/GLAST protein (S4B Fig and S5B). CTL and DS astrocytes lacked expression of the neuronal marker Smi-312 and the oligodendrocyte marker CC1 (S3A Fig). CTL and DS astrocytes also had comparable levels of GFAP and pro-inflammatory markers (*data not shown*), indicating that DS astrocytes do not upregulate markers of glial reactivity. qPCR analysis of DS astrocytes confirmed upregulation of chromosome 21 genes including APP, SOD1, SUMO3, DYRK1A, and S100B (Fig 1G). Thus, DS astrocytes express known markers of differentiated astrocytes and upregulate genes on chromosome 21.

DS ASTROCYTES SHOW NORMAL SPONTANEOUS AND ATP-EVOKED CA^{2+} RESPONSES

A prominent feature of astrocytes is their ability to show spontaneous and evoked intracellular Ca^{2+} elevations which have been associated with numerous functional properties of astrocytes (Perea, Navarrete, & Araque, 2009). To determine if DS astrocytes have altered Ca^{2+} elevations (Scemes & Giaume, 2006), we performed live cell imaging of Ca^{2+} dynamics in CTL and DS astrocytes. CTL and DS astrocytes showed similar spontaneous Ca^{2+} events with no significant differences in the percentage of active cells or changes in Ca^{2+} event amplitude (S3B-C Fig). A recent study demonstrated an increase in spontaneous activity in DS astrocytes (Mizuno et al., 2018). However this was not the

case in this study. It should be noted that a different cell line was used in Mizuno et al. (2018) with an isogenic control. It would be interesting to further examine the potential for line-specific differences in spontaneous Ca^{2+} dynamics in DS astrocytes. To determine if DS and CTL astrocytes differed in evoked Ca^{2+} responses, ATP pulses were delivered to the cells. DS and CTL astrocytes were similar with respect to the percentage of ATP-responsive cells and Ca^{2+} event amplitude (S3D-F Fig). Thus, our results indicate that CTL and DS astrocytes display similar spontaneous and evoked Ca^{2+} dynamics.

GENOME-WIDE TRANSCRIPTIONAL DYSREGULATION IN DS ASTROCYTES

To determine how trisomy 21 affects the transcriptome of astrocytes, we performed RNA-seq on the 3 DS and 3 CTL astrocyte lines. Analysis of CTL astrocytes showed that they expressed many genes found in mature human astrocytes (Zhang et al., 2016) with some residual expression of immature astrocyte genes (S6 Table). This suggests that astrocytes in this study have a transcriptional profile intermediate to fetal and mature astrocytes. We performed differential gene expression analysis of DS and CTL astrocytes which revealed 725 protein-coding genes with altered mRNA levels in DS astrocytes (adjusted p-value < 0.05). Heatmaps of the top 50 differentially expressed genes, as well as PCA analysis and sample clustering were generated for differential analysis (S7 Figure). These analyses highlight the correct segregation of samples from each cell line as well as the variability between the cell lines. Inter-cell line variability has been reported in other studies (Lang et al., 2019; Switonska et al., 2018), and highlights the complexity of working with different human samples which can possess heterogeneous transcriptome profiles. Utilization of isogenic lines from each sample is one approach to reduce such variability.

More detailed analysis of RNA-seq datasets showed that approximately 20% of all chromosome 21 genes were upregulated. This is consistent with a previous microarray study of

multiple regions of DS brain tissue (Olmos-Serrano et al., 2016) (Fig 2A,B). 51 of 52 differentially expressed chromosome 21 genes were upregulated, including known DS targets such as APP, DYRK1A, SOD1, and SUMO3, with a median absolute fold change of 1.56. Interestingly, we observed consistent genome-wide transcriptional changes in DS astrocytes. Of 725 differentially expressed genes, 673 genes (~93%) were located outside of chromosome 21, and were widely distributed across the genome (Fig 2C). Furthermore, whereas chromosome 21 genes were predominantly upregulated (98.1%), differentially expressed mRNAs from other chromosomes were both up and downregulated (51.5% upregulation and 48.5% downregulation) (Fig 2 C,D). These results show that trisomy 21 leads to a robust, genome-wide perturbation of transcriptional activity in astrocytes.

DS ASTROCYTES SHOW STRONG ALTERATION OF GENES INVOLVED IN NEURONAL DEVELOPMENT, CELL ADHESION, AND ECM ORGANIZATION.

Gene ontology (GO) analysis revealed that the 725 differentially expressed genes were primarily involved in nervous system development, axon guidance, regionalization, ECM organization, and cell adhesion (Fig 2E). Differentially expressed genes in DS astrocytes were also enriched in cellular components related to the ECM (Fig 2E). Interestingly pathway analysis also reports alterations to ECM-receptor interactions, ECM proteoglycans and Laminin interactions (S12 A Fig). To rule out potential false-positives of RNA-seq, we validated the change in gene expression by performing qPCR on the top 25 differentially expressed genes associated with GO terms, as well as a few additional genes of interest. BRSK2, CADM2, COL2A1, DCC, FAT4, ITGB8, JAM2, LAMA2, PCDH9, PCDH19 and PCDHB2 showed increases in mRNA expression (S8A Fig) while ACKR3, ADAMTSL4, COL4A4, COL7A1, COMP, FOXG1, LAMA5, LHX2, NGFR, OMD, PCDHA7, PCDHB7, PCDHGC3, PCDHGC4, RELN, SDC4, SEMA3F, SHH, SIRPA and SPON1 showed

reductions in mRNA levels in DS astrocytes (S8B Fig). This revealed strong correspondence between the fold changes in gene expression derived from RNA-seq and qPCR (Pearson correlation coefficient: 0.741) (Fig 2F). PCDHGC4 and RELN were the only mRNAs for which changes determined by the two experimental approaches did not directly correspond. Taken together, these findings demonstrate that trisomy 21 in astrocytes has a profound genome-wide impact on gene expression, with a particularly large effect on genes related to cell adhesion and ECM organization.

ALTERED CHROMATIN STATE IN DS ASTROCYTES.

Recent studies have shown that in addition to causing whole transcriptome dysregulation, trisomy 21 causes changes in epigenetic mechanisms which can alter gene expression. Indeed, changes in DNA methylation has been shown to be associated with DS, and more specifically hypermethylation has been observed in DS brain (Bacalini et al., 2015; Chango et al., 2006; M. J. Jones et al., 2013; Kerkel et al., 2010; Letourneau et al., 2014; Lu et al., 2016; Pogribna et al., 2001). To investigate if chromatin accessibility in DS astrocytes is altered, we performed ATAC-seq (Buenrostro, Wu, Chang, & Greenleaf, 2015) on three CTL and DS astrocyte lines. This revealed approximately fourteen thousand differentially accessible peaks. Differential chromatin accessibility was distributed across the genome, with an even split of increased and decreased accessibility (Fig 3A). Approximately 6% of these sites were located in promoter regions (defined as within 3kb of the transcription start site). Remarkably, we found that upregulated and downregulated genes identified by RNA-seq do not have corresponding differential accessibility at promoters in CTL and DS astrocytes (Fig 3B, S9 Fig). We performed GO analysis to identify the molecular function of genes with altered chromatin accessibility at their promoter regions. This analysis showed that the top five biological processes enriched for genes with differentially accessible chromatin include processes related to cell growth, cell adhesion, and ECM such as: regulation of actin, cell substrate adhesion, and extracellular matrix

organization (Fig 3C). Cellular component analysis also revealed that the products of those genes are largely related to cell adhesion and the ECM. These results reveal complex epigenomic changes in DS astrocytes, especially related to genes involved in cell adhesion and ECM organization. Furthermore, the results suggest that changes to the transcriptome of DS astrocytes are not simply due to direct epigenetic modifications to promoter regions of dysregulated mRNAs.

DIFFERENTIAL CHANGES IN GENE EXPRESSION IN DS NPCS AND ASTROCYTES.

Since trisomy 21 is known to cause alterations to stem cells and precursor cells (B. Liu, Filippi, Roy, & Roberts, 2015), it is possible that the differences in expression of ECM and adhesion genes in DS astrocytes are established at the NPC stage and prior to their differentiation into astrocytes. To determine if this is the case, we analyzed NPCs for the top 25 gene targets found in DS astrocytes and validated by qPCR. DS and CTL NPCs showed similar expression levels of PAX6, SOX1, and SOX2, and DS NPCs upregulated chromosome 21 genes DYRK1A, APP, and SUMO3 (Fig 4A), indicating CTL and DS NPCs have comparable expression of NPC-enriched mRNAs and DS-associated genes, respectively. However, surprisingly, only 5 of 27 validated dysregulated ECM and cell adhesion mRNAs in DS astrocytes showed changes in DS NPCs (Fig 4B, C). Of these, only 2 mRNAs were downregulated in both DS NPCs and DS astrocytes (COL7A1 and PCDHGC3) and 3 were regulated in the opposite direction in DS NPCs and DS astrocytes (ITGB8, PCDH9, and PCDH19) (Fig 4D). These findings indicate that trisomy 21 has selective effects on the expression of ECM and cellular adhesion genes in astrocytes and that the transcriptional changes observed in DS astrocytes are not established in NPCs prior to their differentiation.

ALTERED CELL ADHESION AND RECOGNITION OF DS ASTROCYTES

Considering the transcriptomic and epigenomic changes in DS astrocytes related to genes involved in cell adhesion and ECM organization, we investigated if cell recognition and adhesion properties of DS astrocytes were perturbed. To determine if dysregulation of specific molecules identified in RNA-seq and qPCR analysis led to changes in cell adhesion/recognition, we performed assays involving protocadherins (PCDHs). PCDHs are a family of clustered genes that play critical roles in cell recognition and are important for processes during nervous system development including synaptogenesis and axonal and dendritic growth (Garrett & Weiner, 2009; Light & Jontes, 2017; Molumby, Keeler, & Weiner, 2016), which are processes known to be defective in DS (L. Becker, Mito, Takashima, & Onodera, 1991). RNA-seq analysis showed many PCDHs to be dysregulated in DS astrocytes (Fig 2D, F, S8 Fig). This is consistent with a previous study in which DS fetal cortical lysates were also shown to have downregulated gamma PCDHs (PCDHG) (El Hajj et al., 2016). We first assessed if PCDHG protein levels were changed in DS astrocytes. We utilized a pan-PCDHG antibody to screen for overall changes in expression of this PCDH family using Western blot analysis. We found that PCDHG protein was significantly reduced (3.85 fold) in DS astrocytes (Fig 5A, B, S10 Fig). To investigate if the alteration of PCDHG protein in DS astrocytes caused specific functional changes to PCDHG-mediated homophilic cell recognition/adhesion, we performed cell binding assays on selective PCDH substrates. Interestingly, DS astrocytes showed a selective loss of binding to a PCDHGC3 substrate (Fig 5C), consistent with the downregulation of PCDHGC3 mRNA (Fig 2D, F). In contrast, DS astrocytes showed similar levels of binding to substrates of PCDH10 and PCDH12 as CTL astrocytes (Fig 5C). Thus, DS astrocytes have selective impairments in PCDHG-mediated cellular recognition/adhesion.

During the course of this study, we noted that DS astrocytes appeared larger, more spread and adherent to the surface of the tissue culture vessel/dish than CTL astrocytes. To investigate this, we labelled astrocytes for the membrane bound glutamate transporter EAAT1 and measured the area

covered by individual astrocytes. We found that DS astrocytes were significantly larger than CTL astrocytes (Fig 6A, B). DAPI labeling showed that CTL and DS astrocytes have similar sized nuclei (Fig 6A, B). Flow cytometry further showed that CTL and DS astrocytes have similar sizes when in suspension (Fig 6C, S11A, B). The change in cell size of DS astrocytes did not appear to be caused by differences in cell proliferation or senescence, as the astrocyte cultures were passaged at similar frequencies, and no differences were observed in the expression of senescence markers (Althubiti et al., 2014) (*data not shown*). To assess if DS astrocytes have increased cell spreading ability which affects their size, we performed a cell spreading assay. Astrocytes were fixed and labelled for actin with phalloidin-488 after 30, 60, 90 and 120 minutes (Fig 6D). Cell area at each time point was measured allowing us to obtain the rate of cell spreading. We found that DS astrocytes have a significantly increased rate of spreading (Fig 6E). Thus, the observed increases in the size of DS astrocytes are likely related to their increased cell spreading ability.

To determine if DS astrocytes have alterations in cell adhesion, we performed a series of experiments to examine both cell-cell and cell-substrate adhesion. Cell aggregation assays revealed an altered cell-cell adhesion profile of DS astrocytes. DS astrocytes placed in suspension formed smaller aggregates than their CTL counterparts (Fig 6F, G). Interestingly, this altered cell-cell adhesion property was accompanied by an increased number of astrocytes directly adherent to the uncoated petri dish and a reduced number of non-aggregated DS astrocytes remaining in suspension (Fig 6H-I). Thus, DS astrocytes have an altered cell-cell adhesion profile, showing a preferential attachment to the tissue culture dish. Repeating the experiments using ultralow adhesion plates, in which cells were not capable of adhering to the bottom of the dish, showed no difference in the size of cell aggregates (S11C, D Fig), suggesting that DS astrocytes do not have a cell-cell adhesion deficit but rather a preferential attachment to the tissue culture dish.

Finally, we performed cellular motility assays to investigate whether DS astrocytes have altered motility or dynamics under basal conditions. This was done using live cell imaging and automated cell tracking (Hayer et al., 2016). These experiments revealed that over the course of 4 hours, DS astrocytes plated at the same density as CTL astrocytes have increased cell motility (Fig 7A), as indicated by increased net displacement and migration persistence (Fig 7B-D). Interestingly differences in autonomous cell velocity were not observed (Fig 7E), suggesting that although DS astrocytes move at similar speeds to CTL astrocytes, DS astrocytes cover longer distances and have more persistent directed motility then their CTL counterparts. Altogether, these results suggest that DS astrocytes possess an altered functional adhesion state that impacts cell size, cell spreading, cell-cell and cell-substrate adhesion and cellular dynamics (Fig 7F).

3.4 DISCUSSION

DS is a genetic disorder associated with neurodevelopmental alterations and aging-related brain pathologies, thus presenting complex neurological challenges for affected individuals throughout their lifespan (Lott, 2012). As astrocytes are implicated in a host of neurodevelopmental disorders and neurodegenerative diseases (Phatnani & Maniatis, 2015; Ricci, Volpi, Pasquali, Petrozzi, & Siciliano, 2009; Sloan & Barres, 2014; Verkhratsky, Steardo, Parpura, & Montana, 2016), trisomy 21 in these cells may contribute to multiple aspects of DS etiology (Antonarakis et al., 2004). Indeed, alterations in astrocyte structure and molecular composition have been observed in the developing and adult DS brain (Colombo, Reisin, Jones, & Bentham, 2005; Jorgensen et al., 1990; Mito & Becker, 1993; Zdaniuk et al., 2011). Here, we utilized newly created DS and CTL hiPSC lines to provide a comprehensive analysis of perturbations in gene expression and functional properties of DS astrocytes. Using RNA-seq, we observed a consistent genome-wide disruption in gene expression. Interestingly, while chromosome 21 genes show an expected upregulation (Mao, Zielke, Zielke, &

Pevsner, 2003), their increase represented only a small fraction (~7%) of the overall changes occurring within DS astrocytes. Gene ontology analysis further revealed extensive dysregulation of molecules that participate in cell adhesion and ECM organization. ATAC-seq revealed an alteration in chromatin accessibility in DS astrocytes, and notably showed a preferential dysregulation in chromatin accessibility of promoter regions of cell adhesion and ECM genes. Finally, we investigated the functional properties of DS astrocytes and uncovered changes to cell size, cell spreading, cell-cell adhesion, cell motility, as well as, alterations in PCDHG-mediated cellular adhesion/recognition. These collective results demonstrate a significant perturbation in the molecular and functional cell adhesion state of DS astrocytes.

As triplication of chromosome 21 is the cause of DS, most studies over the last several decades have focused on cellular pathways related to chromosome 21 genes. Recently, however, genome-wide disruptions in gene expression have been identified in various DS tissues and cell types (Briggs et al., 2013; FitzPatrick et al., 2002; Lockstone et al., 2007; Mao et al., 2005; Saran et al., 2003). We now show that trisomy 21 causes a specific genome-wide transcriptome dysregulation in astrocytes which are key cells that contribute to brain development, homeostasis, and function. Our study also identified genome-wide changes in the chromatin state of DS astrocytes, which is in line with other studies suggesting that epigenomic changes may be a common feature in DS and linked to genome-wide transcriptome dysregulation. However, the lack of direct correspondence between mRNAs/genes identified by RNA-seq and ATAC-seq highlights the complex nature of the genetic and transcriptional perturbation found in DS astrocytes. This lack of correspondence has been previously reported in DS fibroblasts (Letourneau et al., 2014). Our results suggest a complex cascade of epigenomic and transcriptional alterations occurring in astrocytes with trisomy 21 that may include dysregulation of transcription factors and non-coding RNAs, as well as, alterations to epigenetic machinery. Our results, along with those from the above-mentioned studies, help to understand the global landscape

of epigenomic and transcriptional modifications in DS, and provide a better comprehension of this complex genetic condition with respect to non-neuronal cells of the brain.

Our transcriptomic analysis revealed extensive dysregulation of molecules that participate in cell adhesion and ECM organization. Remarkably, alterations in cell adhesion and ECM molecules have been reported in various non-nervous system cell types in DS. In DS fetal samples, ECM genes have been shown to be upregulated in cardiac tissues (Conti et al., 2007). Altered expression of ECM molecules (collagen VI and hyaluronan) have also been identified in skin fibroblasts and in the umbilical cord of DS individuals (Grossman et al., 2011; Karousou et al., 2013). In addition, DS individuals have been shown to have an altered ECM composition known to be protective against solid tumors (Benard, Beron-Gaillard, & Satge, 2005; Zorick et al., 2001), which is thought to be the cause of their decreased susceptibility to developing certain types of cancers (Q. Yang, Rasmussen, & Friedman, 2002). The altered expression of adhesion and ECM molecules observed in DS astrocytes may have important repercussions during brain development and also contribute to their response to CNS injury and disease (Wiese, Karus, & Faissner, 2012). The altered adhesion profile of astrocytes could affect their migration, maturation, and subsequent integration into brain microenvironments during development (Rakic, 1972, 2007; Schmechel & Rakic, 1979). Interestingly, recent RNA-seq studies performed on hiPSC-derived DS neurons (Gonzales et al., 2018; Huo et al., 2018) have also shown gene expression changes in ECM and cell adhesion molecules, implying that such changes may occur globally in the DS brain, affecting both neurons and astrocytes. Such changes may have broad implications in early neural circuit wiring and synaptogenesis, which are known to be altered in the DS brain. Importantly, however, it should be noted that while similar results were found by GO analysis, only a small percentage of differentially expressed genes in astrocytes identified in our study are shared with the studies on neurons (S12 B, C Fig). Thus, while cell adhesion and ECM organization are generally altered in hiPSC-derived DS neurons and astrocytes, the specific genes responsible for

such alterations appear to be largely different. Additional factors to be taken into consideration in future studies will be the influence of sex, age, and ethnicity on transcriptional changes in both of these cell types. Understanding the underlying mechanisms for such selective defects in DS in neurons and astrocytes will help dissect the possible causes of intellectual disability and provide additional paths for more specific medical interventions during development.

Genome-wide changes in gene expression in astrocytes caused by trisomy 21 may also affect the ability of astrocytes to respond to CNS injury or disease. Astrocytes play a complex role following injury and in disease, forming glial scars and recruiting other cell types including immune cells (Sofroniew & Vinters, 2010; Tate, Tate, & LaPlaca, 2007). Astrocytes become reactive and motile in order to respond to sites of injury such as in stroke (Wilhelmsson et al., 2006), traumatic injury (Burda et al., 2016) or Aβ plaque deposition (Hou et al., 2011; Olabarria, Noristani, Verkhratsky, & Rodriguez, 2010) and participate in axonal regeneration and remyelination after injury (Clemente, Ortega, Melero-Jerez, & de Castro, 2013; Gotz et al., 1996; Properzi et al., 2005). The altered cell adhesion and recognition properties of DS astrocytes identified in this study may be relevant for DS-associated AD (a pathology known to occur late in the life of DS individuals), where astrocytes become reactive around Aβ plaques (Frost & Li, 2017). Targeting the altered adhesive state and motility of DS astrocytes could be a novel target to mitigate injury-related and progressive AD pathology later in life.

3.5 MATERIALS AND METHODS:

Generation of hiPSCs

Six patient fibroblast cell lines were obtained from the Coriell Institute and one line from American Type Culture Collection (ATCC) (S1 Table) and cultured in DMEM/F-12 supplemented with 20% heat inactivated FBS. Effort was made to match gender, age, ethnicity, and origin of fibroblasts. The fibroblasts were electroporated with episomal vectors containing Oct4, Sox2, c-Myc,

Klf4 as well as a puromycin selection cassette (Alstem). Puromycin selection was performed 48 hours post electroporation. The cells were then cultured for 30 days in TeSR-E7 reprogramming medium (Stem Cell Technologies) with daily medium changes, and hiPSC colonies were picked manually and plated on matrigel (Corning) coated plates. Three clones were selected per cell line and grown and expanded in mTeSR maintenance medium (Stem Cell Technologies). Karyotyping was performed by the Center for Applied Genomics (The Hospital for Sick Children, Toronto).

Production of neural precursor cells and astrocytes

Neural precursors cells (NPCs) were generated from hiPSCs using a double-Smad inhibition protocol (Chambers et al., 2009). The hiPSCs were dissociated and grown as a monolayer on matrigel coated plates in DMEM/F-12 (Sigma) supplemented with N2, B27, BSA (1mg/mL), SB431542 (10μM) and noggin (200ng/mL) for three weeks (neural induction medium; NIM) (Scott Bell, 2019). The cells were then dissociated and grown in suspension in NPC maintenance medium (Stem Cell Technologies) for 7-9 days. Finally, the cells were plated on matrigel and cultured in NPC maintenance medium.

Astrocytes were derived from NPCs using a monolayer differentiation protocol (Fig 1A) adapted from Shaltouki et al, Roybon et al and Serio et al 2013 (Roybon et al., 2013; Serio et al., 2013; Shaltouki et al., 2013). NPCs were passaged onto poly-D-lysine and matrigel coated plates in DMEM/F-12 medium supplemented with FBS (10%; Gibco), EGF (20ng/mL; Genscript) and FGF2 (20ng/mL; Cell Signaling) for 2 weeks and were passaged as needed. The astrocytes were then cultured on tissue culture treated culture dishes in DMEM/F-12 medium supplemented with FBS (10%; Gibco), EGF (20ng/mL) (FGF2 (20ng/mL) and CNTF (5ng/mL; Genscript) (Protocol A), or cultured in DMEM/F-12 medium supplemented with FBS (10%; Gibco), EGF (20ng/mL) and FGF2 (20ng/mL) (Protocol B) for 6 weeks and were passaged as needed. Astrocytes were then matured in

DMEM/F-12 medium supplemented with 10% FBS and CNTF (5ng/mL) for an additional 4 weeks. Many studies have reported a gliogenic shift and an overall increase in the numbers of astrocytes in the DS brain (Busciglio & Yankner, 1995; Guidi et al., 2008; Lu et al., 2011; K. E. Wisniewski, 1990; Zdaniuk et al., 2011). We observe no difference in the efficiency of astrocyte differentiation or astrocyte numbers between our DS and control cell lines. This is likely due to the fact that we are inducing astrocyte differentiation using a specific protocol.

Immunolabeling

Cells were fixed using 4% paraformaldehyde and permeabilized with 0.2% Triton-X100 (Sigma) in PBS for 15 minutes before blocking with normal horse serum (NHS; 2%) in PBS with 0.2% Triton-X100 for 1 hour. Primary antibodies (listed below) were applied for 18 hours in 2% NHS at 4º C. After washing three times, Alexa-conjugated secondary antibodies (Invitrogen, 1:500) were applied for 2 hours before washing three times. Coverslips were mounted using Prolong Gold with DAPI (Life Technologies) prior to imaging. The images were acquired using an Olympus FV-1000 laser scanning microscope and Fluoview FV10 software (Olympus). Primary antibodies were used at the following concentrations: guinea-pig anti-GFAP (Synaptic Systems) 1:500, goat anti-Sox1 1:250 (Thermo Fisher), rabbit anti-Sox2 1:250 (Thermo Fisher), rabbit anti-Sox9 1:500 (Millipore), rabbit anti-Pax6 1:250 (Thermo Fisher), mouse anti-Nestin 1:250 (Thermo Fisher), rabbit anti-Oct4 1:250 (Thermo Fisher), mouse anti-TRA-1-60 1:250 (Thermo Fisher), mouse anti-S100b 1:500 (Sigma), mouse anti-SMI-312 1:500 (Covance), mouse anti-CC1 1:200 (Calbiochem), mouse anti-MAP2 1:500 (Millipore), rabbit anti-EAAT1 1:500 (Abcam), mouse anti-Aldh1l1 1:200 (Neuromab). Analysis of Sox9, Sox1, GFAP and S100b immunolabeling was performed with a DAPI co-label. Cells positive for DAPI and the above-mentioned markers were quantified using the ImageJ cell counter. A total of 10 images per condition were analyzed.

Ca^{2+} imaging

CTL and DS astrocytes (~90 days post-differentiation) were seeded onto coverslips and loaded with the single wavelength calcium indicator Fluo4-AM (Thermo Fisher) dissolved in DMSO. Immediately prior to loading, Fluo4-AM was diluted to 1μM in culture medium. Cell culture medium was replaced entirely with this solution and the cells were incubated for 20-50min at 37ºC 5% CO_2. Coverslips were transferred onto the stage of a customized Olympus FV1000 laser scanning microscope and continuously superfused with artificial cerebrospinal fluid (aCSF; 126mM NaCl, 2.5mM KCl, 1.3mM $MgCl^2$, 10mM d-glucose, 2.4mM $CaCl^2$, 1.24mM NaH^2PO^4, and 26mM $NaHCO^3$ saturated with 95% O2 and 5% CO2). Fluo4-AM was excited at 488nm and images were recorded at a frame rate of 0.5Hz. Spontaneous Ca^{2+} activity was acquired for 10 minutes. We counted all cells in which the F/F0 trace reached values higher than a threshold of 1.30 (30% signal increase over baseline) at any point during the 10 minute baseline recordings as spontaneously active cells. To obtain the fraction of spontaneously active cells, we divided this number by the total number of cells imaged analyzed. To record ATP-evoked Ca^{2+} events, 2 minutes of additional baseline recordings were obtained, after which the cells were superfused with 100μM ATP in aCSF for 1 minute. A total of three, 1 minute 100μM ATP pulses with 2 minutes of baseline in between were recorded. Image analysis was performed using the open source software FIJI. F/F_0 was calculated separately for each pulse by dividing the fluorescence intensity of each image by the average intensity of 10 frames during the preceding baseline. The average F/F_0 intensity over time was measured in manually defined ROIs corresponding to cells.

Quantitative reverse transcriptase polymerase chain reaction (qPCR)

At approximately 90 days post-differentiation, RNA of astrocytes was extracted using the RNeasy plus mini kit (Qiagen), and cDNA libraries were made using the Quantitect reverse

transcription kit (Qiagen). qPCR was performed using Sybr Green Master Mix (Applied Biosystems) on a StepOne Plus thermocycler (Applied Biosystems). Relative levels of mRNA were calculated using the ΔΔCT method with RPL13A as the internal control.

RNA-sequencing and data analysis

At approximately 90 days post-differentiation, RNA was extracted from cells using the RNeasy Plus Mini Kit (Qiagen). Sample quality was assessed using a Bioanalyser (Agilent), and sequenced at 12 samples per lane on an Illumina HiSeq4000 for 100 bp paired-end reads at the McGill University and Genome Quebec Innovation Center. All libraries passed an initial quality control step using the FASTQC pipeline. The Fast-X toolkit was used to trim the first and last 10 bases of each read. TruSeq specific paired-end adapters and low-quality stretches were removed using Trimmomatic (Bolger, Lohse, & Usadel, 2014), while poly-A tails were trimmed using PrinSeq (Schmieder & Edwards, 2011). Surviving paired and orphaned reads were separately aligned using STAR to the GRCh37 human genome (Dobin et al., 2013). HTseq was used to count pairs of reads mapping to specific genes (Anders, Pyl, & Huber, 2015). Using the DESeq2 package, we normalized read counts and applied a variance-stabilizing transformation to the data (Love, Huber, & Anders, 2014).

Differential expression analysis between CTL and DS astrocytes was performed using DESeq2 (Love et al., 2014). Statistically significant genes (adjusted p-value < 0.05) were considered differentially expressed. Shrinkage of log-fold changes was applied using DESeq2 and shrunken estimates were used throughout the analysis. Genes were annotated using Ensembl BioMart and filtered to retain only protein-coding genes (Zerbino et al., 2018). Normalized and variance-stabilized transformed data was used for all heatmaps. Differentially expressed chromosome 21 genes were visualized on the chromosome 21 ideogram using the karyoploteR package (Gel & Serra, 2017).

Gene ontology analysis and functional annotation were performed with enrichGO from the R/Bicoonductor package clusterProfiler (version 3.10.0) (G. Yu, Wang, Han, & He, 2012), using all differentially expressed genes compared to a custom background gene list consisting of all expressed genes (defined as all genes with a baseMean greater than the first decile). FPKM values were calculated using exonic gene lengths with DESeq2 which estimates library size using the median of ratios method. Gene haploinsufficiency data was obtained from Lek et al, 2016 (Lek et al., 2016). Transcription factors were identified from the Animal TFDB 3.0 resource (H. Hu et al., 2019). Targets in each gene ontology category were ranked by fold change, and genes with less than 30 base mean reads were omitted. The top 25 targets were then chosen to be validated by qPCR. Of those 22 targets were validated (3 targets were too low in abundance) 19 of which were in concordance with RNAseq results.

ATAC-sequencing and data analysis

Astrocytes were cultured for 13 weeks as described above, with the exception that they were maintained on Matrigel-coated dishes throughout. ATAC–seq was performed as previously described using 50,000 nuclei (Buenrostro, Giresi, Zaba, Chang, & Greenleaf, 2013; Mayran et al., 2018). Briefly, fresh cell pellets from 50,000 cells were exposed to a hypotonic cell lysis buffer (0.1% (wt/vol) sodium citrate tribasic dihydrate and 0.1% (v/v) Triton X-100) for 30 minutes at 4 °C followed by treatment with normal cell lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl2 and 0.1% (v/v) IGEPAL CA-630) for 30 minutes at 4 °C. To perform the transposition reaction, the nuclei were incubated with 25 μl of Transposase Master Mix (2.5 μl 10× TD buffer, 10 μl H2O and 12.5 μl Tn5 enzyme from an Illumina Nextera kit; FC-121–1031) for 30 minutes at 37°C. DNA was then purified and enriched by PCR using Nextera indexed primers, and the library was recovered with GeneRead Purification columns (Qiagen), eluted in 35ul EB buffer. The library was assessed using a bioanalyzer (Agilent) and sequenced on an Illumina HiSeq4000 instrument to produce 100bp paired-end reads.

Enrichment of open chromatin was verified using qPCR primers amplifying open-chromatin positive (housekeeping genes GAPDH and B2M) versus open-chromatin negative (gene desert) regions. A 11 (B2M)-40 (GAPDH)-fold enrichment was observed (not shown). All libraries passed an initial quality control step using the FASTQC pipeline. Low quality and adapter sequences were trimmed using Trim Galore. Reads were aligned using the GRCh37 human genome using bwa mem with default settings, and duplicates removed using Picard MarkDuplicates. To obtain reads representing nucleosome-free DNA, BAM files were filtered to reads < 100bp, following Buenrostro et al (Buenrostro et al., 2015). Peaks were called using MACS2 callpeak with the default FDR cutoff of 0.05 and used for downstream analysis (Zhang et al., 2008).

Differentially accessible peaks between genotypes were identified using the R/Bioconductor package DiffBind (version 2.10.0) and annotated with nearest gene and genomic compartment using the R/Bioconductor package ChIPseeker (version 1.18.0) (G. Yu, Wang, & He, 2015). Bigwig tracks were generated using deepTools bamCoverage with RPKM normalization and a bin size of 1 (Ramirez et al., 2016). Peaks were defined to be within promoter regions if they were annotated as within 3 kbp of a gene transcription start site by ChIPseeker. Average ATAC-seq signal in 5kbp intervals centered at these peaks were then used to cluster these regions using k-means (k=4) clustering implemented in deepTools. deepTools was used to generate average ATAC-seq signal profile over these peaks for each sample, and to calculate tag density in regions upstream of gene transcription start sites. Gene ontology analysis were performed using enrichGO, applied to genes with differentially accessible peaks within promoter regions.

Code availability

All R code to produce the RNA-seq and ATAC-seq analysis presented in this study will be made available at https://github.com/murailab/Ponroy2020_HMG.

Western blot analysis

At approximately 90 days post-differentiation, astrocytes were lysed in 400µl RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 20 mM Tris pH 8.0, 150mM NaCl and 1mM EDTA) containing 1µg/ml each of leupeptin, aprotinin, pepstatin, 10 mMNaF, 1mM sodium ortho-vanadate and 1mM PMSF. Lysates were diluted with 3X sample buffer and equal quantities of each lysate were run on a 10% polyacrylamide gel and transferred to PVDF membranes following standard protocols. Membranes were blocked for 40 minutes with 5% BSA/TBS-0.1% Tween, and incubated overnight at 4°C with an anti-mouse PCDHG antibody (1:1000, Neuromab), and an anti-rabbit RPL13A antibody (1:2000, Cell Signaling) as the loading control. The next day membranes were incubated for 1 hour at room temperature with secondary antibodies conjugated to horse radish peroxidase. Chemiluminescent signal was obtained using Amersham ECL Plus Western Blotting Detection Reagents (GE Healthcare) and captured on X-ray film. Densitometry was performed using the Gel analysis function in ImageJ.

PCDH adhesion assay

Glass coverslips were coated with PCDHs for 2 hours at the following concentrations: Pcdhgc3 2ug/mL (R&D Systems), Pcdh12 4ug/mL (R&D Systems), and Pcdh10 4ug/mL (RD systems). CTL and DS astrocytes at approximately 2.5 months of age, were seeded onto glass coverslips with and without PCDH coating at $1x10^5$ cells per well and were left to adhere for 30 minutes and subsequently agitated at 1500 rotations per minute for 30 minutes. The astrocytes were plated in astrocyte medium without serum, in order to avoid potential coating by the serum. Astrocytes were then fixed and stained with DAPI using above described immunohistochemistry protocols. Images were acquired using an Olympus FV-1000 laser scanning microscope and Fluoview FV10 software (Olympus). Images were analyzed and cells were counted using FIJI software.

Astrocyte cell size comparison

At approximately 8 weeks post-differentiation astrocytes were stained with a rabbit anti-EAAT1 antibody (Abcam) with a DAPI co-label and the images were acquired using an Olympus FV-1000 laser scanning microscope and Fluoview FV10 software (Olympus). The cell area was calculated using the freehand tool in ImageJ to draw the outline of the cells and the measure function. 45 images per condition were analyzed from 3 independent experiments, in which 5 images were analyzed in 3 cell lines per condition.

Relative size and internal complexity (i.e. granularity) of the cells in suspension were extrapolated from forward scatter (FSC) and side scatter (SSC) parameters acquired using a Cyan ADP flow cytometer and data was analyzed using the FlowJo software (Treestar).

Cell spreading assay

At approximately 8 weeks post-differentiation, 10,000 astrocytes were plated on PolyD-lysine coated coverslips for 30, 60, 90 and 120 minutes in medium without FBS in order to avoid potential coating by the serum. Astrocytes were then fixed and stained with DAPI and Phalloidin-488 (Cell Signaling) using above described immunohistochemistry protocols. 5 images per time point were acquired in all 3 CTL and 3DS cell lines using an Olympus FV-1000 laser scanning microscope and Fluoview FV10 software (Olympus). Images were analyzed and cell area was measured using FIJI software.

Cell aggregation assay

At approximately 8 weeks post-differentiation, 30,000 astrocytes were placed in suspension in non-coated petri-dishes for 2 hours, in low serum containing medium (1% FBS). After two hours the cell clumps were imaged on an Ultraview spinning disk confocal system (Perkin Elmer). The aggregate

area was calculated using the freehand tool in ImageJ to draw the outline of the aggregate and the measure function. 45 images per condition were analyzed from 3 independent experiments, in which 5 images were analyzed in 3 cell lines per condition. The medium containing the cells in suspension was then removed, spun down, trypsinized and resuspended, after which the cells were counted using a cell counter. The cells which had adhered to the petri dish were trypsinized and counted using a cell counter.

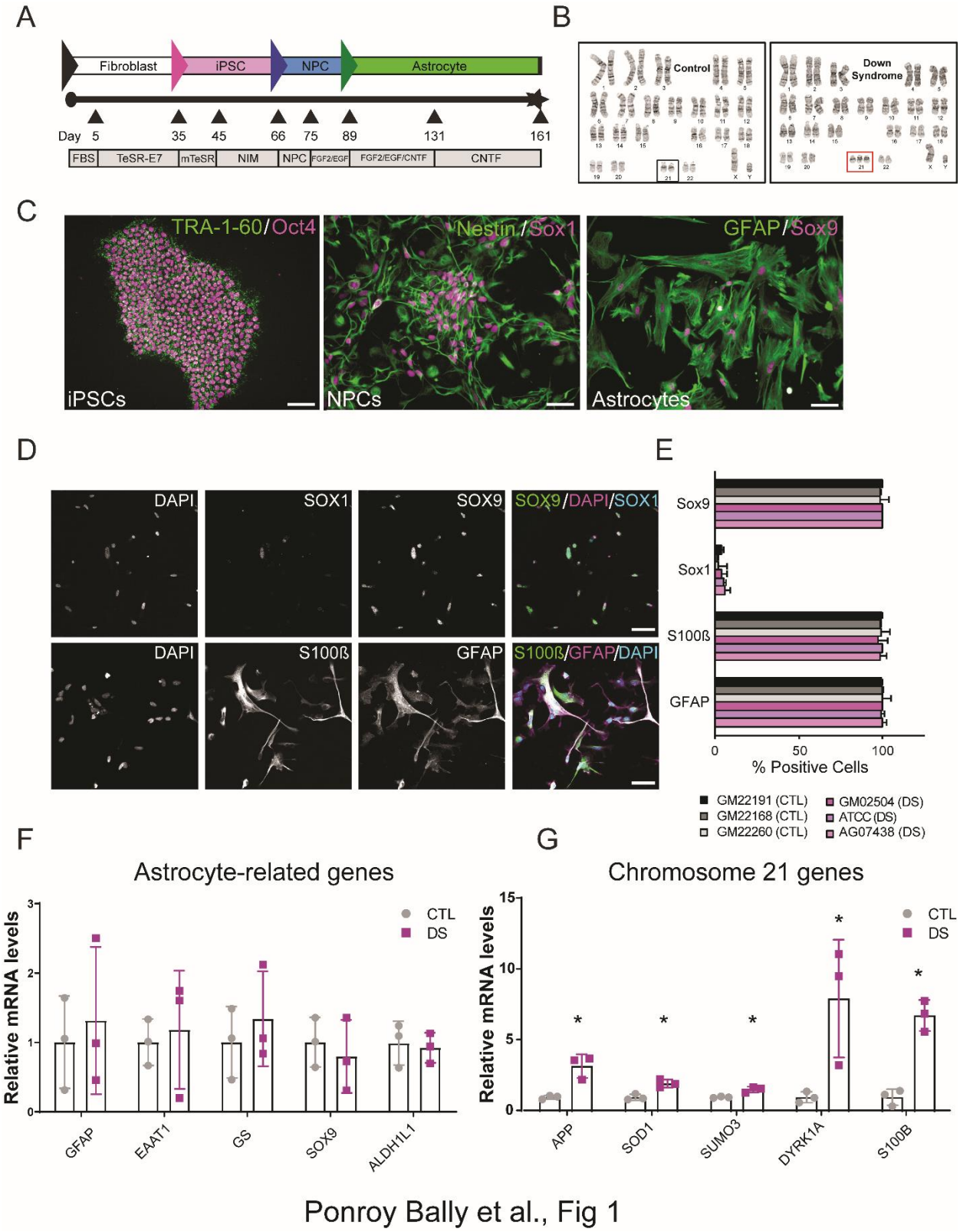
Cell motility assay

At approximately 8 weeks post-differentiation astrocytes were seeded onto plastic tissue culture treated 96 well plates (Corning 3904) at a density of 5000 cells per well and left to adhere for 24 hours. They were then incubated with Hoechst 33342 (0.2μg/ml) to stain cell nuclei for approximately one hour after which the medium was switched for phenol free DMEMF12 with the same previously described supplements, as well as 20mM HEPES (pH 7.4). Time-lapse sequences were then acquired (20 frames, at 10min intervals, 190min total) at 37°C using an automated widefield fluorescence microscope (ImageXpress Micro XL, Molecular Devices), equipped with an Andor Zyla 5.5 sCMOS camera and using a 4x objective. Cell tracking was performed based on the trajectories of segmented cell nuclei using custom-written MATLAB routines, as previously described (Hayer et al., 2016). Cell velocity was averaged over 20 frames, and migration persistence computed as net displacement/total path length over 20 frames.

Experimental design and statistical analysis

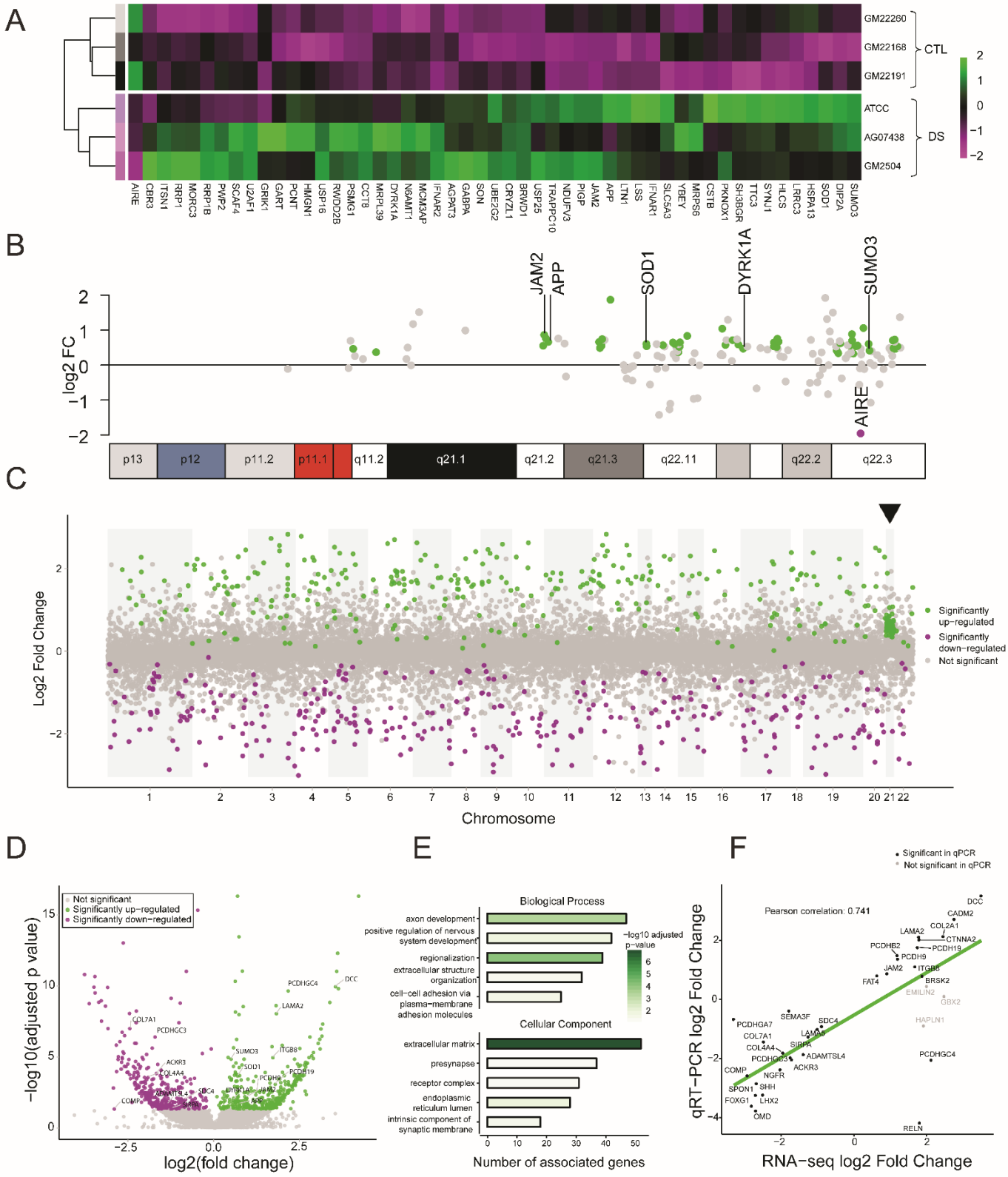
Data are shown as the mean ± SEM. Statistical analysis between two groups was performed using unpaired two-tailed t-test, (using Graphpad software version 5.01). Standard symbols were used to report significance: n.s. - not significant ($p>0.05$), * - $p<0.05$, ** - $p<0.01$, *** - $p<0.001$, **** - $p<0.0001$.

3.6 FIGURES



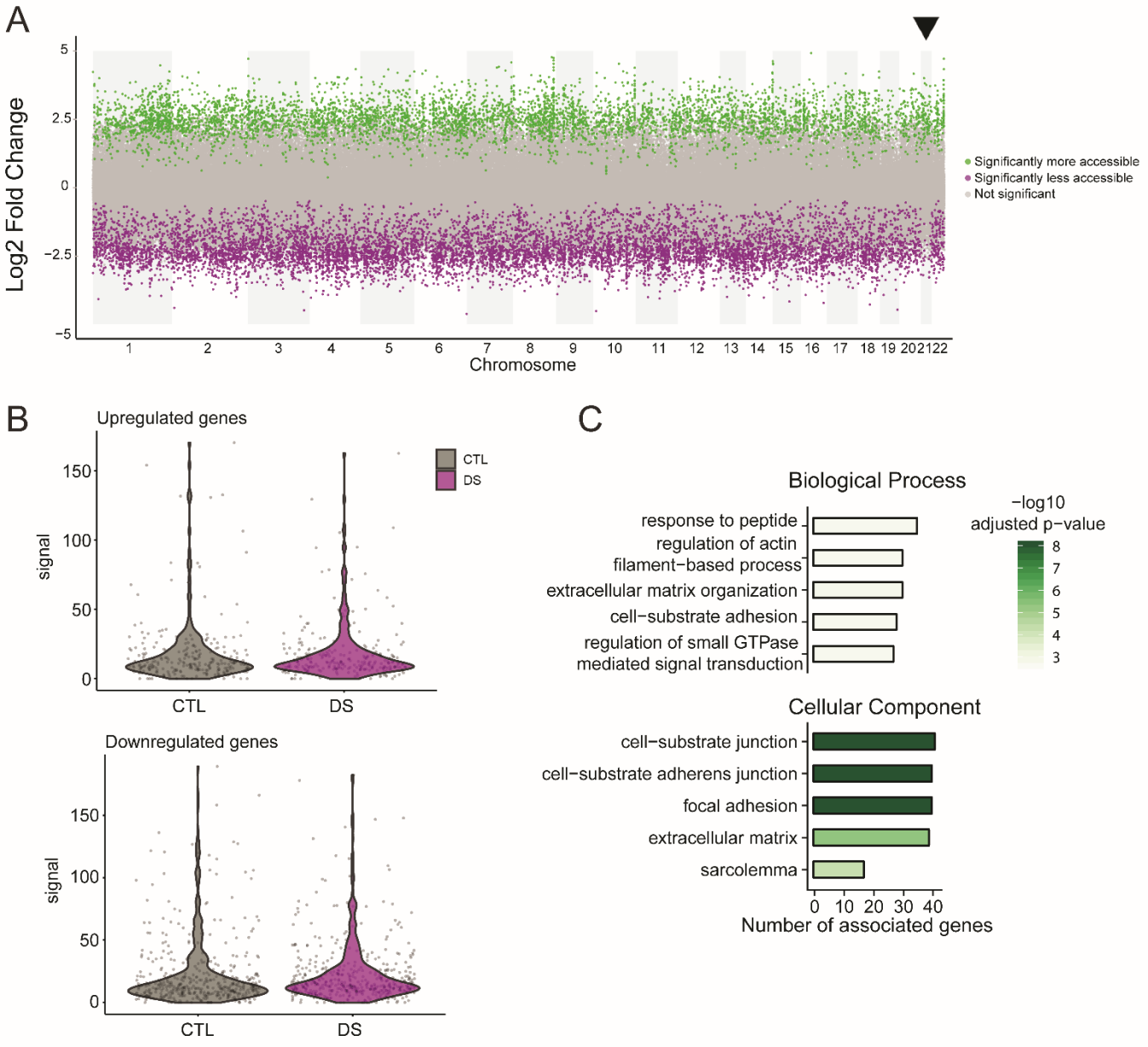
Ponroy Bally et al., Fig 1

Fig 1. Generation and validation of CTL and DS astrocytes from fibroblasts and hiPSCs. (A) Production workflow of hiPSCs, NPCs, and astrocytes. (B) Karyotypes of CTL and DS lines. (C) Expression of pluripotency markers in hiPSCs (TRA-1-60 and Oct4), neuronal precursor cell (NPC markers (Sox1 and Nestin), and astrocyte cultures (GFAP and Sox9). Scale = 20μm in TRA-1-60/Oct4 image and 50μm in all others. (D) At 90 days astrocytes express markers GFAP, S100B, and Sox9, and low levels of the NPC marker Sox1. Scale = 50μm. (E) Astrocyte differentiation causes a reduction of the NPC marker Sox1 and upregulation of astrocytic markers S100B, GFAP and Sox9. (F) CTL and DS astrocytes express equal levels of astrocyte-related genes GFAP, EAAT1, glutamine synthetase (GS), SOX9, and ALDH1L1 (shown by qPCR). (G) Chromosome 21 genes are upregulated in DS astrocytes (shown by qPCR). Data are represented as mean ± SEM. Two-tailed, unpaired t-tests were performed *$p \leq 0.05$, n = 3 (3 experiments each performed in the 3 DS and 3 CTL cell lines) FBS: fetal bovine serum; NIM: neural induction medium; NPC: neural precursor cells.



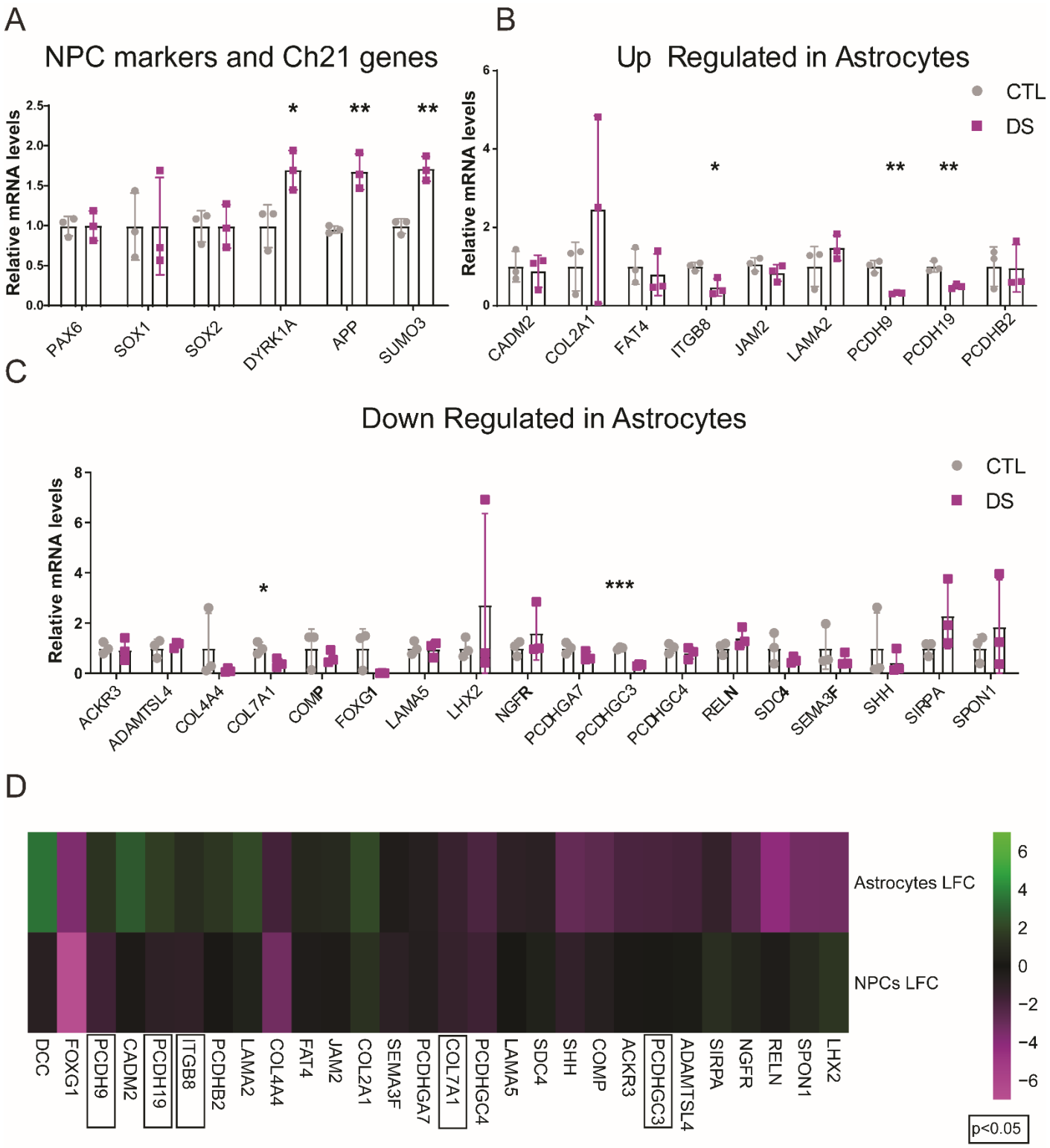
Ponroy Bally et al., Fig 2

Fig 2. DS astrocytes show global transcriptome dysregulation. (A) Heatmap of the differential expression of chromosome 21 genes in DS and CTL astrocytes. Heatmap displays relative expression level of each gene across samples (magenta, lower expression; green, higher expression). (B) Mapping of differentially expressed chromosome 21 genes onto the chromosome 21 ideogram. (C) Manhattan plot showing differentially expressed genes and illustrating global transcriptome dysregulation in DS astrocytes. Significantly, upregulated genes are shown in green and downregulated genes are shown in magenta. Arrowhead indicates chromosome 21. (D) Volcano plot showing symmetrical changes in gene expression, with a similar proportion of up and downregulation in DS astrocytes. (E) Top 5 biological processes and cellular enriched in differentially expressed genes according to GO analysis. Bars encode number of genes associated with the ontology term and are colored by –log10 of adjusted p-value. (F) Correlation between RNA-seq and qPCR data (Pearson's: 0.741, $p \leq 0.01$).



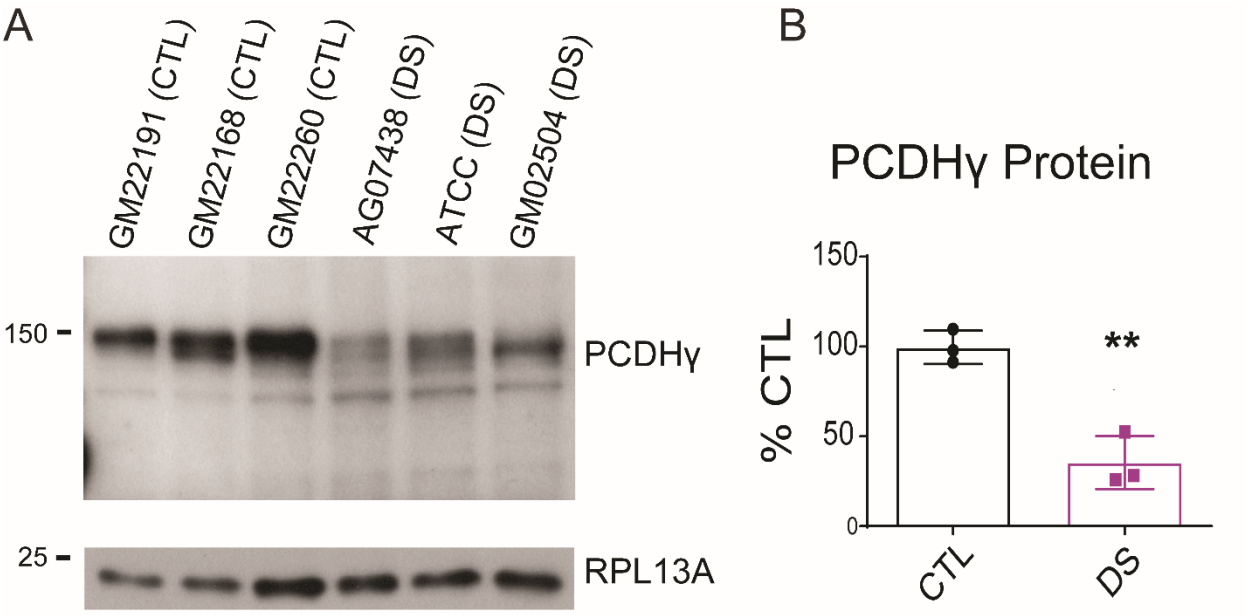
Ponroy Bally et al., Fig 3

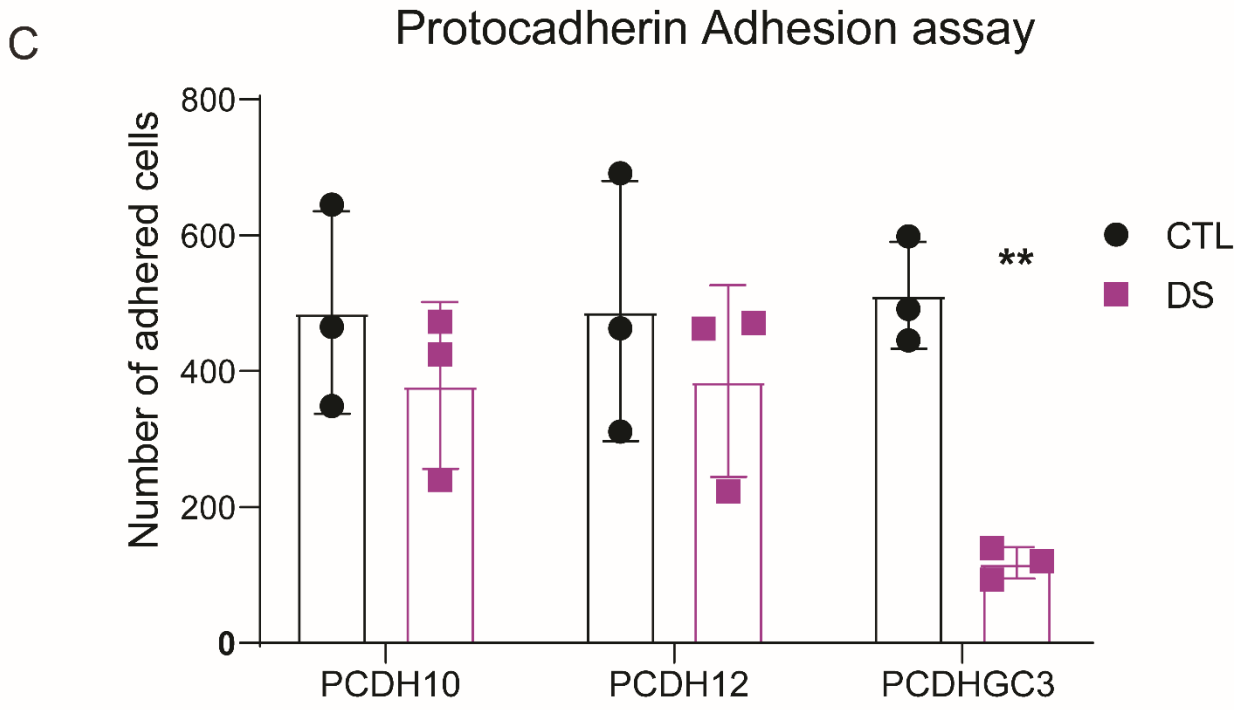
Fig 3: ATAC-seq reveals an altered chromatin state in DS astrocytes, with differential chromatin accessibility in the promoters of cell adhesion and ECM genes. (A) Manhattan plot of the differentially accessible peaks in DS astrocytes revealing both an increase and decrease in chromatin accessibility. (B) Violin plots of the correspondence between ATAC-seq and RNA-seq data sets, show that upregulated and downregulated genes identified by RNA-seq do not have differential accessibility at promoters in CTL and DS astrocytes. (C) Top 5 biological processes and cellular components enriched in genes with differentially accessible promoters according to GO analysis. Bars encode number of genes associated with the ontology term and are colored by –log10 of adjusted p-value.



Ponroy Bally et al., Fig 4

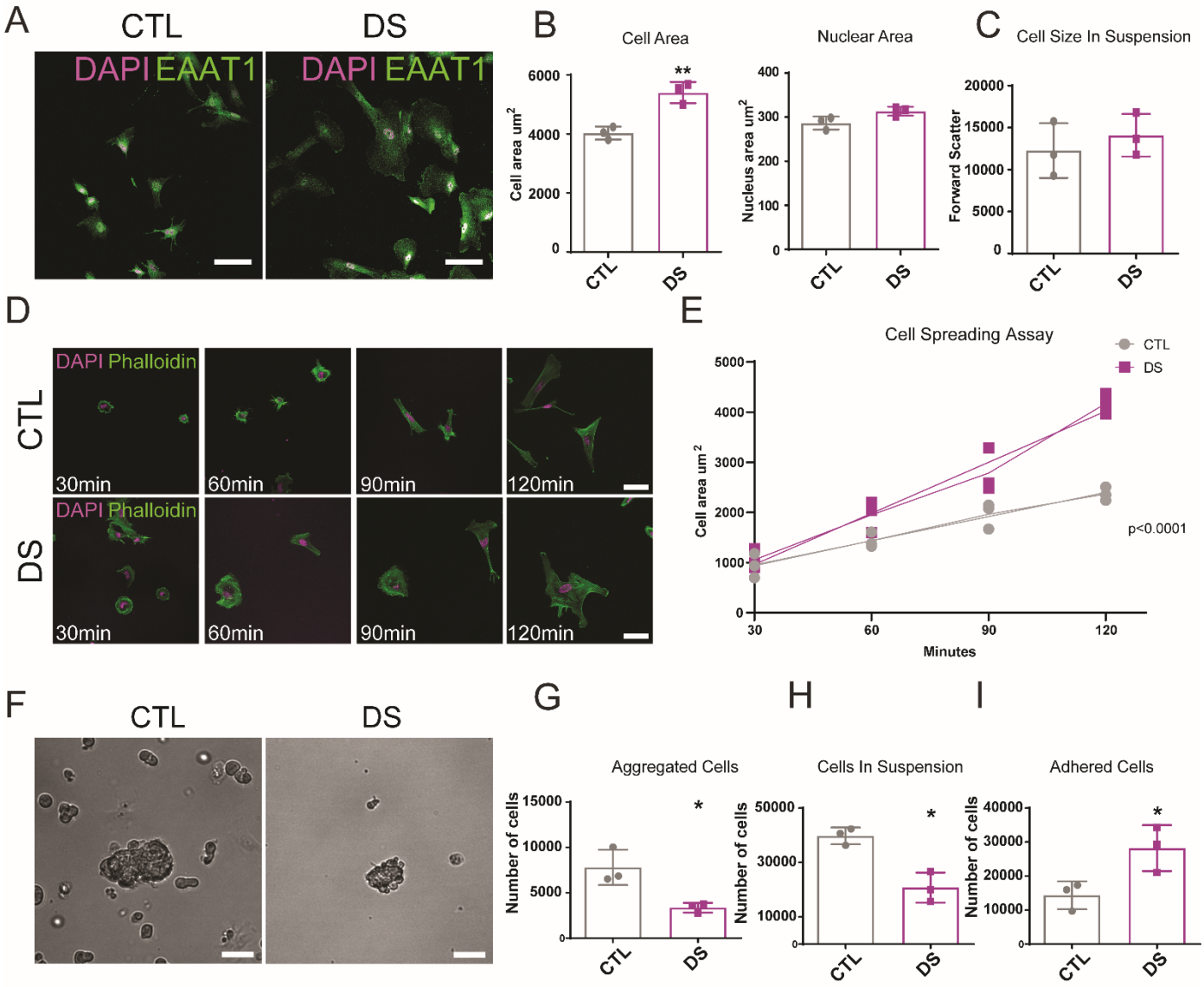
Fig 4: DS NPCs and astrocytes differ in their dysregulation of non-chromosome 21 genes. (A) qPCR demonstrates similar expression of Pax6, Sox1 and Sox2 in CTL and DS NPCs, and upregulation of chromosome 21 genes: DYRK1A, APP, SUMO3 in DS NPCs. Data are represented as mean ± SEM. Two-tailed, unpaired t-tests were performed *p≤0.05, **p≤0.01, ***p≤0.001, n = 3 (3 experiments each performed in the 3 DS and 3 CTL cell lines). (B) ITGB8, PCDH9 and PCDH19 are significantly downregulated in DS NPCs whereas they were upregulated in DS astrocytes; CADM2, COL2A1, FAT4, JAM2, LAMA2 and PCDHB2 are unchanged in DS NPCs whereas they were upregulated in DS astrocytes. Data are represented as mean ± SEM. Two-tailed, unpaired t-tests were performed *p≤0.05, **p≤0.01, ***p≤0.001, n = 3 (3 experiments each performed in the 3 DS and 3 CTL cell lines). (C) COL7A1 and PCDHGC3 were downregulated in both DS NPCs and astrocytes, whereas ACKR3, ADAMTSL4, COL4A4, COMP, FOXG1, LAMA5, LHX2, NGFR, PCDHGA7, PCDHGC4, RELN, SDC4, SEMA3F, SHH, SIRPA and SPON1 are unchanged in DS NPCs and were downregulated in DS astrocytes. Data are represented as mean ± SEM. Two-tailed, unpaired t-tests were performed *p≤0.05, **p≤0.01, ***p≤0.001, n = 3 (3 experiments each performed in the 3 DS and 3 CTL cell lines). (D) Heatmap of the expression of differentially expressed genes in DS astrocytes and NPCs (magenta: lower expression, green: higher expression).





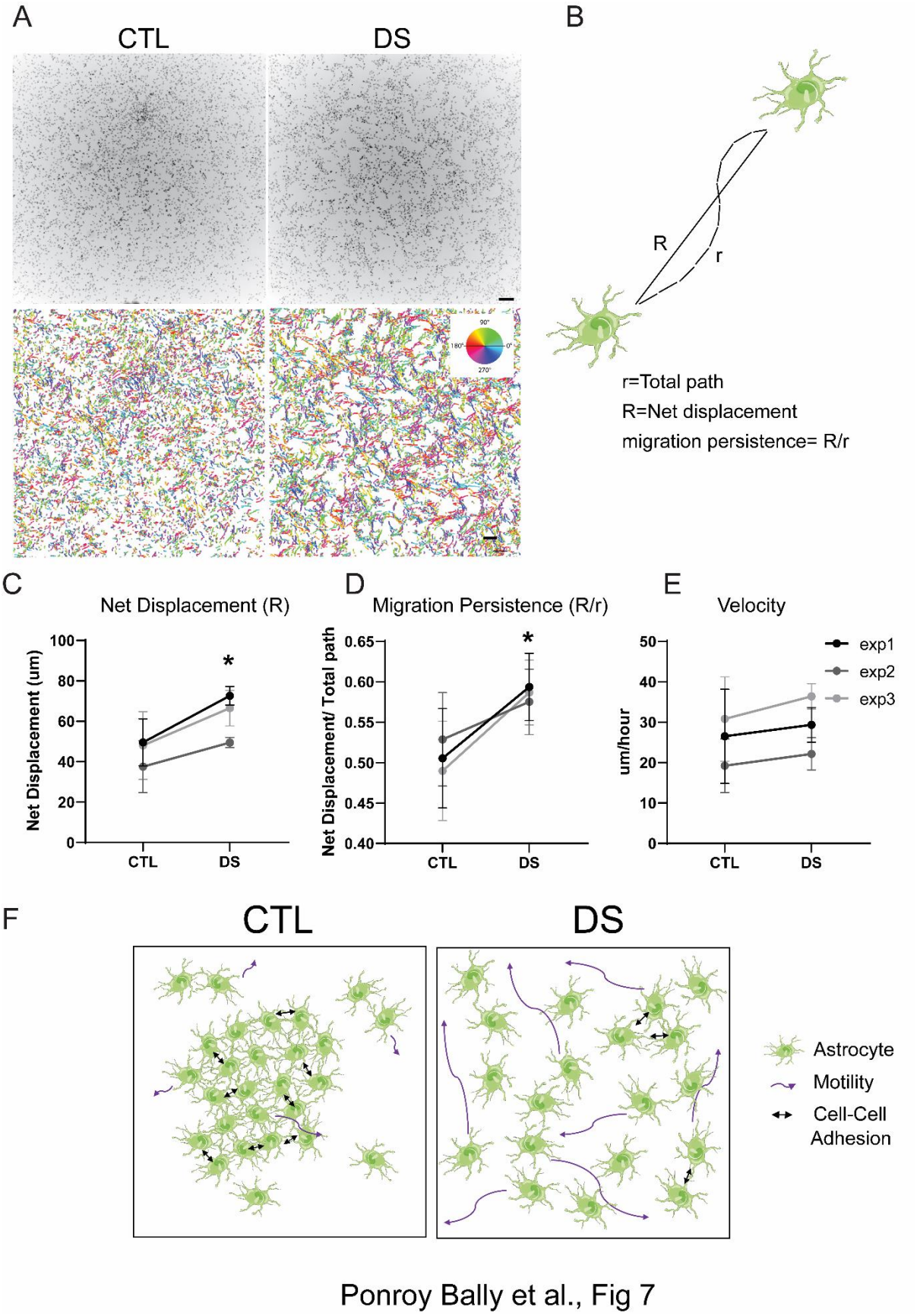
Ponroy Bally et al., Fig 5

Fig 5: DS astrocytes display alterations in PCDH mediated adhesion: (A,B) Western blot analysis and quantification showing the downregulation of PCDHGs in DS astrocytes. Data are represented as mean ± SEM. Two-tailed, unpaired t-tests were performed *$p \leq 0.05$ (n=3), ** $p \leq 0.01$ (n=3 experiments, each performed in 3 CTL and 3 DS cell lines). (C) CTL astrocytes adhere significantly more to PCDHGC3-coated coverslips than DS astrocytes. Data are represented as mean ± SEM. Two-tailed, unpaired t-tests performed *$p \leq 0.05$ n = 3 (3 experiments in which 5 images were taken in all 3 CTL cell lines and 3 DS cells lines).



Ponroy Bally et al., Fig 6

Fig 6: DS astrocytes have increased cell spreading and an altered adhesion profile: (A) DS astrocytes are significantly larger than CTL astrocytes. Composite images of EAAT1 and DAPI-positive astrocytes, Scale = 100μm. (B) Cell area and nuclear area quantifications (8 weeks post-differentiation). N>900 cells from three separate experiments with three cell lines per conditions. (C) Cell size in suspension obtained by flow cytometry (forward scatter). Data are represented as mean ± SEM. Two tailed, unpaired t-test were performed * $p \leq 0.05$ (D) DS astrocyte display increased cell spreading. Composite images of Phalloidin-488 and DAPI-positive astrocytes imaged at 30min, 60min, 90min and 120min of cell spreading, Scale=50μm. (E) Mean cell area calculated for each time point, n=3 (this experiment was performed 3 times in all 3 DS and all 3 CTL cell lines, 5 images were analyzed per cell line at each time point). Linear regressions were performed on the rate of spreading and the slopes were determined to be significantly different from each other $p<0.0001$ (F) DS astrocytes show differential cell-cell and cell-substrate adhesion profiles. After 2 hours in suspension DS astrocytes form smaller aggregates than CTL astrocytes, Scale = 150 μm. (G) Aggregate comparison after two hours in suspension. n=3 (This experiment was performed 3 times in all 3 DS and all 3 CTL cell lines, 5 images were analyzed per cell line at each time point). Data are represented as mean ± SEM. Two-tailed, unpaired t-test was performed * $p \leq 0.05$. (H) The number of cells in suspension is lower for DS astrocytes. n = 3 (This experiment was performed 3 times in all 3 DS and all 3 CTL cell lines,, the number of cells in suspension was counted with a cell counter). Data are represented as mean ± SEM . Two tailed, unpaired t-test was performed * $p \leq 0.05$. (I) The number of cells having adhered is higher for DS astrocytes. n = 3 (This experiment was performed 3 times in all 3 DS and 3 CTL cell lines, the number of cells having adhered was counted with a cell counter following trypsinization). Data are represented as mean ± SEM. Two-tailed, unpaired t-test was performed * $p \leq 0.05$.



Ponroy Bally et al., Fig 7

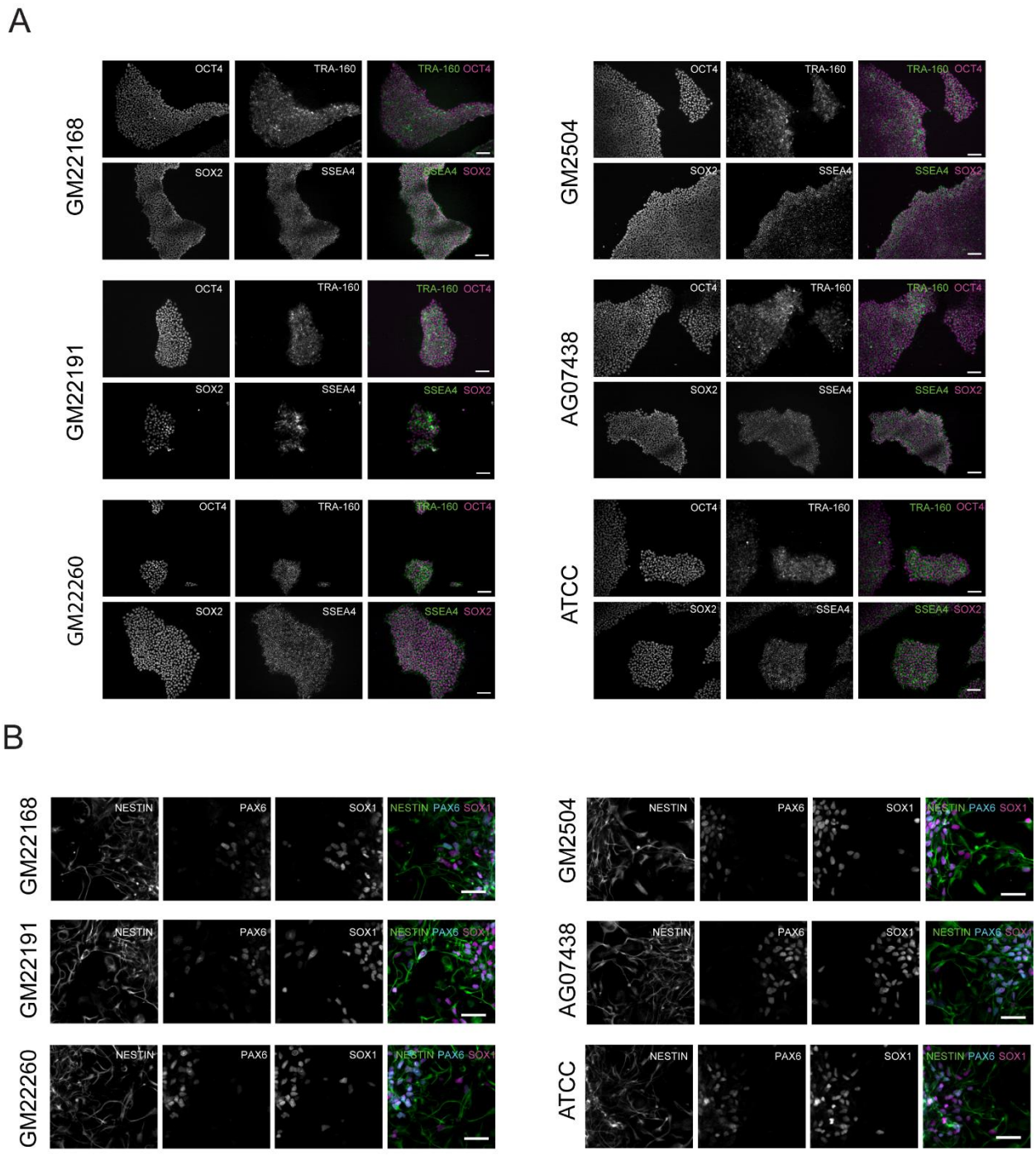
Figure 7: DS astrocytes have increased cell motility: (A)DS astrocytes have an increased motility at basal conditions, as determined by live imaging and nuclear tracking over 190min. Automatically detected nuclear trajectories are colored based of the direction of movement. (Scale bars, 250 μm). (B) Schematic of the measurements used to determine migration persistence: Migration persistence (R/r) is measured through the quotient of the net displacement (R) and the total distance travelled (r). (C-E) Over 190min at basal conditions, DS astrocytes display an increase in net displacement and migration persistence, but not in velocity. (n = 3 independent experiments, in all 3 CTL and DS cell lines , Data are represented as mean ± SEM, Two tailed, paired t-test was performed *p≤0.05 **p≤0.01). (F) Schematic summarizing the results of Figure 6 and 7. DS astrocytes show increased cell size through increased cell spreading, decreased cell-cell adhesion, and increased motility.

3.7 SUPPLEMENTARY FIGURES

Cell line	Disease	Sex	Age	Ethnicity	Cell type of origin	Origin
GM02504	Trisomy 21	Male	1 month	Black	Fibroblast	Coriell Institute
AG07438	Trisomy 21	Male	9 month	Black	Fibroblast	Coriell Institute
AG04823	Trisomy 21	Male	5 years	NA	Fibroblast	Coriell Institute
ATCC-DYP0730	Trisomy 21	Male	Newborn	NA	IPSC	ATCC
GM22260	Control	Male	1 day	Black	Fibroblast	Coriell Institute
GM2191	Control	Male	1 day	Black	Fibroblast	Coriell Institute
GM22168	Control	Male	1 day	Black	Fibroblast	Coriell Institute

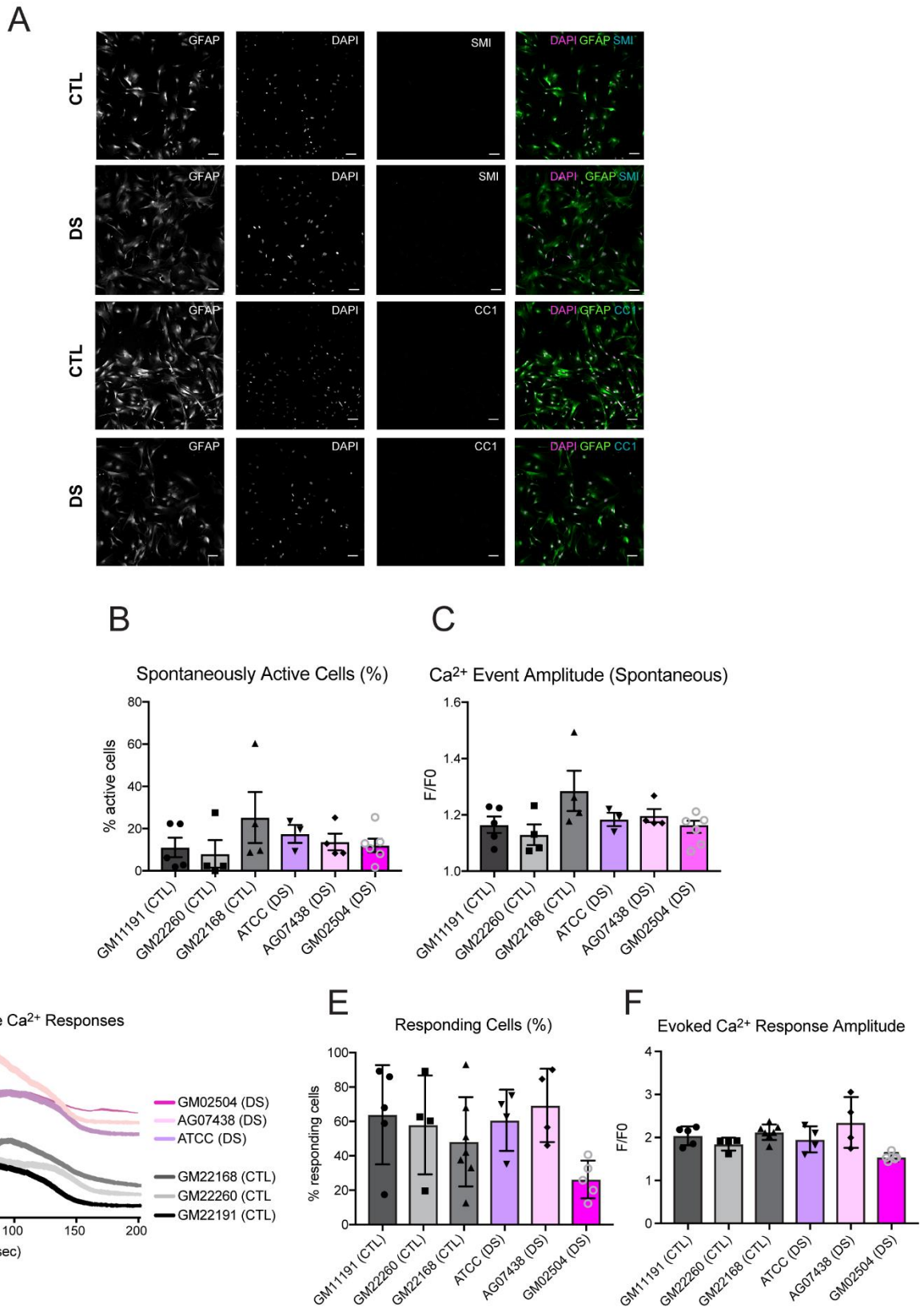
Ponroy Bally et al., S1 Table

S1 Table. Origin and characteristics of fibroblast lines used. NA = not available.



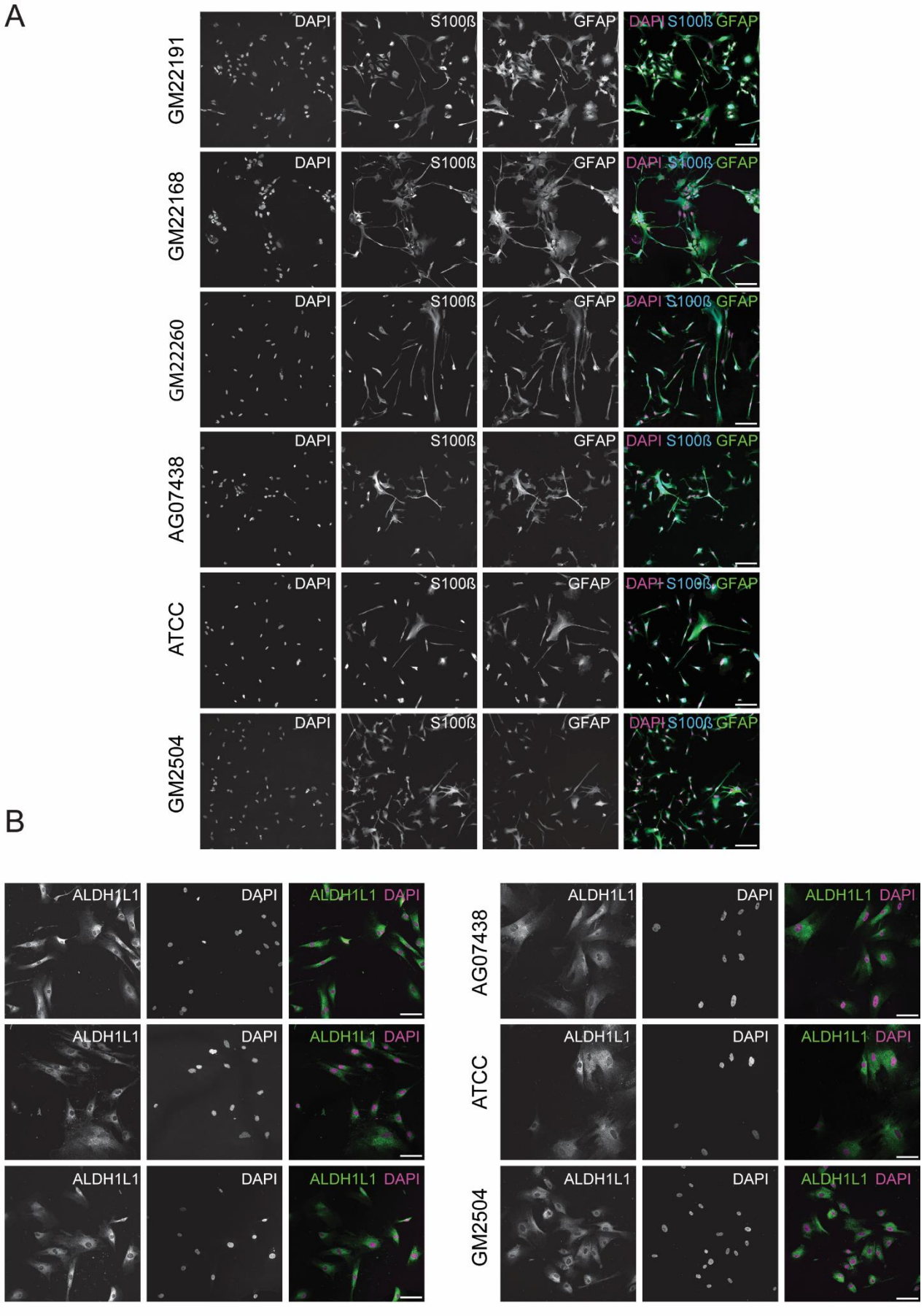
Ponroy Bally et al., S2 Fig

S2 Fig: Expression of IPSC and NPC markers. (A) Expression of pluripotency markers (TRA-1-60 and Oct4, Sox2 and SSEA4) in all hiPSC lines. Scale = 20μm. (B) Expression of neuronal precursor cell (NPC) markers (Sox1, Nestin, and Pax6) in NPC lines. Scale = 50μm.



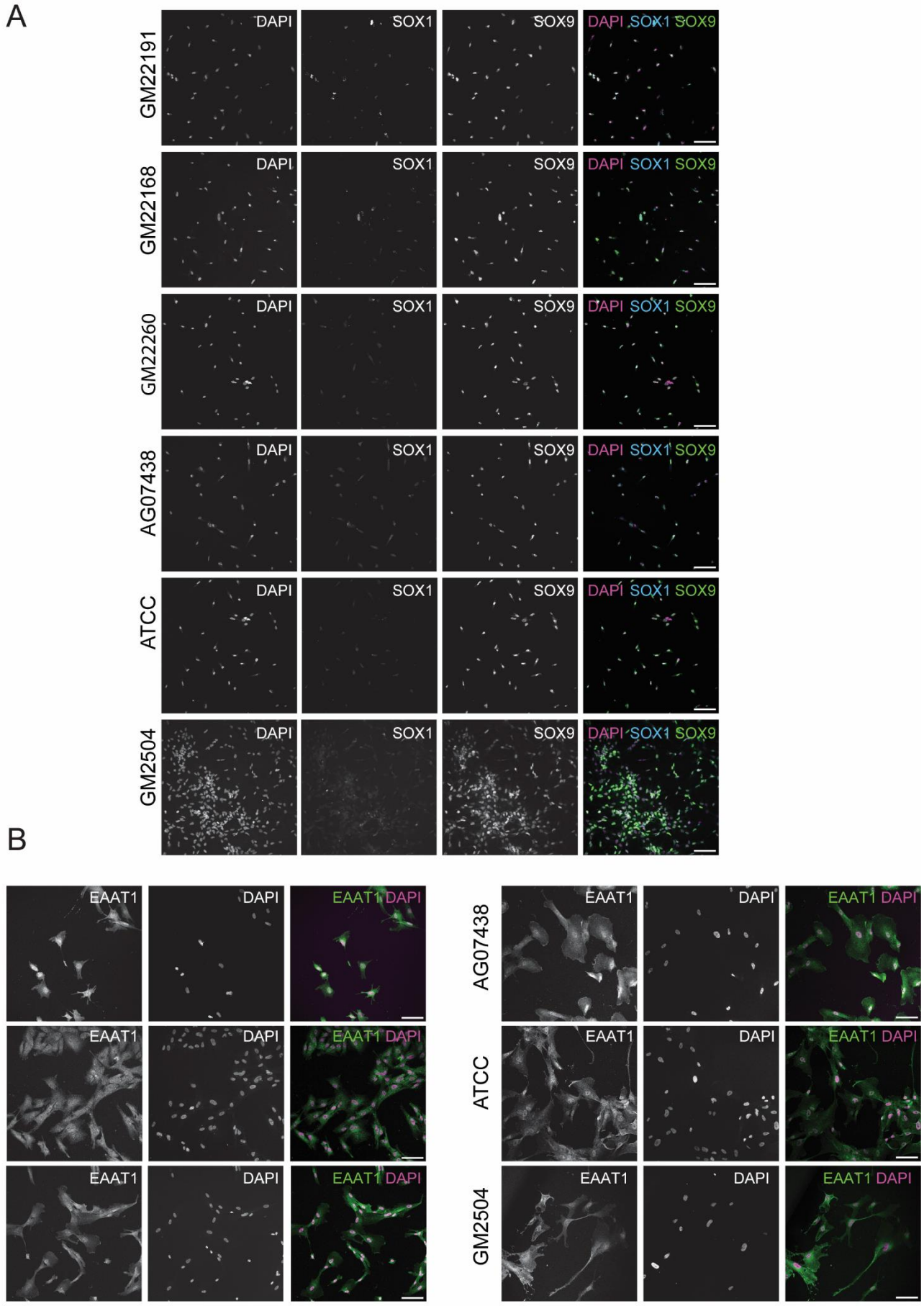
Ponroy Bally et al., S3 Fig

S3 Fig: Characterization of hiPSC-derived DS astrocytes. (A) At 90 days astrocytes express GFAP, but not Smi-312 (neuronal marker) and not CC1 (oligodendrocyte marker). Scale = 100 μm (B-C) Ca^{2+} imaging shows that CTL and DS astrocytes show a similar percentage of spontaneously active cells and have comparable Ca^{2+} event amplitudes. (D) Example traces showing ATP-evoked (100μM) Ca^{2+} responses in CTL and DS astrocytes. (E-F) CTL and DS astrocytes show a similar percentage of active cells and Ca^{2+} events amplitude when stimulated with ATP.



Ponroy Bally et al., S4 Fig

S4 Fig: Expression of astrocytic markers in hiPSC-derived astrocytes. (A) At 90 days astrocytes of each cell line express GFAP and S100B. Scale = 100 μm. (B) At 90 days astrocytes of each cell line express ALDH1L1. Scale = 100 μm.



Ponroy Bally et al., S5 Fig

S5 Fig: hiPSC-derived astrocytes express astrocytic markers and low levels of NPC markers.

(A) At 90 days astrocytes of each cell line express Sox9 and low levels of Sox1. Scale = 100 μm. (B) At 90 days astrocytes of each cell line express EAAT1. Scale = 100 μm.

Immature Human Astrocyte Genes

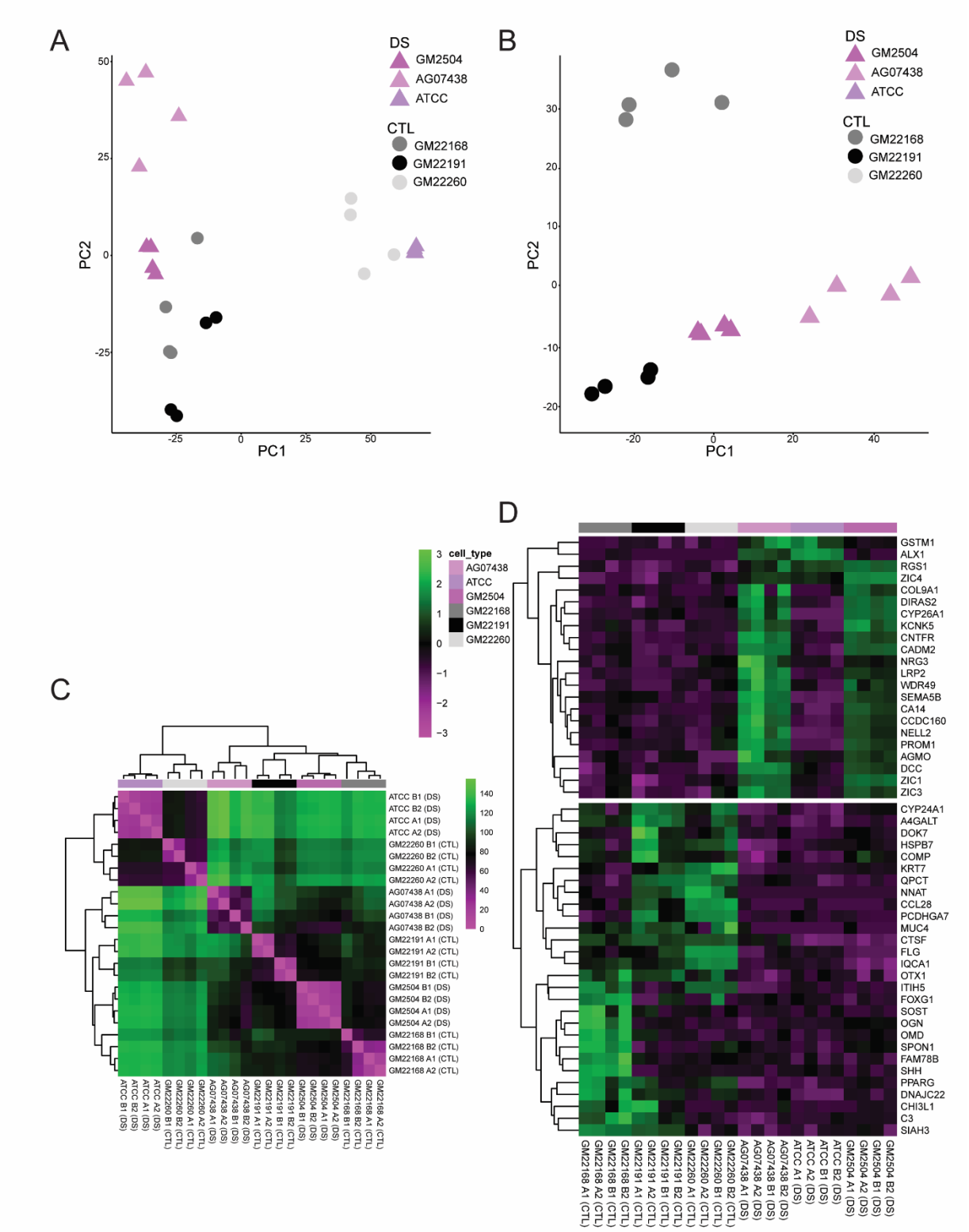
Gene	Fetal Astrocytes *	Mature Astrocytes*	This Study
HIST1H3E	51	0.1	3.825987013
HIST1H3B	176.6	0.6	0.312057375
HIST1H1B	23.4	0.1	0.124149971
PPDPF	26.4	0.2	245.2626714
TPX2	22	0.2	17.93421827
NUSAP1	27.4	0.3	15.68976976
HIST1H2AC	39	0.5	82.9199906
HIST2H2AC	68.9	1.3	0.588336301
TNC	26.6	0.6	160.0008468
KIF15	17.2	0.5	2.201845813
HIST1H2BC	215.2	13.2	18.49184223
FABP5	23.9	1.5	19.25309532
DTYMK	10.3	0.7	23.44242057
RAB11B	10.9	0.8	54.27293107
HES6	21.6	2.1	2.561349403
LRIG3	13.3	1.4	6.642965534
E2F5	10.1	1.4	6.66180485
MPPED2	16	2.3	0.056707511

Mature Human Astrocyte Genes

Gene	Fetal Astrocytes*	Mature Astrocytes*	This Study
S100A1	0.1	84.9	0.390581642
SLC14A1	0.1	29.3	0.653504521
TMEM176A	0.1	27.1	1.598477799
TMX2	0.1	25.9	39.72337804
HHATL	0.1	24.9	0.00151969
PADI2	0.1	26.8	13.08784831
TLR4	0.1	18.7	9.75642949
HSD17B6	0.1	20.5	1.557499588
CHI3L1	0.1	15.2	9.744807014
NUDT3	0.1	13.5	31.12520892
FBXO2	0.1	10.8	12.58826257
ALDH1L1	0.1	10.8	0.002029996
ALDOC	4.8	371	14.29447956
SLC1A2	34.5	1987.9	0.333744013
RYR3	0.3	16	0.064030458
GABRA2	0.9	46.1	2.882354785
CPE	16	657.2	85.6230359
GLUL	21	826.9	19.05975549

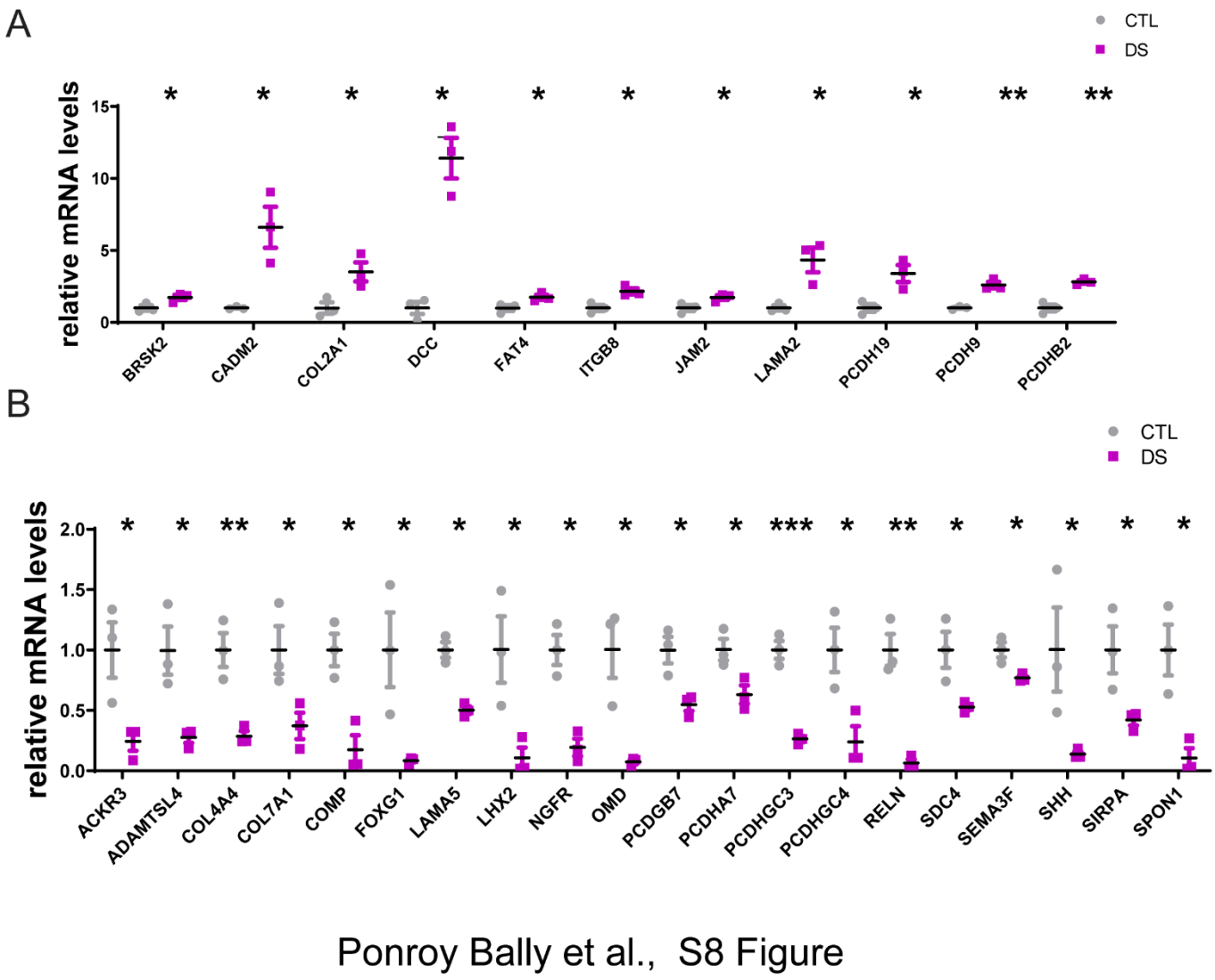
* Zhang, Y., et al., Purification and Characterization of Progenitor and Mature Human Astrocytes Reveals Transcriptional and Functional Differences with Mouse. Neuron, 2016. 89(1): p. 37-53.

S6 Table: Comparison of astrocyte marker expression in our study and those found in Zhang et al. 2016: (1) Top marker expression in fetal astrocytes and mature astrocytes found in Zhang et al (2016) compared to astrocytes in this study using FPKM. (2) Top mature marker expression in fetal astrocytes and mature astrocytes of the Zhang et al. 2016 compared to astrocytes in this study using FPKM.



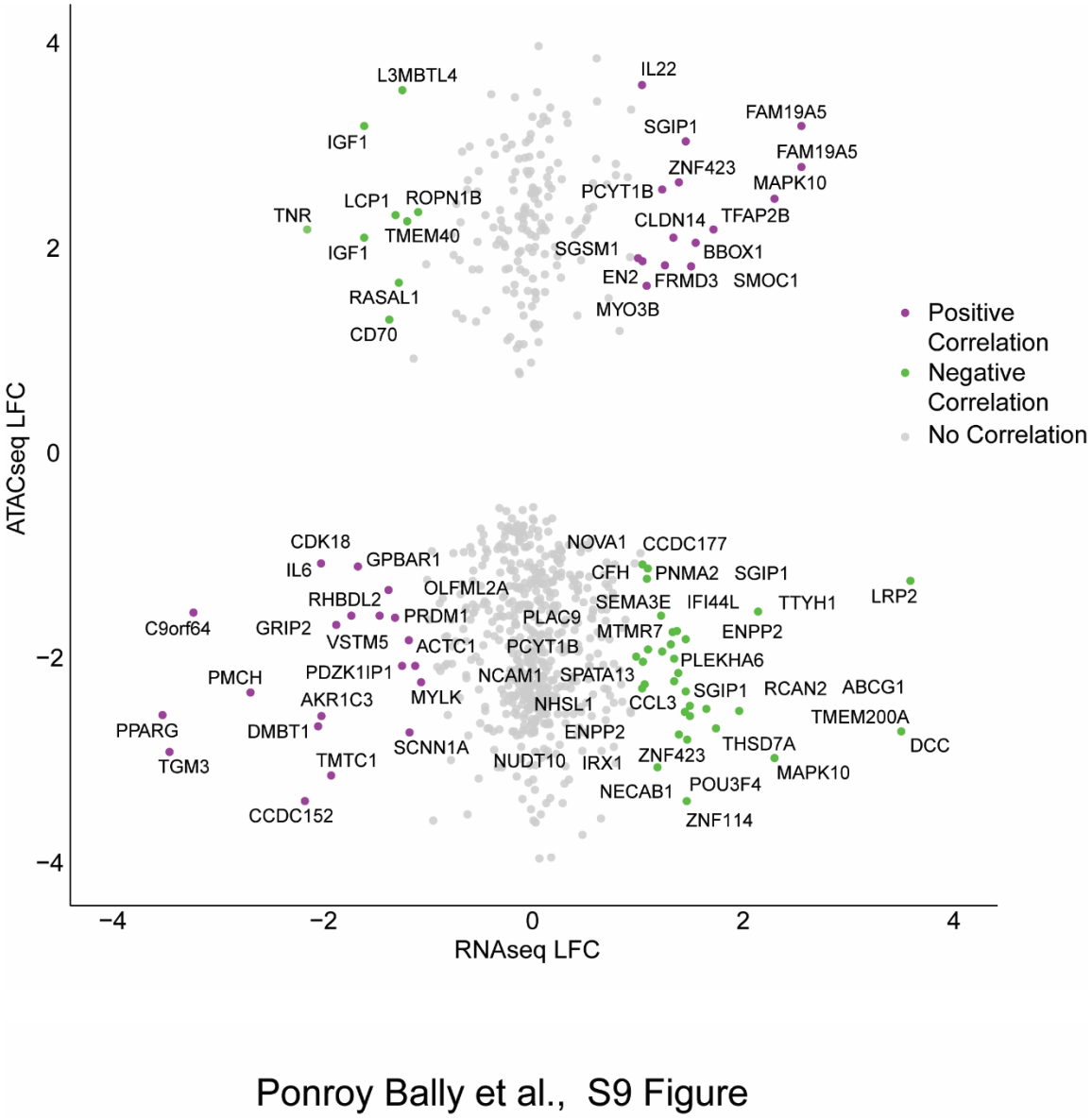
Ponroy Bally et al., S7 Fig

S7 Figure: Differential gene expression analysis. (A) Principal Component (PCA) Analysis of the 3 CTL (grey) and 3 DS (magenta) astrocyte cell lines. (B) PCA Analysis having removed GM22260 and ATCC. (C) Heatmap of unsupervised hierarchical clustering of all CTL and DS cell lines. (D) Heatmap of the top 50 differentially expressed genes in DS astrocytes (magenta: lower expression, green: higher expression).



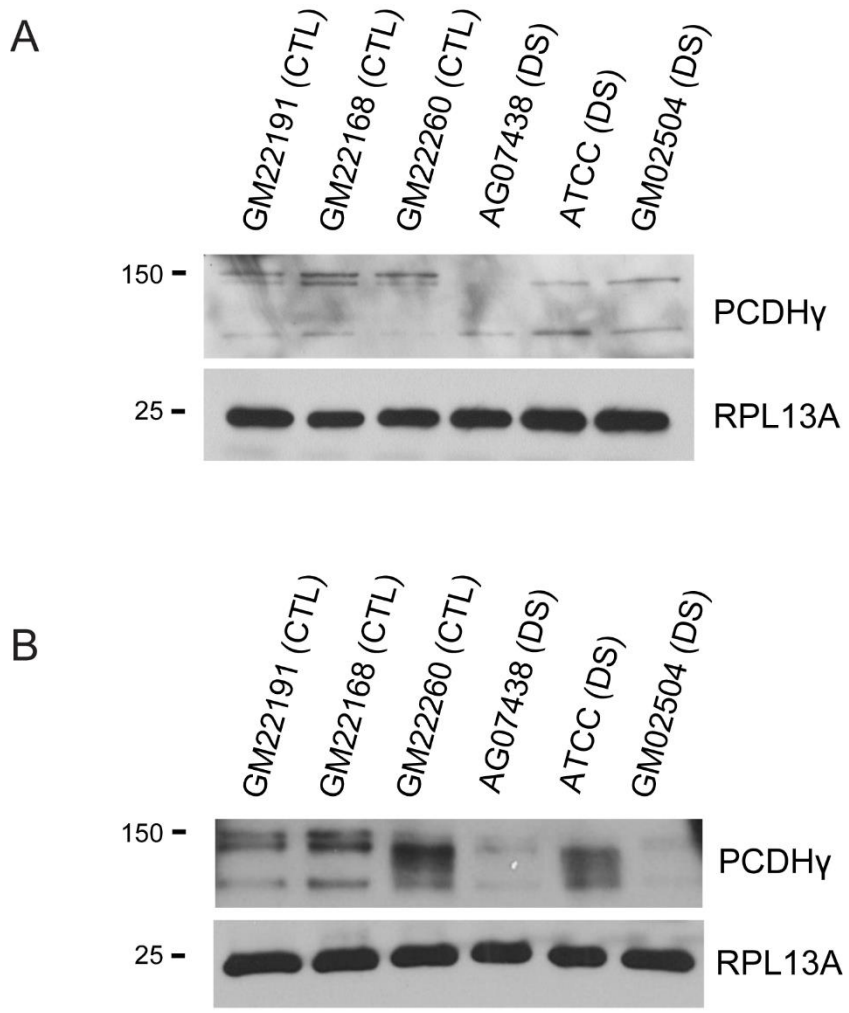
Ponroy Bally et al., S8 Figure

S8 Fig: qPCR validation of differentially expressed in DS astrocytes identified by RNA-seq. (A) qPCR confirms the upregulation of the genes in DS astrocytes: BRSK2, CADM2, COL2A1, DCC, FAT4, ITGB8, JAM2, PCDH9, PCDH19 and PCDHB2. (B) qPCR confirms downregulation of genes in DS astrocytes: ACKR3, ADAMTSL4, COL4A4, COL7A1, COMP, FOXG1, LAMA5, LHX2, NGFR, OMD, PCDHA7, PCDHB7, PCDHGC3, PCDHGC4, RELN, SDC4, SEMA3F, SHH, SIRPA and SPON1. Data are represented as mean ± SEM. N = 3 (3 experiments performed in all 3 CTL and 3 DS cell lines). Two-tailed, unpaired t-tests were performed *p≤0.05 **p≤0.01

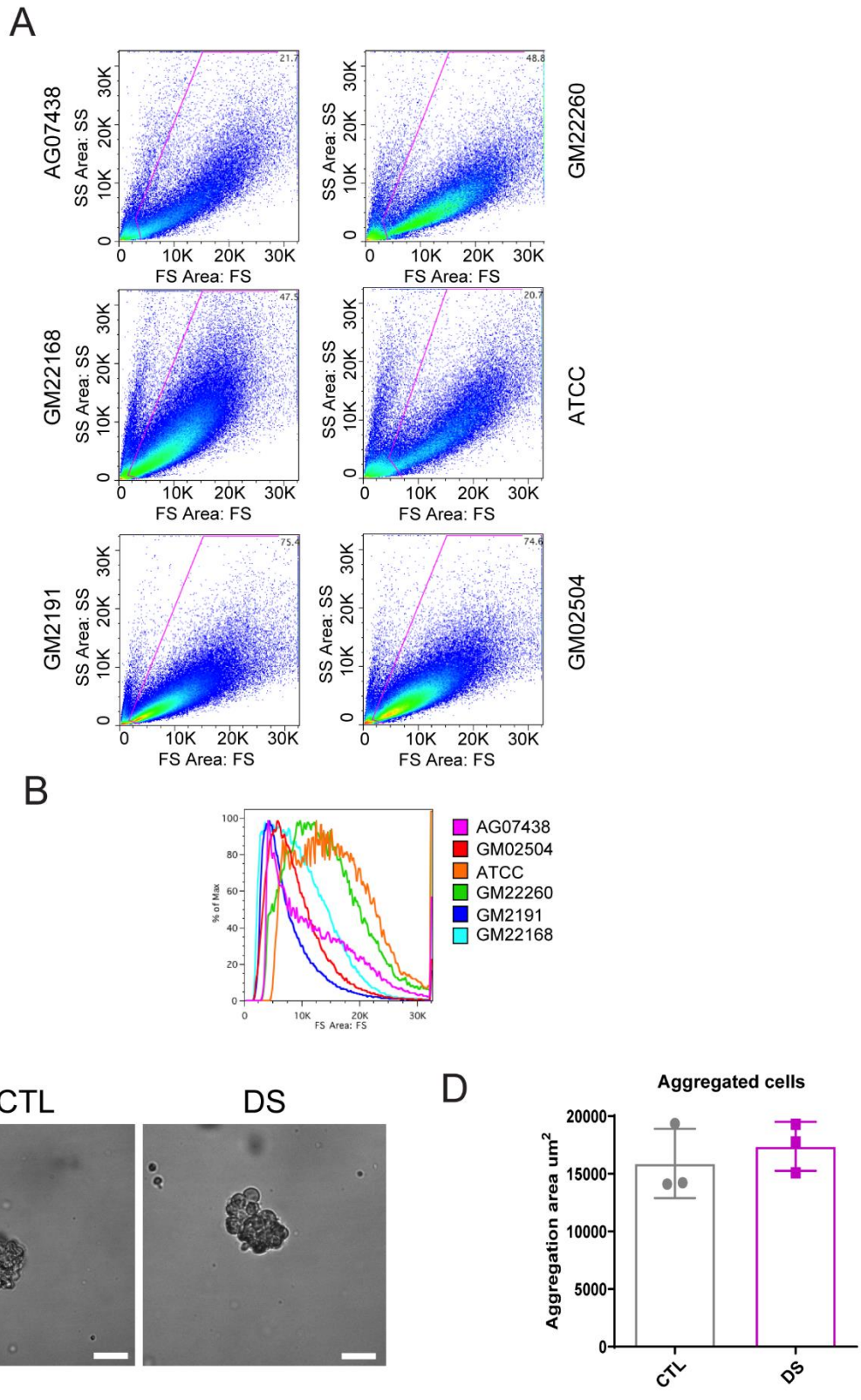


Ponroy Bally et al., S9 Figure

S9 Fig: Concordance analysis between differentially expressed genes identified by RNA-seq and differentially accessible promoters identified by ATAC-seq. Concordance analysis reveals no overall correspondence between individual genes between the ATAC-seq and RNA-seq datasets.

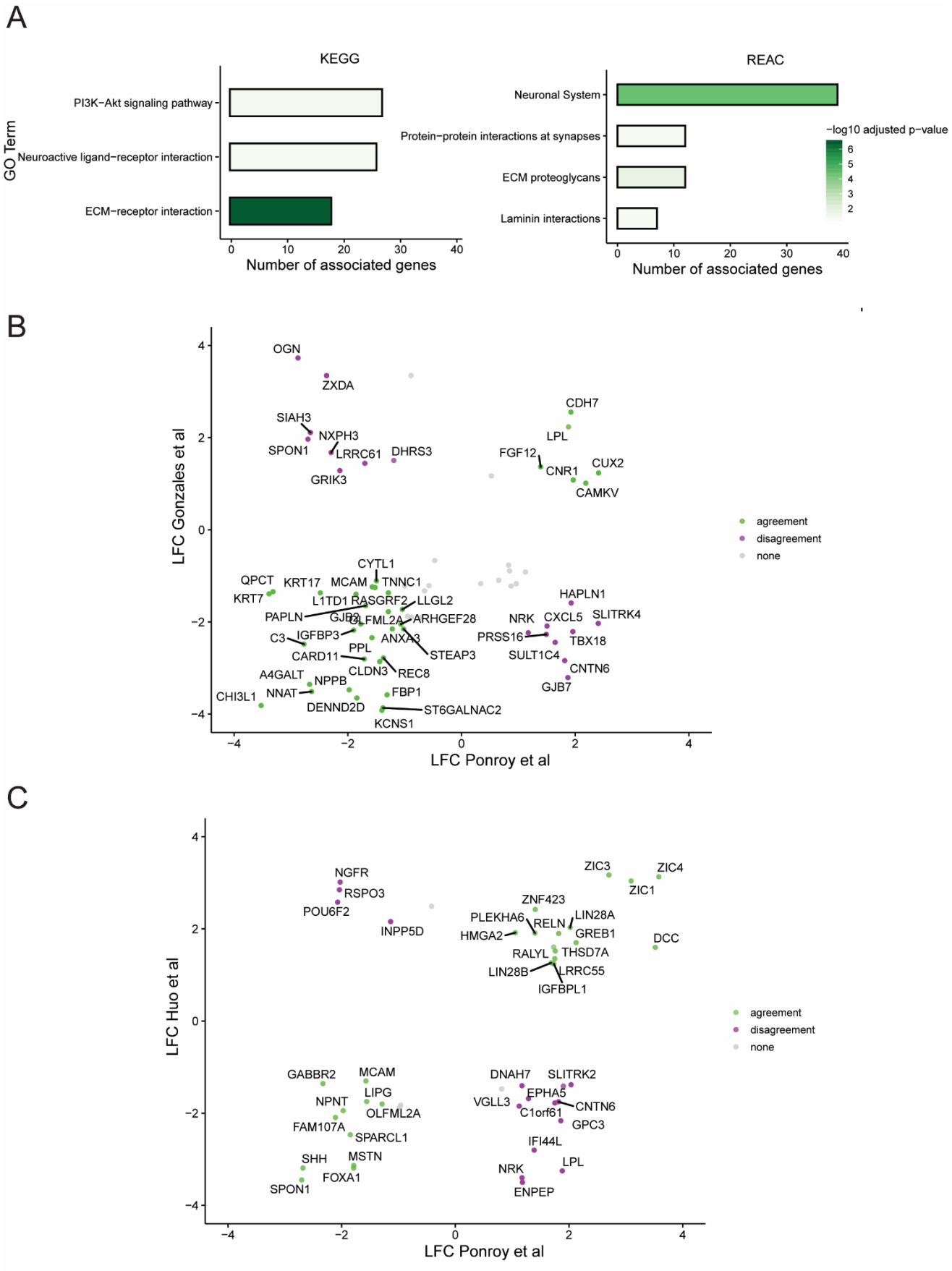


S10 Fig: Additional PCDHG western blots. Western blots demonstrating the downregulation of PCDHGs in DS astrocytes.



Ponroy Bally et al., S11 Fig

S11 Fig: CTL and DS astrocyte size analyzed by flow cytometry. Forward scatter analysis reveals no difference between CTL and DS astrocyte size (cells in suspension). (A) Forward scatter of individual astrocyte lines, showing the cell size distribution of human astrocytes. (B) Forward scatter of all cell lines. (C) After 2 hours in suspension in ultralow adhesion plates DS astrocytes form aggregates of comparable size to those of CTL astrocytes, Scale = 150 μm. (E) Aggregate comparison after two hours in suspension. n = 3 (This experiment was performed 3 times in all 3 DS and all 3 CTL cell lines, 5 images were analyzed per cell line, Data are represented as mean ± SEM. Two-tailed, unpaired t-test was performed.



Ponroy Bally et al., S12 Figure

S12 Fig: Pathway Analysis and Comparison to other datasets. (A) Top pathways enriched in differentially expressed genes according to KEGG and Reactome analysis. Bars encode the –log10 of adjusted p-value and are colored by the number of genes associated with the ontology term. (B) Concordance analysis with the differentially expressed genes of our dataset and Gonzales et al. (C) Concordance analysis with the differentially expressed genes of our dataset and Huo et al.

CHAPTER 4

Progressive Glial Cell Pathology and Age-Related Acceleration of Astrocyte Reactivity in Down Syndrome-Associated AD.

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Keywords: Down syndrome, astrocyte, microglia, Alzheimer's disease, β-Amyloid

4.1 ABSTRACT:

Down syndrome (DS) individuals are at high risk for Alzheimer's disease (AD) and dementia. By age forty, nearly all people with DS show the presence of major hallmarks of AD neuropathology including β-amyloid (Aβ) plaques and neurofibrillary tangles (NFTs). Although much is known about the occurrence of these neuropathological hallmarks, additional attention is needed to understand the alterations to glial cells in DS-associated AD given their importance in brain function/homeostasis. Here, we investigated glial cell alterations associated with AD neuropathology in twenty-one DS individuals, focusing on the timing of Aβ accumulation and the recruitment of activated microglial cells and reactive astrocytes. Analyzing brain samples from older DS individuals (>fifty years of age) demonstrated the abundance of Aβ plaques and severity of tau phosphorylation and NFTs accumulation. Activated microglia and reactive astrocytes formed organized structures around Aβ deposits called reactive glial nets (RGNs) that have been reported in sporadic AD. Monitoring the

development of RGNs in DS samples from individuals ranging in age from thirty-three to sixty-seven years old, we found that RGNs increase their complexity with age. RGN elaboration correlated with Aβ plaque load, but was not associated with a particular Aβ plaque type. Surprisingly, while activated microglial cells showed progressive recruitment to RGNs with age, reactive astrocytes instead showed an age-related pattern of recruitment to Aβ plaques/RGNs that was significantly heightened in DS individuals older than fifty years of age. Altogether, this study demonstrates that DS individuals exhibit progressive AD neuropathology-associated glial cell alterations, form RGN structures similar to those characterized in sporadic AD, and have accelerated astrocyte recruitment to Aβ plaques/RGNs at older ages when there is increased severity of cognitive impairments observed in the DS population.

4.2 INTRODUCTION

Down syndrome (DS) is the most common genetic cause of intellectual disability (affecting 5.8 million people worldwide (Ballard, Mobley, Hardy, Williams, & Corbett, 2016)) and is caused by the triplication of chromosome 21 (trisomy 21). Trisomy 21 affects gene expression in all cells of the body and results in widespread developmental and adult stage alterations in cellular and tissue physiology that impacts facial features, cardiac and gastric system formation, hearing and vision, thyroid function, and brain development/function (Baburamani et al., 2019; Vicente et al., 2020). Although individuals with DS are at greater risk for diseases such as leukemia, the life expectancy of DS individuals has more than doubled in the last thirty years (exceeding sixty years of age (Bittles, Bower, Hussain, & Glasson, 2007)) due to better understanding of the pathophysiology of DS and the effectiveness of medical interventions (Bittles & Glasson, 2004; Esbensen, 2010). With increased life expectancy, age-related comorbidities have become more apparent in DS. This is especially the case with Alzheimer's disease (AD). AD is the most common cause of dementia, accounting for 60-

80% of dementia in the elderly (Wortmann, 2012). AD is characterized by an initial impairment in episodic memory, followed by a more global decline in cognitive abilities such as long-term memory, language skills, and attention (Kelley & Petersen, 2007). Major hallmarks of AD pathology include the accumulation of extracellular Aβ and the formation of intracellular neurofibrillary tangles composed of hyperphosphorylated tau (Ballard et al., 2011). Notably, virtually all DS individuals develop AD neuropathology by age forty, and by sixty years of age, at least seventy percent have dementia (Lai & Williams, 1989; Mann, 1988; Wilcock & Griffin, 2013; K. E. Wisniewski et al., 1985). DS individuals therefore represent the largest group of individuals with early onset AD (Hartley et al., 2015), and dementia is now the leading cause of death in DS (Hithersay et al., 2019). Importantly, there are only limited treatment options available to prevent or slow the progression of AD (Alexander, Emerson, & Kesselheim, 2021; Toyn, 2015).

Post-mortem brain tissue analysis has revealed that DS-associated AD has similar pathological hallmarks to sporadic AD, but these hallmarks can be found decades earlier in life in DS (Mann, 1988; K. E. Wisniewski et al., 1985). Increased gene dosage of amyloid precursor protein (APP) (located on chromosome 21 and triplicated in DS) (Wiseman et al., 2018), and its altered processing to $A\beta_{1\text{-}42}$, has been implicated in DS-associated AD. Aβ deposits have been reported as early as twelve years of age in DS and are abundant by forty years of age (Motte & Williams, 1989; Stoltzner et al., 2000). Interestingly, although Aβ plaque formation occurs much earlier in DS than in sporadic AD, it appears in a similar fashion with the initial formation of diffuse Aβ deposits followed by the presence of mature, dense core plaques that are more closely associated with neurodegeneration (Hartley et al., 2015). NFTs form later (around forty to fifty years of age) and are known to accumulate in the hippocampus, entorhinal cortex and neocortex, similar to what has been detected in older sporadic AD samples (Hyman, West, Rebeck, Lai, & Mann, 1995). Thus, much can be learned about the progression of AD by studying age-related neuropathology in DS.

While neuronal deficits are prominent in DS, alterations to glial cells have also been observed and likely to contribute to AD neuropathology given their critical role in brain function and homeostasis (Araujo et al., 2018; C. Chen et al., 2014; Mizuno et al., 2018; Ponroy Bally et al., 2020). Alterations to microglial cells and astrocytes in AD are implicated in anatomical changes to the brain, breakdown of homeostatic processes, neuroinflammation, and neurodegeneration (McGeer, Itagaki, Tago, & McGeer, 1987; Ojala et al., 2009; Patel et al., 2005; Serrano-Pozo et al., 2013; Shao, Gearing, & Mirra, 1997). Both activated microglia and reactive astrocytes interact with Aβ plaques and have been shown to engulf Aβ peptides in AD (C. Y. Lee & Landreth, 2010; Perez-Nievas & Serrano-Pozo, 2018). Consistent with this, we recently analyzed the three-dimensional structural relationship between astrocytes, microglia, and Aβ plaques in sporadic AD, and found a reproducible glial cell framework around Aβ plaques referred to as reactive glial nets (RGNs) (Bouvier et al., 2016). RGNs have a characteristic inner ring of activated microglia that surround an Aβ plaque. This inner ring is circumscribed by an outer domain of reactive astrocytes. The functional role of RGNs remains elusive, but likely serves as a complex site of communication between microglial cells and astrocytes (Bouvier & Murai, 2015) and as a structural barrier to Aβ plaques, similarly to the glial scar which isolates damaged from healthy tissue following acquired CNS injuries (Burda & Sofroniew, 2014). Whether activated microglial cells, reactive astrocytes, and Aβ plaques show analogous RGN structures in DS-associated AD, as in sporadic AD, remains unknown.

Here, we analyzed brain samples from twenty-one DS individuals and used multi-label, high-resolution microscopy to demonstrate that DS individuals exhibit progressive Aβ-associated glial cell pathology with age, form analogous RGN structures to those detected in sporadic AD, and have accelerated astrocyte recruitment to Aβ plaques/RGNs at older ages. These findings reveal temporal-spatial alterations to glial cells in DS with implications for understanding neuropathological changes in DS and AD.

4.3 RESULTS

EXTENSIVE AB PLAQUE AND TAU PHOSPHORYLATION/NFT ACCUMULATION IN OLDER DS BRAIN

Aβ pathology in DS has been detected as early as twelve years of age (decades earlier than in sporadic AD) and is known to be advanced in nearly all DS individuals over forty years of age (Lai & Williams, 1989; Lemere et al., 1996; Mann, 1988; K. E. Wisniewski et al., 1985). We used antibodies to $A\beta_{1-42}$ and phosphorylated tau (AT8; detecting phosphorylation at serine 202 and threonine 205) and laser-scanning confocal microscopy to detect Aβ plaques and NFTs, respectively, in brain samples of older DS individuals (>fifty years; Table 1). This labeling revealed high levels of Aβ pathology in frontal cortex of DS individuals (Figure 1A) as found previously (Lai & Williams, 1989; Lemere et al., 1996; Mann, 1988; Stoltzner et al., 2000; K. E. Wisniewski et al., 1985). The majority of the Aβ was found as mature dense core plaques (Figure 1A). Extensive tau phosphorylation, especially in close proximity to Aβ plaques, was also observed (Figure 1B and C). The levels of Aβ plaque accumulation and hyperphosphorylation of tau were striking when compared to aged matched controls that had little to no Aβ plaque accumulation (Figure 1A) and tau phosphorylation (Figure 1D). The severity of tau phosphorylation was particularly high, revealing widespread tau phosphorylation that labeled not only NFTs (Figure 1B and C), but also other structures well-beyond NFTs. In addition to extensive Aβ plaques and tau phosphorylation, neuronal pathology in the form of axonal swellings were also evident (Figure 1E). Thus, strong AD pathology was detected in our samples through high-resolution confocal microscopy. This was consistent with other reports of advanced AD pathology in DS individuals.

Reactive Glial Net Formation (RGNs) in DS Brain

Glial cell activation and reactivity is a hallmark of sporadic AD and is implicated in the response to Aβ toxicity and the progression of neuropathology (DeWitt et al., 1998; Kashon et al., 2004; Kato et al., 1998; Nagele et al., 2004; Simpson et al., 2011; Verkhratsky et al., 2010). Glial cell activation and reactivity may also play an important role in the pathophysiology of the DS brain and has been reported to occur as early as 2 days postnatally (Griffin et al., 1989). Interestingly, Flores-Aguilar et al. and Martini et al. have shown gradual increases in the severity of microglial activation in DS brain, ranging from initial moderate microglial activation in young adults to severe activation and cellular dystrophy around age forty to fifty (Flores-Aguilar et al., 2020; Martini et al., 2020). Although previous studies have reported the presence of pathological astrocytes or microglia in close proximity to Aβ plaques in DS (Motte & Williams, 1989; Stoltzner et al., 2000), a deeper understanding of the organization of activated microglia and reactive astrocytes in the DS brain is needed. We recently showed that activated microglia and reactive astrocytes organize into specialized three-dimensional structures around Aβ plaques called RGNs in sporadic AD. RGNs have an organized structural framework composed of an inner sphere of dysmorphic and activated microglia in direct contact with the Aβ plaque and an outer border of reactive astrocytes that appears to partition pathological from adjacent healthier brain tissue (Bouvier et al., 2016). To investigate if RGNs form in DS, we analyzed the distribution of activated microglial cells and reactive astrocytes in proximity to Aβ plaques in multiple brain regions of nine DS individuals over the age of fifty and who all presented advanced AD pathology (Table 1). Activated microglia and reactive astrocytes formed discrete structures around Aβ plaques in older DS individuals in frontal as well as temporal and entorhinal cortex and hippocampus (Figures 2A and B). Activated microglia were identified by Iba1+ staining and an amoeboid morphology, and reactive astrocytes by GFAP+ staining and cellular hypertrophy. We observed an inner ring of activated Iba1+ microglia that was juxtaposed to an outer ring of reactive and dysmorphic GFAP+ astrocytes (Figure 2A). To quantitively analyze the organization of astrocytes and microglia,

we performed detailed three-dimensional analysis of their spatial organization relative the Aβ plaque center in the frontal cortex (Figure 2). Quantitative analysis of the distribution of these cells revealed an enrichment of activated microglia surrounding the Aβ plaque at approximately 10-20μm from the plaque center (Figure 2C). The microglia found at those distances presented a more simplified and amoeboid morphology. This was followed by a high concentration of reactive astrocytes at approximately 40-50μm from the plaque center (Figure 2C). The astrocytes which formed this secondary ring around microglia were polarized towards the plaque center and with processes surrounding the aggregated microglia. Comparing the organization of pathological glial cells in DS samples with those in sporadic AD (Bouvier et al., 2016), indicates that reactive astrocytes and activated microglia form specialized RGNs around Aβ plaques in DS individuals (> fifty years old) that show similar spatial structural properties of RGNs in sporadic AD.

TEMPORAL ANALYSIS OF GLIAL CELL PATHOLOGY AND RGN DEVELOPMENT IN DS

A limitation of analyzing neuropathology in sporadic AD is the late diagnosis of AD at advanced stages of the disease, and the general reliance upon post-mortem brain samples from aged individuals. In contrast, brain samples from DS can be studied at earlier ages with the understanding that all DS individuals eventually go on to develop AD neuropathology. Aβ plaques have been reported as early as 12 years of age in DS (Stoltzner et al., 2000), but brain pathology has generally been observed around thirty years of age, with substantial accumulation of plaques by forty years of age. Interestingly, dementia becomes prevalent around sixty years of age, decades after the first detection of Aβ plaques in DS (Lott & Head, 2019). With this in mind, we were interested in determining how the progression of glial pathology relates to the presence and timing of AD neuropathology in AD. To do so, we tracked the formation of RGNs over time in DS individuals over a time span covering the accumulation of Aβ plaques and tauopathy, as well as, the development of

dementia (thirty-three to sixty-seven years of age divided into four age groups: thirty to thirty-nine; forty to forty-nine; fifty to fifty-nine; sixty to sixty-nine). We labeled frontal cortex post-mortem brain tissue with antibodies to detect astrocytes, microglia, and $A\beta_{1-42}$. We then performed a detailed three-dimensional analysis to reveal the spatial organization of astrocytes and microglia relative to plaques in the four age groups. In these experiments we observed that similarly to what had been reported in previous studies, samples from individuals younger than fifty years of age have an absence or low number of dense core plaques but an abundance of diffuse plaques. This contrasts with samples from individuals fifty years of age and older where there is a high amount of dense core and diffuse plaques. Importantly these experiments also demonstrated the gradual formation of RGNs as DS individuals age (Figure 3). Interestingly, while RGNs were much more formed and complex in the older age groups (Figure 3), we found that in all age groups the accumulation of microglia and astrocytes was present around plaques, this was shown by an increase in microglial cell and astrocyte density 10-20μm and 40-50μm from the plaque center, respectively (Figure 4A,B,D and E). This is similar to what we had observed when looking at aged (50+) individuals (Figure 2).

When examining microglia cell density at two distances (10 and 20μm) between age groups, we found significant differences 10μm away from the plaque center between the 30-39 age group and 60-69 age group, the 40-49 age group and the 60-69 age group, as well as between the 50-59 age group and the 60-69 age group. Significant differences were also observed 20μm away from the plaque center between the 30-39 age group and the 50-59 and the 60-69 age groups, as well as the 40-49 age group and the 60-69 age group (Figure 4B). In order to assess whether the increases observed in microglial density were correlated with age, we compared microglial cell density and age and found a positive correlation between microglial cell density at 10 and 20μm from the plaque and age (R squared= 0.2351 at 10μm and R squared= 0.4929 at 20μm) (Figure 4C). Thus, microglial cell accumulation

begins to occur around plaques in 30-39 age group, with progressive increases in density at 40-49, 50-59, and 60-69 age groups.

We then analyzed astrocyte density from the plaque center (40 and 50μm) between the four age groups and found significant differences observed 40μm away from the plaque center between the 30-39 age group and 50-59 and 60-69 age groups, as well as between the 40-49 age group and the 50-59 and 60-69 age groups. Significant differences are also observed 50μm away from the plaque center between the 30-39 age group and the 60-69 age group, as well as between the 40-49 age group and the 50-59 and 60-69 age groups (Figure 4E). To determine whether the increases observed in astrocyte density are correlated with age, we compared astrocyte cell density and age and found a positive correlation between astrocyte density and age at 40μm away from the plaque but not at 50μm (R squared= 0.3909 at 40μm and R squared= 0.003958 at 50μm) (Figure 4F). These experiments therefore demonstrate that some astrocyte accumulation occurs around Aβ plaques in early age groups, but this accumulation significantly increases in the 50-59 and 60-69 age groups.

RGN STRUCTURE IS NOT AFFECTED BY AB PLAQUE TYPE

By fifty years of age, nearly all DS individuals have significant amounts Aβ dense core plaques and NFTs (Mann & Esiri, 1989; K. E. Wisniewski et al., 1985). However, Aβ accumulation occurs much earlier in DS. Extracellular Aβ deposits in the form of diffuse plaques can be consistently found after thirty years of age and have even been detected in DS individuals in their 20's and teens (Lemere et al., 1996). As stated earlier our experiments showed that in individuals under the age of fifty have an absence or low number of dense core plaques but an abundance of diffuse plaques. While individuals fifty years of age and older possess a high amount of dense core and diffuse plaques. To determine if the RGN-associated glial cell changes are associated with a certain Aβ plaque type (diffuse vs dense core) rather than age of the DS individual, we utilized two labels for plaques that allowed us

to differentiate diffuse and core plaques in our samples. Anti-$A\beta_{1-42}$ was used to label both diffuse and dense core plaques and Thiazine Red (TR) to selectively label dense core plaques and NFTs but not diffuse Aβ plaques (Figure 5A) (Daria et al., 2017; McLellan, Kajdasz, Hyman, & Bacskai, 2003). Importantly, we did not find significant differences in how activated microglial cells and reactive astrocytes organize within the RGN in relation to plaque type (Figure 5B,C). Therefore, we next wondered if the formation of RGNs correlate with plaque load instead of plaque type.

ASTROCYTE RECRUITMENT CORRELATES WITH AB PLAQUE LOAD IN DS

In DS, significant Aβ plaque accumulation begins around 30 years of age and by fifty years of age all DS individuals have sufficient number of Aβ plaques and NFTs to be diagnosed with AD (Mann & Esiri, 1989; K. E. Wisniewski et al., 1985). It is also known that plaque accumulation occurs progressively as DS individuals age. Considering that the differences in the density of astrocytes and microglia between the different age groups was not affected by Aβ plaque type, we investigated if plaque load was a determinant of glial cell pathology and RGN formation. Interestingly, comparing plaque load and age revealed a significant positive correlation between plaque load and age (R squared= 0.5124) (Figure 6B). Moreover, we found a significant increase in plaque load in samples from the two older groups (50-59 and 60-59) when compared to the two younger groups (30-39 and 40-49) (Figure 6A). We then assessed the relationship between astrocytic and microglial cell density with plaque load, however, we did not find a correlation between microglial cell density and plaque load (R squared=0.05115 and 0.1017 respectively) (Figure 6C,D). In contrast, we identified a positive linear correlation between astrocytic cell density and plaque load at 40μm from plaque center (R squared=0.6303) as well at 50μm from plaque center (R squared=0.3282) (Figure 6E,F). In conclusion these experiments demonstrate that plaque load increases with age and while there is a positive linear relationship with age a drastic increase in plaque load appears to occur around age 50.

4.4 DISCUSSION

Improvements in health care coupled with a better understanding of DS pathophysiology have led to increased quality of life of DS individuals. As life expectancy in DS has doubled in the last 30 years, more prevalent age-related comorbidities have emerged in the DS population with AD being the most common. Indeed, virtually all DS individuals are known to develop AD with a rather predictable timeline of events. Studies have reported the initial formation of diffuse Aβ plaques in the twenties and thirties, and by age forty all DS individuals have been reported to have dense core plaques. This is followed by the accumulation of NFTs slightly later life in the fifties, and finally dementia in their sixties (70% of DS individuals). Reports of reactive glial cells in proximity to plaques have also been made anecdotally, however systematic analysis of glial pathology and the timeline for when glial cells become reactive and accumulate around Aβ plaques remains unknown. Treatment options for DS-associated AD, along with AD itself, remain limited.

In this study, we investigated glial cell pathology in DS and its relationship to the progression of AD pathologies including formation of Aβ plaques. Astrocytes and microglial cells formed specialized RGNs around Aβ plaques and showed a conserved architecture to those found in sporadic AD. Analyzing twenty-one DS brain samples across multiple ages (from thirty-three to sixty-seven years of age), demonstrated that glial cell pathology near Aβ plaques changes over time, with a progressive increase in the complexity of RGN architecture. Remarkably, RGN formation showed a strong correlation with Aβ plaque load in DS but was not dependent on Aβ plaque type. Surprisingly, while activated microglial cells progressively increased their association with Aβ plaques in RGNs with age, astrocyte recruitment was significantly accelerated beyond 50 years of age in DS individuals. Thus, DS individuals exhibit progressive microglial cell pathology near β-Amyloid (Aβ) plaques with age. However, the selective increase in astrocyte recruitment to Aβ plaques beyond 50 years of age

correlates with the timing of more pronounced AD-associated cognitive impairments in the DS population.

In our study we aimed to uncover glial pathology in DS with a particular focus on relationships to AD neuropathology. We first were interested in determining whether astrocytes and microglia form complex and specialised structures around Aβ plaques (RGNs), as it had been reported in sporadic AD, and if so, to establish the timeline of the formation of those specialized structures. By analyzing brain samples ranging from individuals aged 33 to 67, we identified the presence of RGNs in DS brain. Furthermore, we showed that the RGNs form over time as the DS individuals age with an increase in microglia and astrocytes contributing to the RGN structure at older ages. Interestingly, RGN formation was not to be affected by plaque type, which was surprising to us since core plaques are more complex then diffuse plaques and are more closely associated with neurodegeneration (Hartley et al., 2015). However, it is important to think about the neuropathology in its globality. As we showed earlier in individuals over the age of 50 the Aβ accumulation is very severe (Figure 1A). Therefore, the diffuse plaques which we analyzed were in close proximity to many other plaques, NFTs and other neuropathologies. In addition, it is known that at this age neuroinflammation is established. Therefore, it is likely that the state of the astrocytes and microglia which form RGNs around diffuse plaques in aged tissue with severe widespread pathology is very different from those forming RGNs in the first diffuse plaques appearing in a relatively healthy young brain tissue. For this reason we next went on to analyse plaque load and found that plaque load in the DS brain was positively correlated with subject age and with RGN formation.

Importantly we identified two very different and distinct patterns in the accumulation of astrocytes and microglia around plaques. Microglia were found to start accumulating around plaques (at 10 and 20μm) early in the thirties and forties and do so in a gradual manner which correlates with

age. Astrocytes however start accumulating later and suddenly around age fifty, and while astrocyte density was not found to correlate with age, it instead correlates with plaque load.

These results are important as they provide a timeline of the formation of the RGNs around plaques which was previously unknown. Indeed, we can now incorporate the changes in glial spatial organization to the pre-existing timelines of other types of AD pathology (Figure 7). This reveals that microglial and astrocytic recruitment to the plaque occur after Aβ accumulation and slightly after the accumulation of NFTs. Importantly these events occur rather late in the disease and therefore offer the possibility of therapeutic intervention. This is especially important considering the fact that an increase in glial cell density around plaques is initially considered beneficial as these cells attempt to isolate the Aβ pathology from the rest of the tissue and are phagocytic (Katsouri et al., 2020; Nicoll & Weller, 2003; Ries & Sastre, 2016). However in the long term, the astrocytes and microglia may exacerbate the disease and contribute to chronic neuroinflammation (Bouvier & Murai, 2015).

This study also allows for a precise timeline of pathological events involving glial cells which would help time possible interventions. Importantly the timeline established in this study also allows us to note that the increase in astrocyte density around the plaques occurs at the same time as the onset of clinical symptoms. This is important since the exact cause of the clinical symptoms remains undetermined, and in DS the appearance of Aβ plaques and NFTs occurs decades before the appearance of dementia. It is therefore possible that the changes in the properties and localization of astrocytes have a direct correlation with the patient's cognitive abilities. Indeed, astrocytes are known to play crucial roles in healthy brain such as the maintenance of homeostasis and metabolism. And prolonged reactive gliosis is known to cause a breakdown of homeostasis and loss of function in astrocytes which further exacerbates neurodegeneration (Matos, Augusto, Oliveira, & Agostinho, 2008; Merlini, Meyer, Ulmann-Schuler, & Nitsch, 2011; Peters et al., 2009). Future research can exploit

these finding to better understand the relationship between accumulation of Aβ and glial cell pathology with changes in cognitive ability in aging DS individuals.

4.5 MATERIALS AND METHODS

Human Tissue Samples

All experiments involving human tissues were conducted in accordance with the guidelines approved by the Douglas Institute Research Ethics Board. Post-mortem brain samples were obtained from the Douglas-Bell Canada Brain Bank (Canada), the Maritime Brain Tissue Bank (Canada), the Bristol Southwest Dementia Brain Bank (UK) and the Corsellis Brain Bank (UK). BRAIN UK Reference number: 15/008.

Preparation of human brain slices

Human brain samples (1 cm^3 dissections) were briefly rinsed in PBS and cryo-preserved in 30% sucrose in PBS for approximately 72 hours. Human samples were embedded in M-1 embedding matrix (Thermo scientific, USA) and cut into 50 μm thick slices on a freezing sliding microtome and stored at −20 °C in a cryoprotectant, anti-freeze solution containing ethylene glycol (30%) and glycerol (30%) in PBS until processed for immunofluorescence.

Immunofluorescence

Tissue sections were stained using a protocol previously established (Bouvier et al., 2016). Briefly the samples were washed 3 times in PBS and placed under a UV light for 16 hours. The samples were then permeabilized in 0.3% Triton-X 100 for 15 minutes and incubated for 1 hour in blocking solution containing 0.3% Triton-X 100, 2% horse serum, in PBS). This was followed by an incubation of the primary antibodies in the same blocking solution for 72 hours at 4°C on a horizontal shaker.

The samples were then washed 3 times in PBS and incubated with fluorescently tagged secondary antibody solution containing 0.3% triton-X 100/PBS at room temperature. The samples were washed 3 times and mounted onto slides using ProLong Gold Antifade reagent (Invitrogen). Primary antibodies were used at the following concentrations: guinea-pig anti-GFAP (Synaptic Systems) 1:500, rabbit anti-Iba1 (Wako) 1:500, mouse anti-Aβ42 (Cedarlane) 1:500, mouse anti AT8 (Thermofisher) 1:500 and mouse anti-SM312 (Covance) 1:500. The dye Thiazine red (Sigma Chemicals) was used to label amyloid plaques at 0.2 μM.

Imaging and analysis

Images were acquired using an Olympus FV-1000 laser scanning microscope and Fluoview FV10 software (Olympus). 10x, 20x and 40x objectives were used (numerical aperture (NA): 0.30, 0.85 and 1.30 respectively). Plaque volumes and inter-distances between center of Iba1+ and/or GFAP+ cells and center of plaques were estimated with Imaris 7.6 software (Biplane) using "Surface Rendering" and "Measurement Points" modules. For all cell density studies, eight plaques were analyzed per subject and therefore the n equals plaque number. For the linear correlation studies n equals subject number. For the plaque load analysis eight images were taken per sample and the mean of those was plotted and therefore the n equals subject number. Statistical analyses were performed with Graphpad (version 5.01). A two-way analysis of variance (ANOVA) was performed to compare the influence of age on cell density (astrocyte and microglia) at different distances from the plaque center. ANOVA analyses were followed by a Tukey/Sidak post hoc test when appropriate. Linear regression analysis was performed in order to assess if linear correlations are present between astrocyte/microglial cell density and age, plaque load and age and finally astrocyte/microglial cell density and plaque load. R squared values were used to measure how close the data points are to the fitted line. A one-way ANOVA was performed to compare the influence of age on plaque load and a

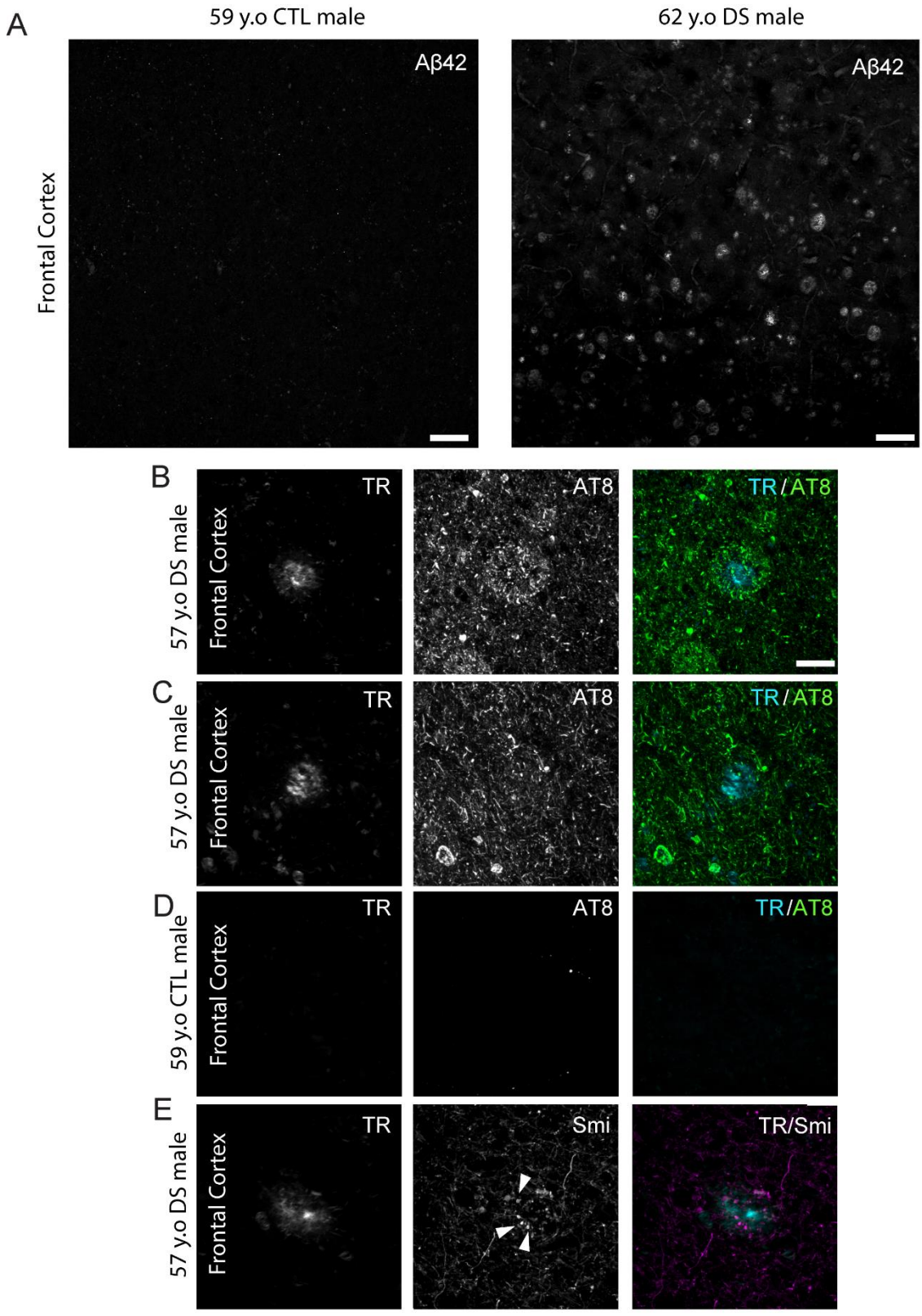
Tukey post hoc test was performed. Standard symbols were used to report significance: n.s. - not significant * $p>0.05$), ** $p<0.05$, *** $p<0.01$, ****-$p<0.001$, ***** $p<0.0001$.

4.6 Figures

Age group	Brain Bank	Individual	Age	Sex	Fixation Date
30-39	Corsellis Brain Bank	218/83	33	F	1983
	Corsellis Brain Bank	74/77	36	F	1977
	Corsellis Brain Bank	174/70	36	F	1970
	Corsellis Brain Bank	2/63	37	F	1963
	Corsellis Brain Bank	168/68	36	M	1968
40-49	DouglasBell Canada Brain Bank	DH88	43	F	1984
	Bristol South West Dementia Bank	563	48	F	1999
	Corsellis Brain Bank	102/85	42	F	1985
	Corsellis Brain Bank	128/74	47	M	1974
	Corsellis Brain Bank	146/78	48	M	1978
	Corsellis Brain Bank	139/76	41	F	1975
	Corsellis Brain Bank	308/78	46	F	1979
50-59	Bristol South West Dementia Bank Dementia Bank	258	50	F	1991
	Maritime Brain Tissue Bank	BB00-008	57	M	2000
	DouglasBell Canada	DH73	59	F	1984
	Bristol South West Dementia Bank	367	59	F	1994
60-69	Bristol South West Dementia Bank	238	62	M	1990
	Bristol South West Dementia Bank	252	63	F	1991
	Bristol South West Dementia Bank	325	64	F	1993
	Bristol South West DementiaBank	364	64	M	1994
	Bristol South West Dementia Bank	472	67	F	1997

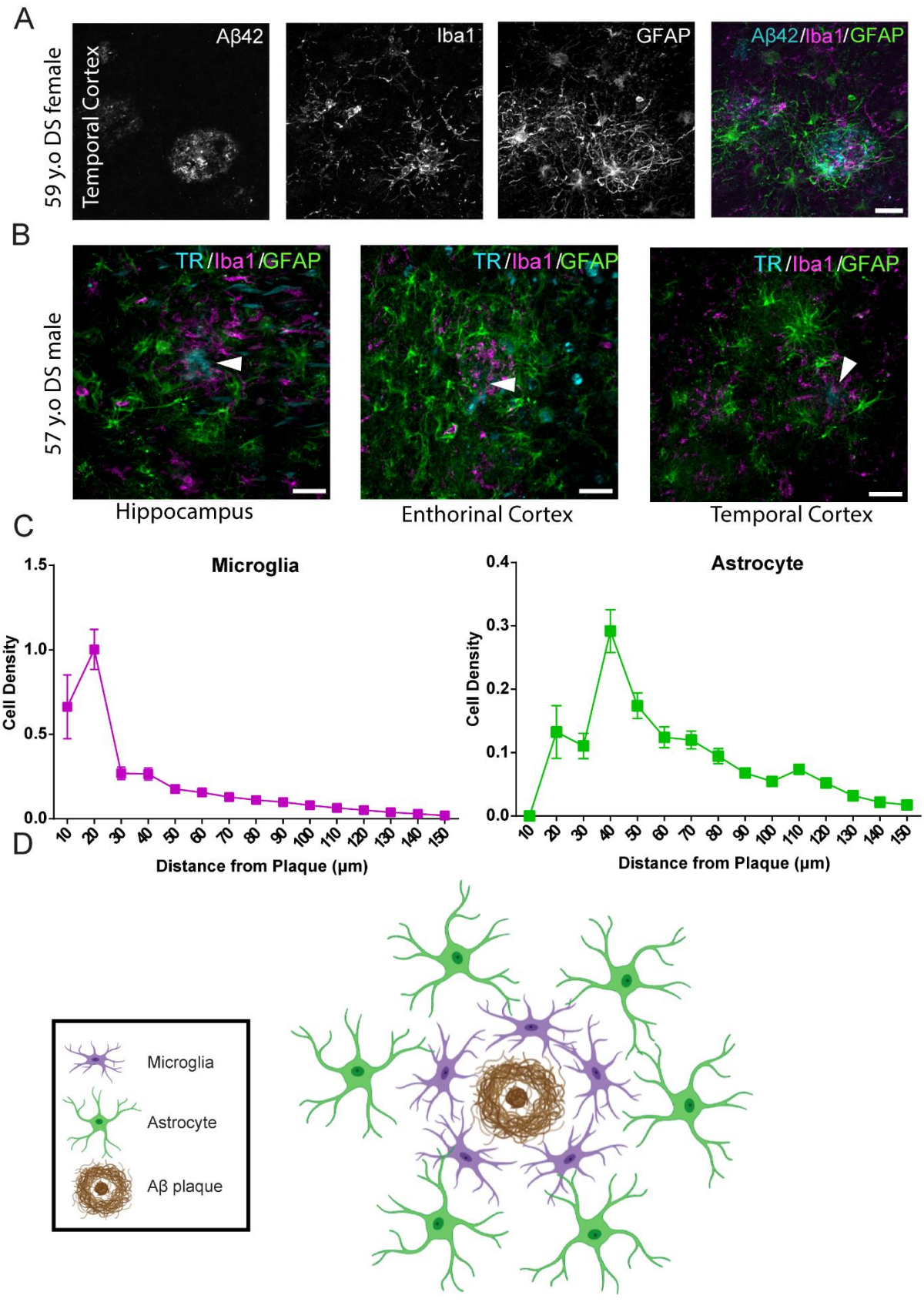
Ponroy Bally et al, Table 1

Table 1: Table of the DS post-mortem human tissue samples used in this study.



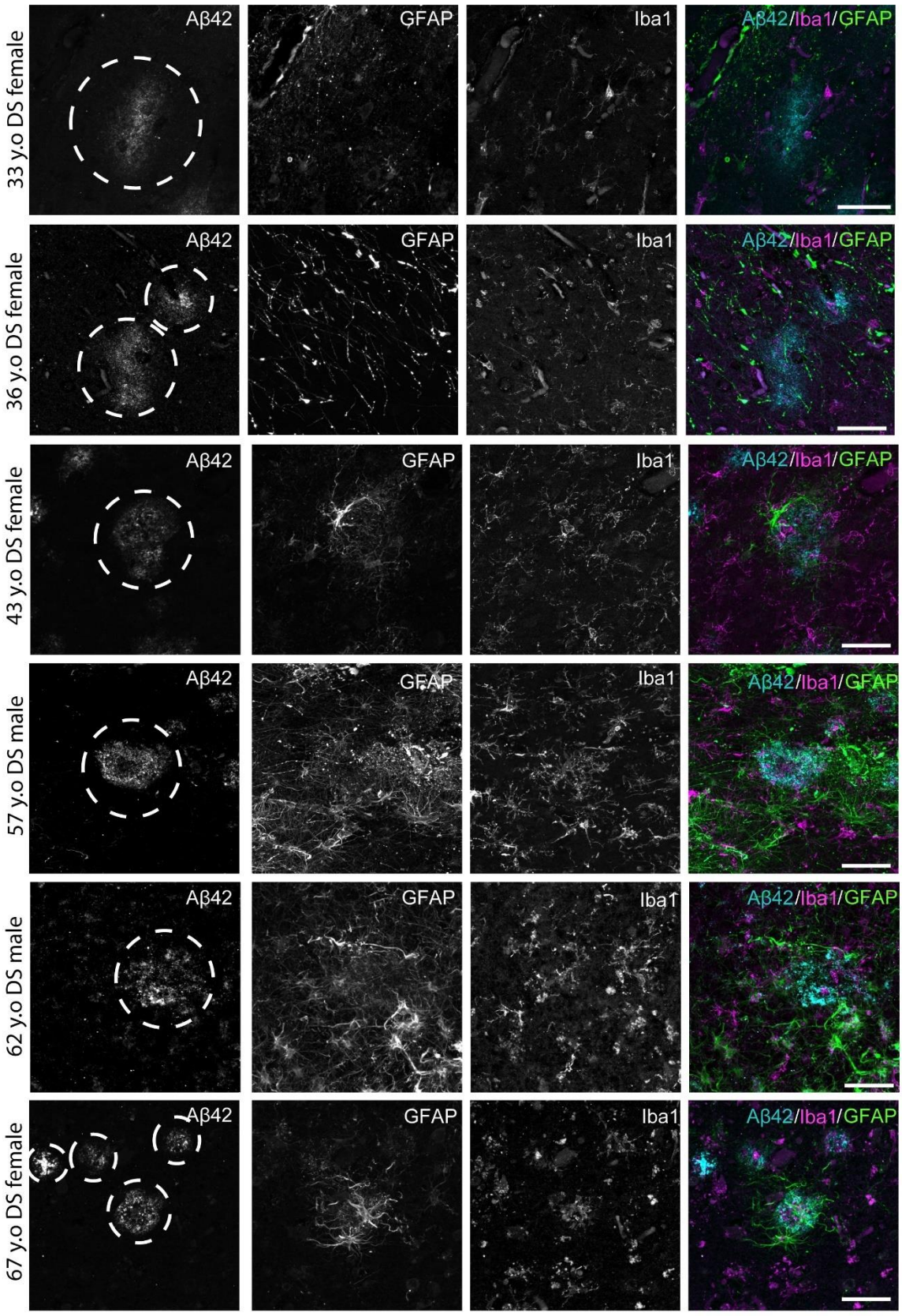
Ponroy Bally et al, Figure 1

Figure 1: Aged DS individuals present advanced AD A) Severe plaque load (Ab42 white) is found in the frontal cortex aged DS individuals (illustrated here in a 62 year old DS male) compared to an absence of plaques in aged matched control (illustrated in a 59 year old male) (scale bar= 100 μm). B,C) Aged DS individuals possess high levels of phosphorylated Tau both in the frontal cortex (AT8 green) located in close proximity to core plaques (TR cyan) but is also widespread throughout the tissue (57 year old DS male, scale bar = 50μm). D) In comparison in aged matched controls there is a total absence of Tau phosphorylation and plaque deposition (59 year old CTL male, scale bar = 50 μm). E) Presence of neural pathology in the form of axonal swellings (SMI312 magenta) near plaques (TR cyan) (57 year old DS male, scale bar = 30μm).



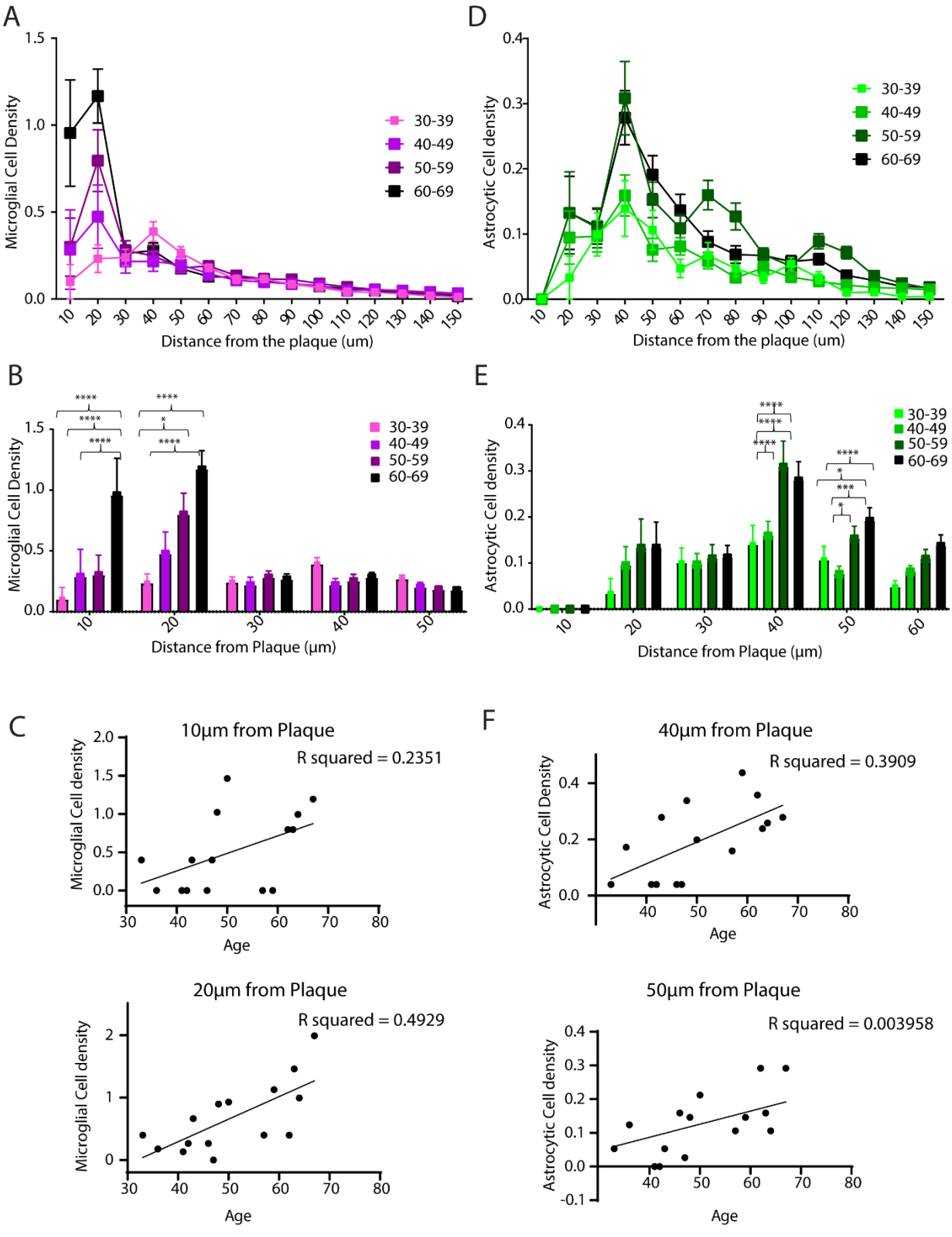
Ponroy Bally et al, Figure 2

Figure 2: Specialized Reactive Glial Nets (RGNs) form around beta-amyloid plaques in the cortex of aged DS individuals. A) Composite images of Aβ42 positive plaques (cyan) surrounded by Iba1 positive microglia (magenta) and GFAP positive astrocytes (green) in a 59 year old DS female, Scale= 50μm. B) RGNs are found around core plaques in various brain regions such as the hippocampus, temporal cortex and entorhinal cortex. Indeed core plaques are present in all brain regions (stained with TR in cyan) and are surrounded by microglia (magenta) and astrocytes (green) (57 year old male, scale bar bar= 30μm). C,D) Quantification of the positioning of microglia and astrocytes relative to the plaque (n=72 plaques) D) Schematic of the RGN: Activated Iba1+ microglia form an inner ring around the Aβ-plaque to which is juxtaposed an outer ring of reactive and dysmorphic GFAP+ astrocytes. (Created with Biorender).



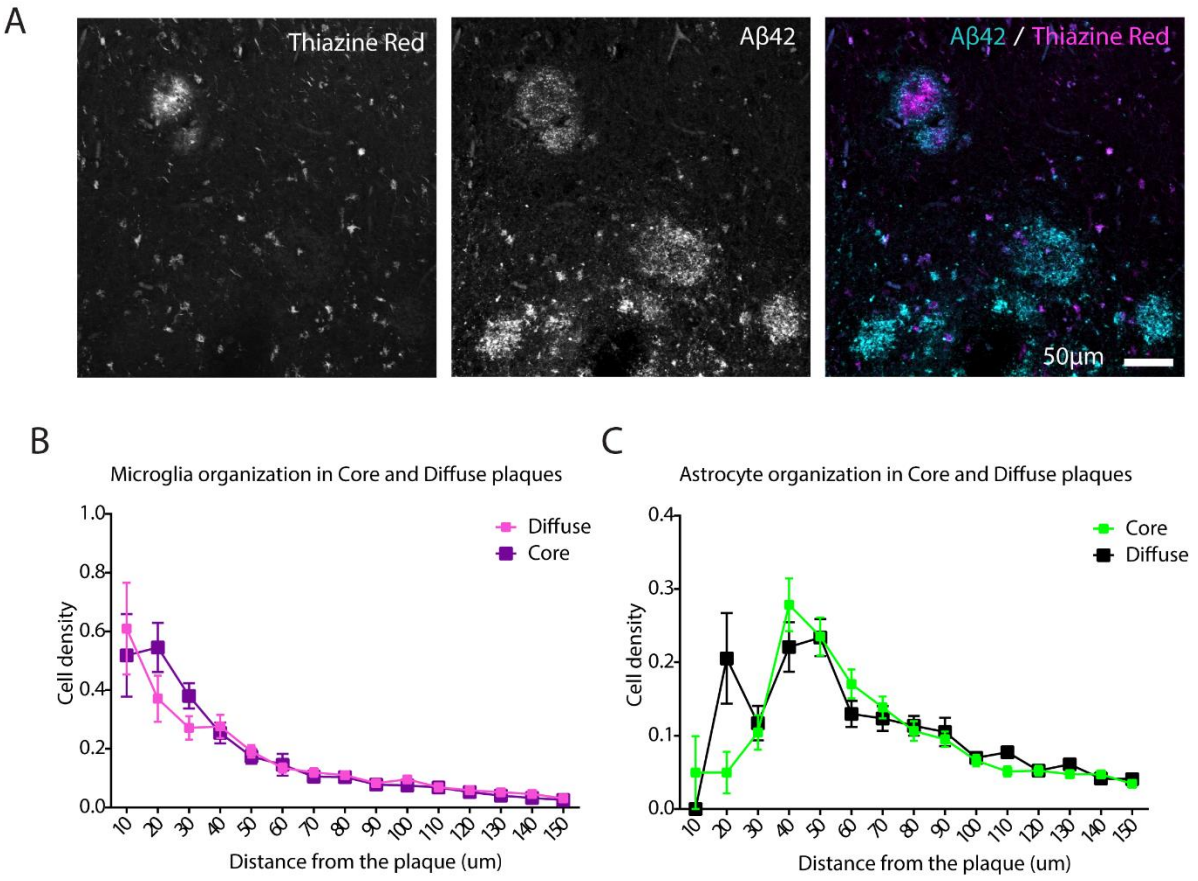
Ponroy Bally et al, Figure 3

Figure 3: RGNs form gradually as DS individuals age. Composite images of Aβ42 positive plaques (cyan) surrounded by Iba1 positive microglia (magenta) and GFAP positive astrocytes (green) in DS individuals ranging from age 33 to 67 illustrate the gradual increase in GFAP positive astrocytes and Iba1 positive microglia around plaques, and therefore the gradual formation of the RGN. (33 year old female, 36 year old female, 43 year old female, 57 year old male, 62 year old male, 67 year old female, scale = 50μm)



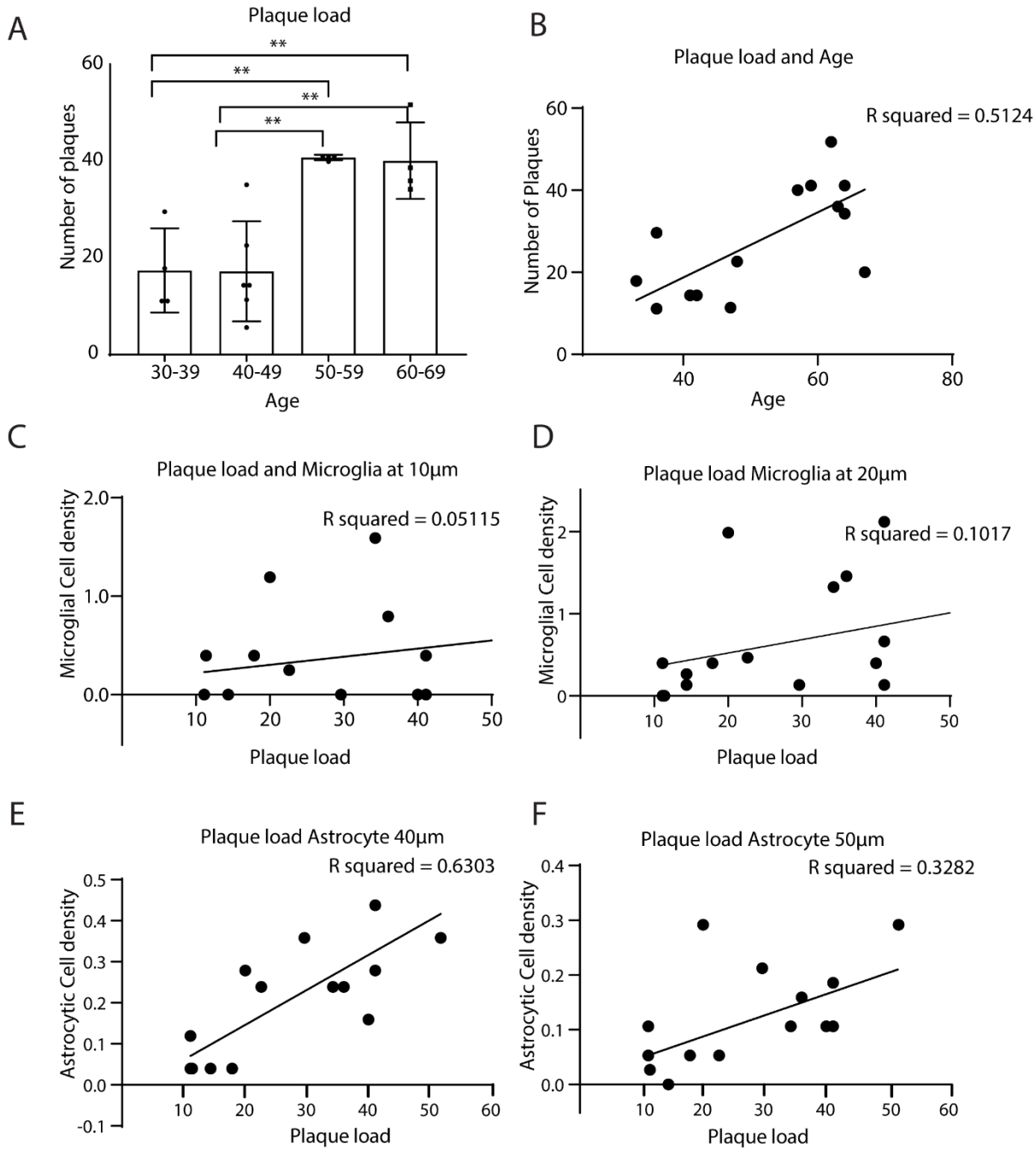
Ponroy Bally et al, Figure 4

Figure 4: Analysis of RGN formation as DS individuals age. A,E) Quantification of the positioning of astrocytes and microglia relative to the plaque in individuals ranging from 30 to 69 years of age. (n=32 plaques, 56 plaques, 32 plaques, 40 plaques, respectively) B) Microglial density at 10μm and 20μm from the plaque increases with age. Significant differences are observed 10 μm away from the plaque center between the 30-39 age group and 60-69 age group, the 40-49 age group and the 60-69 age group, as well as between the 50-59 age group and the 60-69 age group. Significant differences are also observed 20 μm away from the plaque center between the 30-39 age group and the 50-59 and the 60-69 age groups, as well as the 40-49 age group and the 60-69 age group. (Two way Anova, Sidak's Multiple comparison test) (n=32 plaques, 56 plaques, 32 plaques, 40 plaques, respectively). C) Presence of a positive correlation between microglial cell density at 10 and 20μm from the plaque and age (R squared= 0.2351 at 10μm and R squared= 0.4929 at 20μm). E) Astrocyte density at 40μm and 50μm from the plaque increases with age. Significant differences are observed 40 μm away from the plaque center between the 30-39 age group and 50-59 and 60-69 age groups, as well as between the 40-49 age group and the 50-59 and 60-69 age groups. Significant differences are also observed 50 μm away from the plaque center between the 30-39 age group and the 60-69 age group, as well as between the 40-49 age group and the 50-59 and 60-69 age groups. (Two way Anova, Tukey's Multiple comparison test) (n=32 plaques, 56 plaques, 32 plaques, 40 plaques, respectively). F) Presence of a positive correlation between astrocyte density and age at 40μm away from the plaque but not at 50μm ((R squared= 0.3909 at 40μm and R squared= 0.003958 at 50μm).



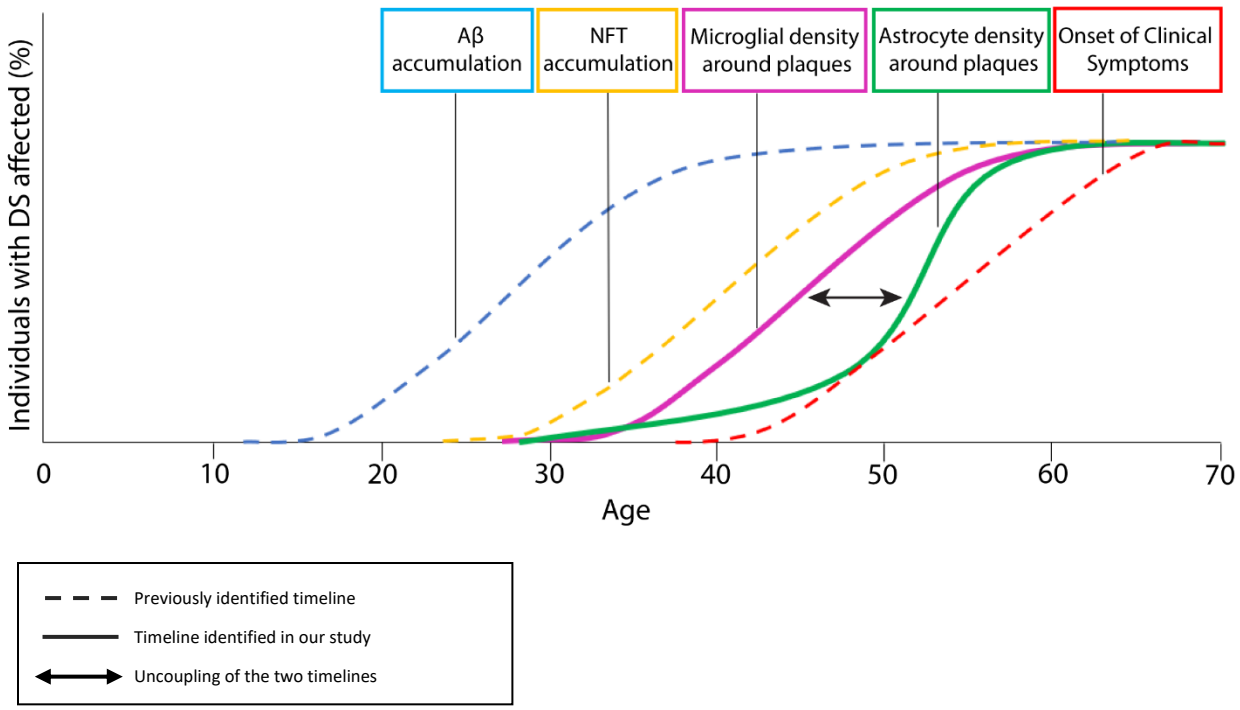
Ponroy Bally et al, Figure 5

Figure 5: The RGN is not affected by plaque type. A) Composite images of Thiazine Red staining of core plaques (magenta) and Aβ42 staining of all plaques in a 64 year old DS male, Scale= 50μm B,C) Quantification of the positioning of astrocytes and microglia relative to the plaque in individuals ranging from 50 to 69 years of age in core and diffuse plaques. Astrocytic and microglial density are not significantly different between core and diffuse plaques. (n=64 plaques) (Two-way Anova, Tukey's Multiple comparison test)



Ponroy Bally et al, Figure 6

Figure 6: Astrocyte density around Aβ plaques is positively correlated to plaque load. A) Plaque load in the four age groups shows a sudden increase in plaque load around age 50. Significant differences are observed between the 30-39 age group and the 50-59 and 60-69 age groups as well as between the 40-49 age group and the 50-59 and 60-69 age groups. (n=5 individuals, 7 individuals, 4 individuals, 5 individuals, respectively) (One-way Anova, Tukey's multiple comparison test) B) Presence of a positive linear correlation between plaque load and age (R squared=0.5124) C,D) Absence of a linear correlation between plaque load and microglial density at 10 and 20μm away from the plaque (R squared=0.05115 and R squared=0.1017) E,F) Presence of a positive linear correlation between plaque load and astrocytic cell density at 40 and 50μm away from the plaque (R squared=0.6303 and R squared=0.3282).



Ponroy Bally et al, Figure 7

Figure 7: Schematic representation of the evolution of the RGN across the lifespan, added to previously identified timelines of the evolution of Aβ deposition, NFT accumulation and appearance of neurological symptoms, adapted form Lott and Head (Lott & Head, 2019).

CHAPTER 5
Conclusions and Future Directions

Over the past few decades, astrocytes have gone from being considered as passive connective elements of the CNS, to being appreciated as complex, heterogeneous cells which play many critical roles in brain development and function. Moreover, it is now well-recognized that astrocytes are involved in many CNS disorders/diseases where they are important regulators and, an in some cases, drivers of disease. Because of this, astrocytes may be useful therapeutical targets for these conditions. Related to this, new discoveries are challenging the concept that impairments to neurons in DS fully account for the alterations observed in brain development and function. There is now a stronger appreciation that other brain cell types, including astrocytes, are actively participating in DS. In Chapter 1, I presented a literature review highlighting important roles which astrocytes play in the brain and their participation in various CNS disorders/diseases. In Chapter 2, I reviewed the literature that highlights the known alterations found in DS astrocytes and the implications which these have on the developing, adult and ageing brain. While this review sheds light on some key studies, it also exposes the relatively low number of studies that have been performed in this area.

For this PhD thesis, I aimed to further uncover the role of astrocytes in DS. I performed to two main studies, one which identified whole-genome dysregulation of the transcriptome and chromatin structure of human DS astrocytes *in vitro* (Chapter 3) and one which showed the progressive pathology of glial cells in DS human brain samples (Chapter 4). I hope that these studies will spark further work to be performed on astrocytes in DS, and potentially, lead to development of targeted therapies for correcting specific alterations to astrocytes in DS.

In Chapter 3, I demonstrated that hiPSC-derived astrocytes present whole-transcriptome alterations with especially strong dysregulation of ECM and cell adhesion molecule genes. Previous studies had also found whole-transcriptome alterations in other cell types such as neurons, hiPSCs and fibroblasts, as well as in human brain tissue (Olmos-Serrano et al., 2016). This has led to an important shift in the DS field which is now incorporating studies that provide a more global picture of changes occurring in DS. Our study was the first to demonstrate the presence of such alterations in DS astrocytes and identified over 700 misregulated genes. I believe this study can serve as an important resource for the research community by providing valuable hiPSC lines (control and DS) that can be used in a variety of experimental contexts, along with unique datasets that can be further studied to better understand the cellular effects of trisomy 21.

Interestingly, the alterations to ECM and cell adhesion molecule genes we found were specific to astrocytes. NPCs of the same hiPSC lines from which the astrocytes originated, did not present the same alterations to these genes. This is consistent with studies that have shown significant alterations to ECM and cell adhesion molecule genes in other cell types and tissues including hiPSC-derived neurons (Gonzales et al., 2018; Huo et al., 2018). However, there is little concordance between the differentially expressed ECM and cell adhesion molecule genes previously identified (Gonzales et al., 2018; Huo et al., 2018). Therefore, while cell adhesion molecule and ECM genes are generally altered in hiPSC-derived DS neurons and astrocytes, the genes responsible for such alterations appear to be largely different. Expanding this work to include other cell types, including those outside the CNS, would be helpful in determining if there are any conserved alterations in transcriptional pathways. I also identified genome-wide alterations in the chromatin structure in DS astrocytes, with especially strong alterations of the chromatin structure of ECM and cell adhesion molecule genes. These studies are in agreement with a study that revealed alterations in the whole-genome epigenetic landscape in hiPSCs derived from DS individuals (Letourneau et al., 2014). In the future, an interesting experiment

would be to perform ATAC-seq on several cell types in parallel including hiPSCs, NPCs, neurons, and astrocytes in order to identify common and distinct changes to the whole genome. Furthermore, analysis of DNA methylation state in DS astrocytes, for example through bisulfite sequencing would likely help pinpoint the precise changes to the genes affected (Y. Li & Tollefsbol, 2011; Rauluseviciute, Drablos, & Rye, 2019). Moreover, it would be interesting to determine if the epigenetic changes can be normalized, for example by exogenously expressing a chromatic remodeling protein or applying small molecules that are known to regulate the epigenetic landscape (Finley & Copeland, 2014; Y. Li & Tollefsbol, 2011; Zhu, Wei, Cai, & Jin, 2020).

Following the identification of transcriptomic and epigenomic changes to ECM and cell adhesion genes, I performed experiments to test cellular adhesion and motility properties of DS astrocytes. DS astrocytes displayed an increase in cell size which was attributed to an increase in cell spreading. Furthermore, DS astrocytes presented altered adhesive preferences, as they displayed decreased homophilic cell adhesion and increased adhesion to uncoated surfaces. DS astrocytes also showed an increase in overall cell motility. In addition to these more generalized alterations in cell adhesion and dynamics, DS astrocytes displayed specific impairments in PCDHγC3-mediated adhesion which was caused by decreased expression of PCDHγC3 at the mRNA and protein level. PCDHs are an important and diverse family of proteins which play critical roles in cell recognition and regulate important processes during development such as synaptogenesis and axonal and dendritic growth (Garrett & Weiner, 2009; Light & Jontes, 2017; Molumby et al., 2016). These processes known to be defective in DS (L. Becker et al., 1991). It is therefore possible that some of the defects in PCDH-mediated adhesion are responsible for such deficiencies in DS. Validating changes in PCDH expression in DS brain samples and functionally testing the impact of alterations in PCDH-mediated adhesion in a DS mouse model (such as in the Ts65dn) would be useful to explore this pathway. Gene

delivery methods could be used to correct PCDH expression followed by analysis of astrocyte development and other developmental processes such as synaptogenesis and dendritic growth.

In addition to following up on specific alterations identified with PCDHγC3-mediated adhesion, more work in general is needed to understand the impact of trisomy 21 on astrocyte cell adhesion and motility on the brain. As described in Chapter 1, astrocyte reactivity accompanies CNS injury and disease. In some conditions like stroke (Wilhelmsson et al., 2006), TBI (Burda et al., 2016) or AD (Hou et al., 2011; Olabarria et al., 2010), astrocytes become motile and migrate to damaged areas and contribute to glial scar formation. Alterations in cell adhesion and recognition properties of DS astrocytes identified in Chapter 3 may be important for these processes (Frost & Li, 2017) and have important implications for the development of glial cell pathology and the formation of RGNs in DS as described in Chapter 4. Future experiments should aim to validate the expression of ECM and cell adhesion genes in the DS brain, for example, through immunolabeling experiments. Studies could be performed at different ages to determine whether the expression of ECM and cell adhesion molecule genes change over the lifespan of DS individuals. Once strong candidates are identified through these experiments, *in vivo* testing could be performed in animal models. One novel way to do this would be to transplant DS hiPSCs or immature astrocytes in mice. This has been performed successfully in several disease models including in DS and would allow for the study of human DS astrocytes in an *in vivo* context (Real et al., 2018). This could show how human DS astrocytes migrate, develop and maintain their architecture *in vivo*, and would also allow for the study of their interactions with other cell types such as neurons. Genetic manipulations could be performed *in vitro* prior to cell transplantation and provide insight into potential therapeutic approaches for correcting astrocyte perturbations.

Previous studies reported the presence of reactive astrocytes or activated microglia near plaques in DS. However, a systematic analysis of glial cell pathology was never performed. Thus, there

was an opportunity to describe glial cell pathology in finer detail and determine whether RGNs are present in DS. In addition, most studies reporting AD neuropathology in DS were performed in the 1980s using traditional methods such as Thioflavin S and Bodian staining to visualize plaques and NFTs, respectively. Thus, I was able to add to the characterization of AD neuropathology in DS using more modern approaches including multi-label immunofluorescence and laser-scanning microscopy. This revealed severe AD neuropathology in the form of Aβ plaques (detected via $A\beta_{1\text{-}42}$ antibodies), tau hyperphosphorylation and NFT accumulation in samples over the age of 50. Interestingly, RGNs were present and structured similarly to those we had identified in sporadic AD (Bouvier et al., 2016). By gathering a large collection of post-mortem brain samples of DS individuals ranging from age 33 to 67, I showed that RGNs form gradually as DS individuals age. Surprisingly, while the density of activated microglial cells associated with Aβ plaques correlated positively with age, changes in reactive astrocyte density dramatically increased after 50 years of age in DS individuals. This large shift occurring at age 50 correlated well with plaque load and with the known timing for significant cognitive impairments in the DS population. Future experiments are required to investigate the specific purpose of RGNs and their relationship to neuroinflammation and neurodegeneration. Genetic manipulations to inhibit the activation and proliferation of astrocytes and microglia in animal models, similarly to what has been done in the Sofroniew Laboratory (Voskuhl et al., 2009), could be performed to demonstrate whether RGN formation is protective or detrimental to brain health. However, such studies are not currently feasible with DS animal models as they do not form Aβ plaques (Hunter, Bimonte-Nelson, Nelson, Eckman, & Granholm, 2004; Seo & Isacson, 2005). More effective DS animal models are needed to model DS-associated AD (Choong, Tosh, Pulford, & Fisher, 2015) such as the recent cross of the J20 APP transgenic AD mouse model with the panel of DS mouse models. These crosses have led to the significant accumulation of Aβ in the brain and formation of plaques

(Wiseman et al., 2018) and could provide better insight into the development of DS-related glial cell pathology and RGN formation *in vivo*.

An important aspect of Chapter 4 is that it adds key information regarding glial cell pathology that can be integrated into the timeline for the development and progression of AD-associated events in DS such as Aβ plaque formation, NFT accumulation, neuroinflammation and the appearance of cognitive symptomology. However, a limitation with the results presented is the absence of a cognitive assessment of the individuals which donated the brain samples. The human post-mortem samples used in the study were collected in the 1960s, 70s, 80s and 90s. The fact that the samples were preserved for such a long time period and were still suitable for analysis was remarkable. However, little information about the medical condition of the subjects was available and no information was included about their cognitive state or types of therapies subjects received throughout their life. These factors are known to vary greatly in the DS population and may affect glial cell pathology and formation of RGNs with age. We know today that 70% of DS individuals over the age of 60 have been diagnosed with dementia, and new and improved cognitive tests adapted to the DS population have been developed in the last few years which allow for more precise assessment of cognitive abilities and decline (Startin et al., 2019). Performing the study in Chapter 4 using subjects which had frequently performed cognitive assessments during their lifetime would allow for a better comparison of changes in the organization of microglia and astrocytes in the DS brain, and the appearance of dementia. Along these lines, exciting research initiatives are emerging, such as in Sant Pau Barcelona, where the Alzheimer-Down Unit was formed in 2014 under the direction of Dr Fortea. The purpose of the Unit is to assess the clinical and cognitive abilities of one of the largest adult DS cohorts while, in parallel, performing biomarker and brain imaging research (Fortea et al., 2020). Research studies integrating such multi-modal analysis (from biomarker to brain imaging to cognitive assessment) are

likely to generate powerful datasets to more precisely understand the timing of AD pathology and cognitive impairments in DS.

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