

Investigating Neurovascular Unit Aging in a Mouse Model of Autism

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Thesis submitted to the University of Ottawa in partial fulfillment of the requirements for the
Master of Science degree in Neuroscience.

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Abstract

Ageing is the primary risk factor for most neurodegenerative diseases. While neurodevelopmental disorders, neuropsychiatric conditions, and neurodegenerative diseases are clinically expressed at different points during the lifespan and present distinct clinical features, these conditions share common cerebrovascular abnormalities. Growing evidence link cerebrovascular dysfunction to neurodegeneration, yet the early origins of these vascular impairments remain unclear. Identifying early changes is crucial for designing effective and/or preventive therapeutic strategies.

This thesis aims to investigate neurovascular unit (NVU) ageing in a 16p.11.2 deletion (*16p.11.2^{df/+}*) mouse model of autism spectrum disorder (ASD). While we previously demonstrated that *16p.11.2^{df/+}* mice exhibit early-life NVU alterations, whether these initial deficits alter NVU ageing trajectory remains unknown. To answer these questions, we used multiple approaches to assess morphological and molecular NVU ageing. Using transmission electron microscopy to assess NVU characteristics in the cerebral cortex, we found that aged *16p.11.2^{df/+}* mice exhibit a pronounced, age-related decrease in the number of astroglia endfeet, a premature endfoot hypertrophy, and basement membrane thickening. Furthermore, immunohistochemistry for Glial Fibrillary Acidic Protein showed increased levels of reactive astrocytes in the stratum radiatum and stratum lacunosum-moleculare layers of the hippocampus CA1 region in middle-aged *16p.11.2^{df/+}* mice. Moreover, Aquaporin-4, a marker for water channel present in astroglial endfeet, displayed a premature decrease on vessels from aged *16p.11.2^{df/+}* mice. Collectively, our findings suggest a modified trajectory of ageing in this ASD mouse model and provide novel insights on NVU structural and morphological decline.

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Acknowledgment

I would like to express my deepest gratitude to Dr. Baptiste Lacoste for welcoming me into his laboratory and granting me the opportunity to pursue my M.Sc. in Neuroscience. Thank you for taking a chance on a prospective student with limited technical experience. Your expertise, knowledge, and insight have made this project possible. I would also like to thank him for his invaluable guidance, mentorship, and continuous support, which have allowed me to grow both academically and personally.

My sincere gratitude also goes for Dr. Moises Freitas-Andrade for his indispensable support and vast technical expertise. I am incredibly grateful for your advice and unique perspectives, which have helped me grow both as a scientist and as an individual. Thank you for patiently training me and for your help in troubleshooting countless experiments. Your continuous encouragement and positive spirits always made a difference in the laboratory.

I would also like to extend my gratitude to present Lacoste lab members Nicole, Pavel, Taj, Leya, Abigail, James, Paniz and past members Juile, Joanna, Nour, Sara and Maheen. Thank you for creating such a supportive, collaborative, and lively environment, and for all the insightful discussions and laughs we shared along the way.

Additionally, this project would not have been possible without the assistance of the Electron Microscopy (EM) Core Facility at the University of Ottawa in processing and ultrasectioning the samples. I am also grateful to Dr. Maxime Rousseaux and Dr Mireille Khacho for acting as my Thesis Advisory Committee members, and for providing constant feedback on this project.

To my friends, thank you! To my parents, Afaf and Abdulrahman: thank you for your unwavering belief, your love, and for always standing by my side. A special thanks also goes to my lovely sisters, Ghada, Salha, Sarah, and Halah, for their constant motivation, inspiration, and for being my biggest supporters.

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List of Abbreviations

AD	Alzheimer's disease
ADHD	Attention-deficit/hyperactivity disorder
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
AQP4	Aquaporin-4
ASD	Autism spectrum disorder
BBB	Blood-brain barrier
CBF	Cerebral blood flow
CCAC	Canadian Council on Animal Care
CNS	Central nervous system
CNV	Copy number variation
CSF	Cerebrospinal fluid
CRP	C-reactive protein
DSM-5	Diagnostic and Statistical Manual of Mental Disorders, 5th Edition
ECM	Extracellular matrix
FPE	Female protective effect
GFAP	Glial fibrillary acidic protein
IBA-1	Ionized calcium-binding adapter molecule 1
ID	Intellectual disability
IHC	Immunohistochemistry
IHD	Ischemic heart disease
IL-6	Interleukin-6

IP	Intraperitoneal
IQ	Intelligence quotient
LTD	Long-term depression
LTP	Long-term potentiation
MDD	Major depressive disorder
MS	Multiple sclerosis
NDD	Neurodevelopmental disorder
NMDA	N-methyl-D- aspartate
NO	Nitric oxide
NVC	Neurovascular coupling
NVU	Neurovascular unit
PD	Parkinson's disease
PFA	Paraformaldehyde
ROS	Reactive oxygen species
SLM	Stratum lacunosum-moleculare
SR	Stratum radiatum
TNFR-1	Soluble tumor necrosis factor receptor-1
TEM	Transmission electron microscopy
VEGF	Vascular endothelial growth factor
WT	Wild type

1. Introduction:

1.1. Ageing:

Ageing is the primary risk factor for most neurodegenerative diseases. This is primarily because ageing shares common features with neurodegenerative conditions. Ageing can be defined in multiple ways according to the scientific field studied. For instance, psychologists may define ageing as the changes in cognitive abilities, coping, personality, and emotional well-being ¹. While sociologists may define ageing as the changes in individuals' roles and engagement in social networks and resources ². Importantly, one definition of biological ageing is that it is the time-related progressive degeneration in physiological function essential for animals' survival and reproduction ³. This definition can be further reduced into molecular ageing, where it can be characterized by the gradual decline and progressive accumulation of cellular and DNA damage ⁴. Consequently, the multifaceted and complex perspectives on ageing highlights the importance of understanding the underlying mechanisms to improve health, quality of life and reduce the societal burden of age-related diseases.

Moreover, population ageing is occurring worldwide. According to 2022 Canada census data, the ageing population in Canada represents one of the fastest-growing age groups in the country and is projected to triple in number in the next 20 years ⁵. This shift is expected to be associated with an increase in age-related chronic medical conditions that will naturally exert pressure on the Canadian healthcare system. In Canada, it is estimated that one over three Canadian seniors is living with one or two chronic medical conditions ⁶. Cardiovascular diseases are among the most prevalent chronic medical conditions affecting Canadian seniors, with hypertension being the most commonly reported condition. Moreover, according to 2017 statistics, about 27% of Canadian seniors were living with a diagnosis of ischemic heart disease (IHD), a condition characterized by

a reduction in blood flow and oxygen supply to heart ⁶. In tandem with other age-related vascular pathologies, stroke occurs when an acute reduction or total blockage of blood flow deprives brain tissue of oxygen and nutrients, resulting in a brain damage. The severity of this damage depends mainly on the type of stroke, whether it is ischemic or hemorrhagic. In Canada, around 9.5% of seniors were reported to have a diagnosis of stroke in 2017 ⁶. Nevertheless, this highlights the involvement of the vasculature in many age-associated medical conditions and underlies the importance of studying vascular ageing mechanisms.

Vascular ageing is the time-related accumulation of structural and functional changes in blood vessels ⁷. Vascular changes include increased vessel stiffness, vessel wall remodeling, reduction/loss of formation of new blood vessels, and endothelium-dependant vasodilation dysfunction. Molecularly, vascular ageing can be the result of telomere attrition and accumulation of senescent cells in the vascular system ⁸. Another driver of vascular ageing is mitochondrial dysfunction through the impairment of cellular energy pathways and the increased production of reactive oxygen species (ROS). Additionally, Nitric Oxide (NO) plays a crucial role in the vasculature through its involvement in vasodilation. During ageing, age-associated elevation in ROS production reduces NO bioavailability and plays a role in its inactivation ⁹. ROS scavenging on NO, which results in reducing its bioavailability, was previously suggested as a possible underlying mechanism of hypertension ¹⁰. In addition to oxidative pathways, systematic inflammation represents another driving mechanism of vascular ageing. Clinically, soluble tumor necrosis factor receptor -1 (sTNFR-1), Interleukin -6 (IL-6) and C-reactive protein (CRP) are common systemic inflammation markers ¹¹. In humans, increased CRP levels was found to be associated with arterial stiffness ¹². Lastly, extracellular matrix (ECM) remodeling represents another vascular ageing hallmark. ECM remodeling involves the permanent alteration in the

structural and molecular properties of vessel walls. This is due to the excessive replacement of cellular and matrix elements with Interstitial collagenous matrices containing different types of collagen and various other molecules ¹³. Nevertheless, the consequences of vascular ageing extend beyond the cardiovascular system and can affect multiple organs. The brain in particular, possesses a tightly regulated cerebrovascular network and demands a substantial amount of energy. Consequently, any alterations in cerebral homeostasis may result in considerable neurobehavioral and cognitive changes.

1.2. Brain Ageing

One of the most common alterations caused by brain ageing is the associated changes in cognitive abilities. Aged population tends to have a slower processing speed, a decline in conceptual reasoning, executive functioning and memory ^{14,15}. Additionally, elderly individuals have an increased prevalence of sleep disturbances including sleep apnea, circadian rhythm disorders, and insomnia ¹⁶. Sleep disruptions combined with age-related cognitive decline exacerbates the progression of cognitive impairment and can accelerate the rate of neurodegeneration ¹⁷.

Several structural changes have been proposed to explain the overall reduction in cognitive abilities in elderly populations. Primarily, reductions in the volume of cortical regions associated with cognition. A previous neuroimaging study demonstrated a reduction in cortical volume in the lateral occipital and lingual regions of the brain of elderly people ¹⁸. Similarly, shrinkage in hippocampus volume was shown to be associated with cognitive decline in the aged population ¹⁹. Reduction in cerebral white matter integrity has also been linked to the observed decline in mental acuity in the older population ²⁰.

At a cellular and a molecular level, the cognitive deficits observed in ageing are not driven solely by sudden loss of brain volume, but also by progressive disruptions in synapse connectivity and the resultant neuronal dysfunction. Broadly, ageing impacts neuronal function and alters the efficiency of neuronal circuits to transmit and process information. For example, ageing involves alterations in the electrophysiological connectivity of the brain. Previous studies found that ageing is associated with a reduction in beta waves associated with active thinking ²¹. This in turn was found to be correlated with lower cognitive scores. Another hallmark of neuronal ageing is the reduction of dendritic spine density ²². Moreover, long-term potentiation (LTP) and long-term depression (LTD) are two complementary processes by which synapses encode and eliminate memories in the brain. With advancing age, the homeostatic balance between these two processes shifts and results in a reduction in the maintenance and induction of LTP. That is, ageing is associated with an increased bias towards LTD ²³. Crucial to both LTP and LTD is the N-methyl-D-aspartate (NMDA) receptor, Ca^{2+} influx through synaptic NMDA receptors activates either LTP or LTD. This in turn, result in the redistribution of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors which potentiate or weaken the synapse. During ageing, several NMDA receptor subunits were found to be reduced in expression in rats ²⁴. It was also found that ageing results in a reduced Reelin interaction with NMDA receptors, resulting in less LTP regulation ²⁵. Neuronal high metabolic demands place a greater burden on brain mitochondria and alter the neuronal aging timeline. One hallmark of neuron ageing is the accumulation of damaged mitochondria ²⁶. This is mainly due to the observed decline in mitophagy in aged animals ²⁷. Importantly, these damaged mitochondria Increase the production of ROS and have been shown to impair LTP function ²⁸. Additionally, age-associated reduction in frontal lobe

glucose metabolism has been previously observed ²⁹. Consequently, this results in lower ATP production and hinders neuronal functions.

1.3. Neurovascular Unit Ageing

The brain's distinct environment as well as its unparalleled structure and cellular composition results in a unique process of ageing. This is due to the isolated nature of the brain from the rest of the body, as the cerebrovasculature is specifically designed to provide structural and functional barriers to tightly control the brain microenvironment. The brain account for almost 2 % of body mass, but consumes approximately 21 % of the body's energy expenditure ³⁰. This is amplified by limited brain energy storage, and the primary dependency on glucose as energy source ³¹. Thus, it is paramount for the brain to have a healthy vasculature to support its metabolic demands and promote healthy brain ageing.

The brain contains around 100 billion vessels that maintain the substantial metabolic demands of brain cells by continuously supplying oxygen and nutrients and efficiently eliminating metabolic waste. Brain cells and vasculature structurally and functionally integrate into one multicellular system called the neurovascular unit (NVU). NVU regulates the blood-brain barrier (BBB) and neurovascular coupling (NVC), two key features that determine neuronal function and health. NVU is primarily composed of neurons, astrocytes, mural cells, and endothelial cells. Any perturbation to one or a few components of the NVU leads to a cascade effect of neuronal activity dysfunction and eventually may result in neuron death.

Significant impairments in several components of the NVU have been previously associated with brain ageing. NVC is one key brain process that is affected during ageing. NVC occurs when local cerebral blood flow (CBF) responds to changes in neuronal activity ³². This

process is essential in ensuring that the brain meets its demand for oxygen and energy. As a result, small-scale perturbations to such processes are enough to affect brain health and, particularly, neuron health. In an aged mouse model, and using laser speckle contrast imaging, it was found that NVC response to whisker stimulation is reduced compared with those of adult mice ³³. In addition, Zhang et al. [33] found that aged mice have slower Ca^{+2} kinetics and signalling in vascular mural cells compared to adult mice. Moreover, Bennett et al. [34] found that the brains of healthy aged mice exhibited a significant increase in arteriole tortuosity within the somatosensory cortex and medial prefrontal region. This is particularly interesting as ageing has been strongly associated with decreased CBF. Thus, increased vessel tortuosity will result in increased blood flow resistance ³⁴. Prior data have found that blood flow is lower in aged mice in comparison to young mice ³⁵. Together, these deficits may severely affect the brain's energy supply, particularly in deep cortical layers.

Additionally, ageing is associated with impaired angiogenesis (the sprouting of new capillaries from preexisting vessels), and such impairment is particularly dangerous as microcirculation remodeling may give rise to pathological conditions ³⁶. Interestingly, vascular endothelial growth factor (VEGF), a potent angiogenic factor, was found to be upregulated in aged mice choroid plexus in comparison to young adults ³⁷. Moreover, the basement membrane is a specialized ECM that surrounds the endothelium. Its main function is to provide structural support and protection to vessels. Interestingly, BBB disruption is closely linked to the structural remodeling of the basement membrane ³⁸. Additionally, pericytes are contractile mural cells that wrap around the surface of capillaries. Pericytes are uniquely embodied within the basement membrane of vessels. Functionally, pericytes provide protection to the vessel, maintain the BBB, and more recently have been associated with blood flow regulation ³⁹. Selective reduction in

capillary pericyte density has been previously observed in the brains of aged mice. Such reduction was restricted to the thin and mesh pericytes subtypes in the entorhinal cortex ³⁴. Also, pericyte remodeling is an active mechanism where pericyte distal processes dynamically extend to reposition pericyte-endothelial contact following an insult ⁴⁰. In aged mice, capillary pericytes failed to regain complete capillary coverage 21 days post focused two-line scan pericyte ablation, resulting in increased capillary dilation ⁴¹.

Furthermore, ageing has been strongly correlated with increased brain neuroinflammation ⁴². Neuroinflammation is defined as the inflammatory response within the brain and or the spinal cord that is mediated by the production of chemokines, cytokines and ROS. Neuroinflammation is defined as the inflammatory response within the brain and/or the spinal cord that is mediated by the production of chemokines, cytokines, and ROS. Neuroinflammation mediators are produced mainly by resident central nervous system (CNS) glia cells such as astrocytes and microglia. Mast cells are also native brain immune cells that are localized near blood vessels in the meninges and brain parenchyma ⁴³. Peripherally derived immune cells such as infiltrating leukocytes, can also be present in the brain to help during an inflammatory response. Microglia are specialized immune system cells that are resident in the CNS and represent approximately 10% of CNS cells ⁴⁴. Their primary function is to maintain brain homeostasis by clearing up cellular debris and defending against pathogens. Microgliosis refers to the process in which microglia are activated, and their cell shape is transformed from a resting branched shape, into enlarged amoeboid state or dystrophic shape in response to an injury, infection, or neurodegeneration. This shape transformation enhances microglia's ability to clear debris and fight pathogens. Interestingly, the microglial immune system response to injury is also age-dependant. Damani et al. [45] found that microglial response in aged mice to a laser-induced focal tissue injury is less dynamic compared to young

mice ⁴⁵. Additional research also showed that aged microglia are functionally compromised during the immune system response to an inflammatory condition ⁴⁶. Interestingly, studies indicate that age-related changes in microglial numbers are region-dependant in mice ^{47,48}. This suggests that microglia population undergoes a distinct spatial redistribution during ageing. Astrocytes on the other hand, are the most abundant type of glial cells in the brain. Structurally, astrocytes are uniquely positioned to interact with synapses at one end to modulate neurotransmitter release and with vessels at the other end to regulate blood flow. Astrocytes are also involved in waste clearance, synaptic formation, maintenance, and BBB formation. Astrocytes interact with synapses through the perisynaptic astrocytic processes to actively engage in neuronal communication through the formation of the tripartite synapse. During an inflammatory response, astrocytes are morphologically transformed into an activated state through a process called astrogliosis. Astrocytes become hypertrophic, where the cell body thickens and increases in branching. Reactive astrocytes are also characterized by the overexpression of a cytoskeletal intermediate filament protein known as glial fibrillary acidic protein (GFAP). Earlier studies found upregulation of genes associated with A1 reactive astrocytes in the brains of naturally aged mice ⁴⁹. Elahy et al. [51] found increased expression of GFAP in the cerebral cortex of 24-month-old wild-type mice ⁵⁰. In the hippocampus, aged mice showed increased levels of GFAP-positive cells and a reactive astrocyte morphology ⁵¹. Importantly, astrocytes extend perivascular projections known as astrocytic endfeet to fully wrap around blood vessels under healthy conditions. Functionally, astrocytic endfeet are involved in regulating the BBB, CBF, and waste clearance. Given that these are essential functions in brain network health, astrocytic endfeet are packed with water and ion channels, dense mitochondria, scaffolding and adhesion proteins that anchor the endfeet to blood vessels ⁵². Astrocytic endfeet also undergo morphological changes during ageing. In rodent model, it was previously found that aged mice undergo a region-

dependant endfeet enlargement ⁵³. In addition, Aquaporin-4 (AQP4), a water channel present on endfeet membrane, has been reported to lose its perivascular localization in aged cognitively intact humans ⁵⁴. Together, these age-related alterations contribute to astrocyte reduced functionality during ageing and in neurodegenerative diseases.

The NVU is also responsible for the formation and regulation of the BBB between endothelial cells. Brain endothelial cells are unique due to the presence of specialized junctional complexes that completely seal the space between the endothelium and separate the brain from other body compartments. These complexes are comprised of tight junctional proteins (e.g., Claudin-5 and Occludin) that mainly seal the interendothelial cleft, adherence junction proteins (e.g., VE-cadherins) that are responsible for cell adhesion, and gap junctions (e.g., Connexin-43) that are responsible for intercellular communication ⁵⁵. During normal conditions, the BBB allows for very low levels of transcytosis and has tightly controlled and very selective efflux and influx transports, both features impose tight control over what goes in and out the brain.

BBB impairment has been rigorously established as a hallmark of neurodegenerative diseases. However, the mechanism and extent of BBB dysfunction in normal ageing are still debated. For instance, several studies showed that natural ageing alters the expression of multiple basement membrane and tight junction proteins in rodents ^{37,56}. Another study showed increased IgG extravasation in 22-month-old mice ³⁴. In non-pathological human ageing, the BBB leakage rate was found to be higher in older individuals, particularly in brain regions such as the cerebral white matter which is known to be vulnerable to ageing ⁵⁷. A recent study suggests that healthy ageing does not result in structural impairment in BBB (Increased leakiness) but rather alters several regulatory and signalling properties that increase BBB susceptibility to insult ⁵⁸. This result

is particularly significant in relation to other neuropsychiatric and neurodevelopmental conditions that are hypothesised to prime the BBB for structural impairment upon injury.

1.4. Neurovascular Continuum

While brain ageing is associated with neurovascular alterations, life-long developmental and psychiatric conditions may affect brain ageing as well. Neurodevelopmental disorders (NDD) are group of conditions that develop/diagnosed during early infancy, childhood or adolescence, leading to impairments in social, personal, academic, or occupational functioning. NDDs are lifelong conditions, their effects span multiple domains including memory, learning, communication, emotion, and motor skills. Although the clinical classifications of NDDs are constantly being updated, disorders such as autism spectrum disorder (ASD), attention deficit hyperactivity disorder (ADHD), intellectual disabilities (ID), and motor disorders are commonly diagnosed NDDs. In addition, psychiatric disorders refer to medical conditions that severely disrupt an individual's behaviour and psychological well-being. According to the diagnostic and statistical manual of mental disorders (DSM-5), these conditions involve significant disturbance in cognition, emotional regulation, and mood ⁵⁹. Fundamentally, these conditions are driven by underlying biological or developmental dysfunction.

Several neurodevelopmental and psychiatric conditions have been associated with accelerated ageing and/or neurodegenerative diseases ⁶⁰. A previous study showed that individuals with high autistic traits are prone to a faster pace of ageing ⁶¹. Additionally, major depressive disorder (MDD) and anxiety have been widely associated with accelerated ageing, which is mainly driven by the prolonged activation of the hypothalamus-pituitary axis (HPA), which exacerbates inflammation. Previous reports showed evidence of a decreased putamen volume in MDD patients

⁶². Interestingly, individuals with schizophrenia have been shown to be at a higher risk of the development of dementia ⁶³. Also, a systematic review on the association between individuals with chronic mid-life anxiety and the development of dementia revealed a significant positive association ⁶⁴. At a cellular level, one underlying mechanism of the higher risk of developing neurodegenerative conditions in individuals with chronic psychiatric conditions is alteration in telomere length. In individuals with schizophrenia, leukocyte telomere length was found to be significantly shorter ⁶⁵. Surprisingly, while schizophrenia and Parkinson's disease (PD) are traditionally viewed as opposite to each other from a biological standpoint, a study found that both PD and schizophrenia share many common genetic underpinnings ⁶⁶. Taken together, these studies imply that psychological and physiological stress imposed by neurodevelopmental and psychiatric conditions alters biological ageing and possibly leads to neurodegeneration.

In the context of cerebrovasculature, it is increasingly recognized that early-life NVU disruptions are key contributors to later neuronal degeneration ^{67,68}. While NDDs, neuropsychiatric conditions, ageing and neurodegenerative diseases are clinically expressed at different points in individuals' life span and present distinct clinical features, these conditions share some common cerebrovascular abnormalities ⁶⁷. Interestingly, several NDDs and neurodegenerative diseases are characterized by dysregulated CBF. ASD, ageing, PD and multiple sclerosis (MS) have all been found to be associated with regional decrease in CBF ⁶⁹⁻⁷². Notably, several tight junction proteins (Claudin-1,5, ZO-1) have been found to be dysregulated in ageing, Alzheimer's disease (AD), schizophrenia and ASD ^{37,73-75}. These dysregulations in important junctional proteins could alter the molecular make-up of the BBB, leading to a BBB breakdown. Evidence from human and rodent models suggest a decline in BBB function and increased permeability are associated with schizophrenia, ageing and AD ⁷⁶⁻⁷⁸. Therefore, it is reasonable to suggest that developing early-

life vascular deficits predisposes NVU to reduced resilience. This in turn, can change ageing trajectory and may advance to become a neurodegenerative condition. To the best of our knowledge, no studies have been done to investigate NVU ageing in a mouse model of a neurodevelopmental disorder.

1.5.16p11.2 deletion syndrome as mouse model for ASD

ASD is a neurodevelopmental disorder characterized by difficulty in social interactions, abnormal sensory processing, intellectual difficulties and may present with motor disorders. According to a 2018 report, about 1:66 Canadian youth aged 5-17 years old (15.2 per 1000) are diagnosed with ASD ⁷⁹. Another 2019 survey conducted in 2019 by Canadian Health Survey on Children and Youth (CHSCY) suggested that 1:50 Canadian youth aged 1-17 years old had been diagnosed with ASD in 2019 ⁸⁰. The increasing rate of ASD diagnosis in Canada is largely due to the advancement in medical screening, a broader ASD definition, and increased public awareness. In Canada, ASD diagnosis is based on the criteria set in the DSM-5, where a child must show a persistent deficit in social interaction and communication, repetitive and restricted behaviours that cause a functional impairment ⁸¹. Many children diagnosed with ASD are diagnosed with other conditions such as anxiety, ADHD, and learning disability ⁸⁰. According to the CHSCY 2019 report, males are four times more likely to be diagnosed with ASD than females. While the mechanisms underlying differences in ASD rates of diagnoses between males and females are still poorly understood, several hypotheses provide insights. In brief, one hypothesis to explain the sex-dependant differences in ASD diagnosis is the extreme male brain hypothesis. This hypothesis argues that male brains are wired toward high systemizing and low empathizing ^{82,83}. At the molecular level, this concept suggests that male brains are configured to lean towards the ASD

phenotype. More recently, the female protective effect (FPE) hypothesis has been recognized as possible driver of the sex differences in ASD diagnosis. The FPE proposes that females have an inherited biological and genetic resilience that requires greater environmental ASD-linked genetic mutations to manifest the condition ⁸⁴. Interestingly and in support of this hypothesis, Andersen et al [85]. found that the X-chromosomal gene ZFX escapes X-linked chromosome inactivation in females ⁸⁵. Thus, resulting in overexpression bias in females over males. Importantly, it was found that such overexpression helps in boosting the expression of a network of genes that is usually diminished in ASD. Thus, at baseline, females can buffer the effects of ASD-linked mutations.

The etiology of ASD is highly heterogeneous and can arise from different genetic mutations. ASD can be caused by a monogenic mutation (3-5%) such as in Fragile X syndrome, or from a de novo mutation in single genes (~30% of cases) such as in *SYNGAP1*, or from a copy number variation (CNV) (~10-20% of cases) such as the *16p.11.2* deletion syndrome ⁸⁶⁻⁸⁸. Nonetheless, individuals diagnosed with ASD exhibit a reduced life expectancy and elevated risk of premature mortality ⁸⁹. This is metrically quantified by a study done in the UK, where it was found that males diagnosed with autism and intellectual disability have a reduced life span of 7.28 years, while females had a reduced life span of 14.59 years ⁹⁰. CNV in particular, refers to structural variation in the human genome where regions of the genome are either deleted or duplicated. The advancement of microarray chromosomal analysis and karyotyping have led to the identification of several CNVs found in ASD patients including 15q11-13, 16p.11.2, and 22q11-13. ⁹¹. Several studies have highlighted the importance of studying CNVs in ASD due to its prevalence and the mutation's large effect size ^{86,92}. Therefore, this thesis is going to focus on the 16p.11.2 microdeletion as a mouse model of ASD.

16p11.2 copy number variation (CNV) is one of the most common genetic risk factors for ASD, in which affected individuals carry either a deletion or duplication of the *16p11.2* locus ⁹³. The prevalence rate of the 16p.11.2 duplication is 1:1100, while the prevalence rate of the deletion is 1:2000 in the general population ⁹⁴. In humans, the 16p11.2 deletion syndrome has a higher penetrance in comparison to the duplication ⁹³. Also, the 16p.11.2 deletion predominantly occurs (60-90% of cases) through a de novo mutation event ⁹³. In clinical populations, approximately 2% of individuals carrying the deletion or duplication are formally diagnosed with ASD. ⁹³. Importantly, the human 16p11.2 deletion associated with ASD involves the loss of ~ 550 kb of the genome in the *16p11.2* locus, resulting in the haploinsufficiency of approximately 26 genes. Within the 16p.11.2 deletion locus reside genes that regulate neuronal differentiation and maturation (*Taok2*, *Kctd13*), endothelial cells maturation and migration (*Mapk3*), endothelial cells apoptosis (*Tmem219*, *Coro1A*), neuronal excitability (*Doc2A*, *Prrt2*), vascular growth (*Maz1*), energy production regulation (*Aldo1A*) ⁹⁵. Given that these genes control important aspects of cellular function and survival, their involvement in the ageing process is highly intriguing.

Similar to ASD, repetitive and restrictive behaviours are observed in individuals with 16p.11.2 deletion syndrome ⁹⁶. Patients with 16p.11.2 haploinsufficiency also present with emotional dysregulation, reduced social communication, social interaction and maladaptive behaviours ⁹⁶. Moreover, children with the deletion often clinically present with language delays, with a particular struggle in expressive language skills ⁹⁷. Cognitive impairments and delays are other symptoms found in children with 16p11.2 deletion syndrome. This was reported by Henson et al. [96], where they found that patients with the deletion syndrome showed a lower verbal and nonverbal intelligent quotient (IQ) ⁹⁶. Additionally, the 16p11.2 deletion is associated with fine and gross motor impairments and delays ^{96,98}. Children with the deletion syndrome often report

high clumsiness and poor motor coordination is found in 50% of deletion carriers ⁹⁹. Notably, 16p.11.2 deletion carriers commonly present with sleep disturbances including difficulty falling asleep, teeth grinding, and dreaming while still awake ¹⁰⁰. Moreover, almost 24-40% of children with the deletion experience neurological issues such as seizures and 18% met the criteria for epilepsy ^{101,102}. In addition, head circumference analysis revealed that children with the deletion syndrome are clinically characterised by a larger than average head size ¹⁰². This occurs in addition to observed widespread aberrant alterations in white matter tract microstructure ¹⁰³. The 16p.11.2 deletion is also associated with other chronic and non-chronic medical conditions such as obesity (75% of cases), cardiac malformations (6%), and hearing loss (11%) ¹⁰⁴. Collectively, the developmental alterations and associated clinical manifestations linked to the 16.11.2 deletion syndrome, may place substantial physiological stress on multiple biological systems. Hence, influencing the trajectory of the brain ageing process.

A mouse model of the deletion was successfully developed by flanking the sites of the previously identified mutated genes in human patients, resulting in the deletion of ~ 440 kb of genomic DNA on mouse chromosome 7qF3. Important to the context of this thesis, a previous study showed that *16p11.2^{df/+}* altered CBF responses to evoked stimulus in adult male mice ¹⁰⁵. They also showed that male mice displayed decreased vessel density and branching as well as defective angiogenic activity. Furthermore, *16p11.2^{df/+}* mice were found to have a significantly reduced number of mitochondria in endothelial cells of brain capillaries, as well as elevated brain glucose uptake ¹⁰⁶. Interestingly, *16p11.2^{df/+}* astrocytes exhibited early-life metabolic and functional alterations ¹⁰⁷. Additionally, the BBB of adult male *16p11.2^{df/+}* mice was found to be vulnerable to insults in comparison to the wild-type (WT) (Pavel Kotchetkov, unpublished). Together, these data indicate that NVU is

compromised during early stages in *16p11.2^{df/+}* life span. Therefore, we postulate that these alterations may affect the trajectory of NVU ageing in the mutant mouse.

1.6. Overarching hypothesis

Ageing and neurodegeneration are continuous progressive processes rather than random chronological events that occur without prior priming. This priming effect can be a result of normal physiological processes such as telomeric shortening over time or small natural decay from mitochondrial dysfunction. Crucially, sustained physiological stress from a chronic lifelong neurodevelopmental disorder or a psychiatric condition may be poised to modify the ageing process. Hence, the explored *16p11.2* cerebrovascular deficits represent an early-life insult to the organism, that could in turn adjust the process and sequence of ageing in the brain. Therefore, the overarching hypothesis of this thesis is:

Cerebrovascular deficits associated with 16p11.2 deficiency alters the trajectory of NVU ageing in 16p11.2^{df/+} mice compared to the WT.

To study this hypothesis, we will use the male *16p.11.2* deficient mouse as a mouse model of ASD. The overarching hypothesis will be examined by the two following objectives:

Objective 1: ***Investigating NVU morphological differences associated with ageing between WT and 16p11.2^{df/+} mice.***

Objective 2: ***Investigating molecular changes associated with NVU ageing between WT and 16p11.2^{df/+}.***

2. Materials and Methods

2.1. Animals

Prior to experiments, all animal procedures were approved by the University of Ottawa Animal Care Committee and were conducted in accordance with the guidelines of the Canadian Council on Animal Care (CCAC).

Mouse Husbandry: Mice used in this study were all bred in-house and housed in a cage with environmental enrichment and free access to water and food. To obtain hemizygous *16p11.2^{df/+}* male offspring and WT littermates, males *16p11.2^{df/+}* (Jackson Laboratory, stock #013128; mixed B6/129 mixed background) were crossed with WT females with B6/129 background (Jackson Laboratory #013128). Based on Jackson Laboratory recommendation, *16p11.2^{df/+}* breeding cages were supplemented with breeding chow (#2019, Envigo Teklad) and DietGel(#76A, ClearH₂O) up to weaning age. At P21, WT and *16p11.2^{df/+}* littermates are moved to experimental cages and housed under standard housing conditions until the collection date. Given that ASD is more commonly diagnosed in males and previous NVU alterations were primarily observed in male *16p11.2^{df/+}* mice, this study utilized brain tissue from male mice only. In order to compare aging progression, mice were euthanized at four distinct time points based on the Jackson laboratory life phase equivalences: 2-3 month-old (young adults), 6-7 month-old (mature adults), 12-13 month-old (middle-aged), and 17-19 month-old (aged).

Genotyping: WT and *16p11.2^{df/+}* littermates were genotypes using the following primers: 5'-CCTCATGGACTAATTATGGAC-3' (forward) and 5'-CCAGTTTCACTAATGACACA-3' (reverse). *16p11.2^{df/+}* mice PCR product weighted 2.2Kb.

For all experiments, brain tissues were collected after administering an anesthetic mixture consisting of ketamine/xylazine (1mg/kg) injected to the intraperitoneal space (IP). Mice were then

ethanized via cervical dislocation. Each brain tissue collected was regionally divided to allow for a comprehensive assessment of brain ageing. Figure 1 illustrates the brain regions collected for each planned experiment.

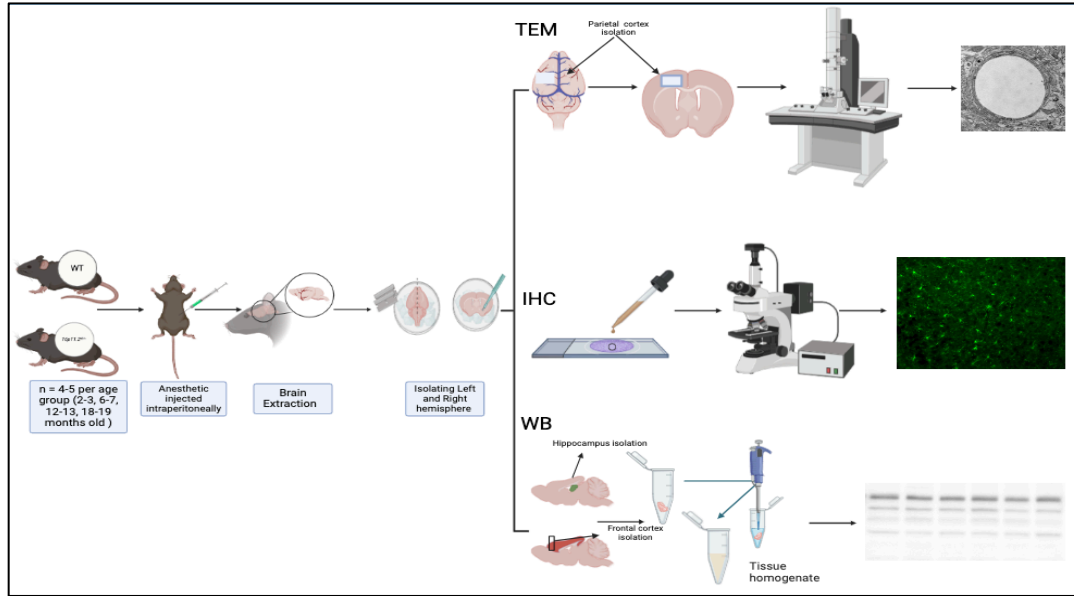


Figure 1: Overview of experimental methodology.

The figure shows the collected brain regions in our study per mouse sample. A) Transmission Electron microscopy (TEM) (Parietal cortex of the left hemisphere). B) Immunohistochemistry (IHC) (Right hemisphere). C) Western blot (Hippocampus and prefrontal cortex of the left hemisphere).

2.2. Immunohistochemistry (IHC):

At the time of sample collection, the right hemisphere of male mice were dissected and immediately placed in 4% paraformaldehyde (PFA) for overnight fixation at 4°C. In preparation for 2D imaging, tissues were rinsed 3x (5 min each) in 1x PBS and then placed in 30% sucrose overnight at 4°C. On day three, tissues were embedded in OCT blocks in preparation for later cryosectioning. Tissues were cut coronally into 25 µm-thick serial sections using a cryostat (HM525 NX, ThermoScientific) with the temperature set within the range of -21°C to 25°C and then mounted on positively charged slides. Collected sections were then stored at -80°C. For IHC staining, sections were stained following the Lacoste lab staining protocol with slight amendments

to reduce autofluorescence in aged sections¹⁰⁵. On day one of staining, sections were blocked with a blocking solution containing 10% Donkey serum, 0.5% PBT (0.5% Triton X-100 diluted in PBS), and 0.5% cold water fish gelatin for 90 minutes in a humid chamber. Examined primary antibodies, including GFAP (1:500, Abcam), IBA-1 GT (1:300, Abcam), AQP4 (1:500, Synaptic System), CD31 (1:200, BD Bioscience), PDGFR- β (1:200), were added and incubated overnight in a humid chamber at 4°C. The following day, sections were washed 3x (5 min each) with 0.5% PBT and then incubated with species-specific Alexa Fluor secondary antibodies for 2 h at room temperature. Sections were subsequently washed 3x with 1xPBS, then 150 μ l of diluted TrueBlack® Lipofuscin Autofluorescence Quencher (Cell Signaling #92401) (1:20 – 1:40 in 70% ethanol) was added for ~ 30s in a humid chamber. Sections were then washed 3x (5 min each) in 1x PBS, followed by 2x washes in 0.1M PB (5 min each), and then coverslipped in Fluoromount G. Sections were then left to dry for at least one hour before image acquisition.

2.3. Image Acquisition and IHC Quantification:

Stained sections were examined using a Zeiss Axio Imager M2 microscope equipped with a digital camera (AxioCam 506 mono), in addition to the Zeiss ApoTome.2 module for optical sectioning. To acquire 2D images of target cells, we used the 20x objective to obtain deep z-stack tiled images of target regions. Tiled images were immediately converted into Apotome images after acquisition. Prior to quantitative analysis, the z-staked tiled images were processed using the extended depth of focus function, followed by generating a maximum intensity image for analysis. For **% coverage** quantification, the image threshold was first determined using Fiji (ImageJ). In order to reduce bias, an auto-threshold was used to determine the built-in stain-specific thresholding algorithms that yield an optimal proportion of the image containing the signal of interest. Images were then converted to binary images, followed by obtaining the % coverage

using the analyze particle function, with size set to stain-specific. For CD31–AQP4 **overlap quantification**, CD31 and AQP4 channels were thresholded individually to generate binary masks for colocalization analysis. This was followed by using the image calculator plugin with the “AND” function to generate an overlapped image. The % coverage of the overlapped image was then obtained using the aforementioned analyze particle method. In Excel, the % coverage of the overlapped image was normalized against AQP4 % coverage and then multiplied by 100 to obtain the AQP4-CD31 overlap percentage. To **count individual microglia**, the cell body of individual microglia associated with nuclei DAPI staining were counted manually using the built-in cell count tool in Fiji-Image J.

2.4. Transmission Electron Microscopy (TEM):

During dissection, the parietal cortex was surgically isolated from the left hemisphere using the flattened cortex method ¹⁰⁵. Then, the parietal cortex is immediately immersion-fixed overnight in a solution containing 4% PFA, 0.2 M Sodium Cacodylate buffer, 2% Glutaraldehyde, and Milli-Q water. The following day, the tissue was rinsed 2x (5 min each) with 0.1 M Sodium Cacodylate, then stored in 0.1 M sodium cacodylate overnight. During the third day, the cortex is embedded in 2% agarose and coronally sectioned (50 µm-thick sections) in 1x PBS using a Leica VT1000s Vibratome. Sections were then stored at -20°C in an antifreeze solution (PBS, glycerol, and ethylene glycol) until processing. For processing, samples were submitted to the University of Ottawa TEM core facility for osmium-thiohydracarbazine (TCH)-osmium (OTO) post-fixation. Briefly, sections are transferred from antifreeze solution to 1X PBS and washed 3x (10 min each). Sections were then incubated in 1.5% potassium ferrocyanide and 2% aqueous osmium tetroxide in 0.1 M phosphate buffer for one hour at room temperature. Subsequently, sections are washed 3x with ddH₂O for (3 min each). Sections are then incubated in 1% w/v

thiocarbohydrazide for 20 minutes at room temperature. After incubation, sections are washed again with ddH₂O (5x 3 minutes each) and then incubated in 2% aqueous osmium tetroxide. Before dehydration, sections are washed 5x with ddH₂O (3 min each). Sections are then dehydrated using increasing concentrations of ethanol (10 -100%). This is followed by placing the sections in propylene oxide. Sections are then embedded in Durcupan resin (Sigma-Aldrich) between ACLAR sheets for 3 days at 55 °C. Small tripzone sections are collected and mounted on a block for ultrathin sectioning. Ultrathin sections were obtained using a Leica UC7 Ultramicrotome at ~ 65 nm. For each animal, 10 capillaries were imaged randomly in the isolated sections of the parietal cortex. These images were obtained using a FEI Tecnai Spirit G2 transmission electron microscope operating at 80kV and equipped with a Hamamatsu ORCA-HR digital camera (10 MP). To capture each capillary and surrounding NVU features, low-magnification images were taken at 4000x. Additionally, images at 10000x-12000x were photographed to obtain higher-resolution images of capillaries.

2.5. TEM Image Analysis:

All images were quantified using Fiji-imageJ software. **Blood vessel perimeter** was calculated using the segmented line tool to trace the perimeter of the capillary and then using the measure function in imageJ. For **astrocytes endfeet count**, astrocytic endfeet around capillaries were counted manually using the built-in cell count tool in ImageJ. **Astrocytes endfeet area** was quantified by drawing an ROI around each astrocytic endfoot in close contact with the capillary using the polygon selection tool, then by utilizing the measure function to calculate the area. In Excel, the sum of endfeet areas around a blood vessel was calculated and normalized to the blood vessel perimeter. **Basement membrane thickness** was measured using the straight-line selection tool and by drawing lines from the edge of the basement membrane near the endfeet to the edge of the basement membrane close to endothelial cells, then by using the measure function. For each image, 10 measurements were collected from different regions of the vessel and

averaged to generate a single average thickness per image. Different **astrocytic endfeet mitochondrial** measurements were also quantified. Mitochondrial numbers were first counted manually for each endfoot. For **mitochondria density**, the mitochondria number per endfoot was normalized against the sum of astrocytic endfeet area. **Mitochondrial average maximum length** was calculated using the straight-line tool by drawing a line along the longest axis of a mitochondrion and then obtaining the average per endfoot. For **mitochondrial average area size**, the boundaries of individual mitochondrion were delineated by drawing an ROI (polygon selection tool) and using the measure function to calculate the area before obtaining the average area size. For **mitochondrial area fraction**, the sum of the mitochondria area was normalized against endfeet total area.

2.6. Western blots:

Protein Extraction: During dissection, the prefrontal cortex (PFC) and hippocampus were isolated and placed in Eppendorf tubes, immediately frozen in dry ice, then stored at -80°C until protein extraction. To lyse tissues, RIPA buffer (NaCl 150 mM, Sodium Deoxycholate 0.5%, SDS 1%, NP-40 1% in Tris 50 mM pH 8.0) was vortexed with protease and phosphatase inhibitors, then added to tissues at approximately a 1:10 (w/v) ratio. Samples are homogenized using a handheld homogenizer, then vortexed and placed on ice for 5 minutes. Samples were then sonicated for 5 minutes (highest setting) in an ice bath to prevent overheating and protein degradation. Sonicated samples were placed on ice for 10 minutes, then transferred to -80°C for 1 day. On day 2, samples were cold-centrifuged for 5 min at 13,000 rpm, and the supernatants were transferred to new tubes and stored at -80°C until protein quantification.

BCA Assay: Protein concentrations were determined using the Pierce BCA Protein assay (kit #23227, ThermoScientific, Illinois, USA). In brief, BSA standards dilutions were prepared using

the dilution scheme provided in the kit, while the Working Reagent was determined using the following formula: (#standards + # unknowns) x (# replicates) x (200 μ l) = total working reagent volume required. Samples were then diluted at a 1:15 ratio in RIPA buffer. Then, 25 μ L of BSA standards and samples are loaded into a 96-well plate in duplicate, followed by the addition of 200 μ L of Working Reagent. The working plate is then incubated at 37 °C for 30 min. Absorbance was measured at 562 nm using a BioTek microplate reader.

2.7. Immunoblotting:

30 μ g of protein was loaded into the wells of a manually casted SDS-acrylamide gel (GX Stain-free FastCast 12% Bio-Rad gels, Bio-Rad, ON, CAN, #1610184). Proteins were separated at a constant current of 150 V for 75 minutes in a running buffer (25 mM Tris, 192 mM glycine, and 0.1% (w/v) SDS). Subsequently, the Stain-Free gel was transferred to the ChemiDoc for 5 minutes of UV activation. Proteins were then transferred to a PVDF membrane via the wet transfer method using cold transfer buffer (25 mM Tris, 192 mM glycine, and 20% (v/v) methanol) for 1:45 h at 400 mA. Immediately following transfer, the gel and PVDF membrane were imaged to visualize the total protein transferred. The membrane was then blocked with 5% non-fat dry milk (Blotting Grade Blocker, Bio-Rad, #1706404) in TBS-T buffer (10 mM Tris, 150 mM NaCl [pH 8.0], and 0.05% (v/v) Tween-20) for 90 minutes on the shaker (Corning LSE Platform Rocker Shakers) at room temperature. Following blocking, the membrane was incubated with primary antibodies Claudin-5 (1:1000, Invitrogen) or Occludin (1:1000, Abcam) in 1% milk-TBST overnight at 4 °C on the shaker. On day two, the membrane was washed 2x with TBS-T (5 min each) before incubating for 60 minutes at room temperature with species-specific secondary HRP-conjugated antibody (Promega, 1:3000) in 5% milk-TBS-T. The

membrane was then washed 3x with TBS-T (20 min each). Protein bands were visualized using Pierce ECL Western blotting substrate (#32106) and the SuperSignal West Femto trial kit, following the manufacturer's instructions, and were captured using the University of Ottawa ChemiDoc. Using Image Lab software, semiquantitative optical density western blot analysis was performed and normalized to the loading control.

2.8. Statistical analysis

All data analysis, data visualization, and statistical tests were performed using GraphPad Prism 11 software. All data are presented as mean $\pm$ standard error of the mean (SEM), and statistical significance is defined as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. For all data, statistical significance was determined using a two-way analysis of variance (ANOVA) with genotype and age designated as independent factors. Additionally, a post hoc Tukey's multiple-comparison test was applied to detect genotype, age, or interaction effects.

3. Results

3.1. Objective 1: Investigating NVU morphological differences associated with ageing between WT and *16p11.2^{df/+}* mice.

Objective 1A: Morphological Visualization of NVU Across Mice Lifespan (Immunohistochemistry (IHC))

3.1.1. Neuroinflammation

Microglia are important immune cells in the CNS, and their activation reflects the neuroinflammatory status of the brain's environment. Thus, to obtain an overall characterization of the neuroinflammatory landscape under baseline conditions, we first assessed cortical microglial coverage and density using the microglia marker (IBA-1) between WT and *16p11.2^{df/+}* during the life span of mice. Quantification of IBA-1 % area coverage and cell count showed that microglia levels remained relatively stable between WT and mutant at 2- and 6-month time points (Figures 2A–C and Supplementary Figures 1A and B). While quantification of IBA-1 coverage and cell count did not reveal significant differences between genotypes in middle-aged and aged mice, analysis of cell counts and microglial coverage showed a significant age-dependent decrease in both WT and mutant 12- and 18-month-old mice.

Next, we sought to assess astrocyte coverage using GFAP, a marker of reactive astrocytes in adult mice. Initially, the aim was to visualize astrocyte reactivity in the cortex and assess differences between genotypes across ageing. However, assessing astrogliosis in the cortex can be challenging, as cortical GFAP expression is relatively low under non-injured conditions. Therefore, we elected to evaluate astrocyte reactivity under baseline conditions in the stratum radiatum (SR) and stratum lacunosum-moleculare (SLM) layers of the hippocampal region CA1. This is because a previous report indicated that GFAP levels were altered in the CA1 region of the hippocampus of aged mice¹⁰⁸. While no significant differences in astrocyte reactivity levels were observed in 2- and 6-month-old mice between WT and mutant, reactive astrocytes' area coverage was higher in 12-month-old *16p11.2^{df/+}* mice in comparison to age-matched WT

mice (Figures 3A and B and Supplementary Figure 2). Additionally, GFAP % area coverage quantification showed increased reactive astrocytes levels in aged WT mice, while *16p11.2^{df/+}* astrocytes' area coverage exhibited a slight trend of increase from middle-aged *16p11.2^{df/+}* mice levels. Overall, GFAP levels in the WT mice displayed a non-significant trend of progressive decrease from 2 to 12 months of age before increasing at 18-month-old age mice. While reactive astrocyte levels in the *16p11.2^{df/+}* mice displayed an age-associated non-significant trend of progressive increase as mice aged.

3.1.2. Gliovascular Interaction

Given that biological ageing is closely linked to alteration in astrocytic endfeet and vessel localization ¹⁰⁹, we transitioned to investigate endfeet perivascular localization in *16p11.2^{df/+}* mice. Using Aquaporin-4 (AQP4), a marker for water channel present in astrocytic endfeet, we measured AQP4 vessel coverage to assess differences between WT and mutant. Analysis of AQP4 – CD31 overlap in 2-month-old and 6-month-old *16p11.2^{df/+}* and WT mice showed no significant differences in the parietal cortex (Figures 4A and B and Supplementary Figure 3). However, AQP4 vessel coverage decreased in *16p11.2^{df/+}* mice in comparison to WT at the middle-age life stage. While AQP4 vessel coverage levels relatively stabilized at 18-month-old *16p11.2^{df/+}* mice, AQP4-CD31 co-localization decreased significantly in WT mice. Overall, both WT and mutant groups exhibited an age-dependent pattern of AQP4 – CD31 co-localization. AQP4 perivascular localization displayed no significant changes from 2 to 6 months in both groups; however, at 12 months, mutant animals showed a decrease, whereas WT animals exhibited a slight increase. By 18 months, co-localization remained relatively stable in *16p11.2^{df/+}* mice, whereas it decreased in WT animals. Together, these data show that aging and genotypes affect AQP4 vessel coverage in mice.

Objective 1B: Assessing BBB integrity Across Mice Lifecycle (Transmission Electron microscopy [TEM])

NVU impairment has been associated with ageing, psychiatric disorders, neurodevelopmental and neurodegenerative diseases^{58,67}. In humans, earlier research showed a higher NVU functional decline in the parietal cortex of males than in females during ageing¹¹⁰. Furthermore, previous studies showed that early life induction of traumatic brain injury in the parietal cortex did not disturb the BBB but rather induced its modification in adult rats¹¹¹. Importantly, it was previously demonstrated that most vascular changes (lower vascular branching, vessel density and failed characteristics endothelial remodeling) in *16p11.2^{df/+}* mice occur in the parietal cortex¹⁰⁵. Collectively, these data suggest that the parietal cortex undergoes NVU modifications during ageing and injury. Therefore, making the parietal cortex highly relevant region of interest to assess NVU features using ultrastructure imaging between the mutant and WT mice groups during mice life cycle.

Using ultrastructural imaging, we first assessed the number of astrocytic endfeet surrounding capillaries across time. While no significant overall trajectories were observed in aged WT mice, our quantification showed a significant trend of progressive decline in astrocyte numbers as *16p11.2^{df/+}* mice aged (Figures 5A and B and Supplementary Figure 4A). No significant differences between genotypes were revealed when comparing astrocytic endfeet counts in 2- and 6-month-old mice. However, it was observed that endfeet numbers are significantly lower in the *16p11.2^{df/+}* than in WT at 12 months. At 18 months, astrocyte count decreased slightly in WT while the count remained relatively constant in *16p11.2^{df/+}* mice. Moreover, this reduction in astrocytes' endfeet numbers was accompanied by an overall increase in astrocytic endfeet size as animals reached older age (Figures 5A and C and Supplementary Figure 4B). While there was no significant difference in astrocytic endfeet size between WT and mutant at 2- and 6-month-old mice, it was found that *16p11.2^{df/+}* middle-aged mice exhibited significantly enlarged

astrocytic endfeet in comparison to aged-matched WT. Such astrocytic endfeet enlargement remained significantly higher in aged *16p11.2^{df/+}* mice, while endfeet size slightly increased as WT mice reached 18-month-old.

Given that mitochondrial dysfunction is associated with different morphological abnormalities, multiple mitochondrial morphological features were quantified across ageing. While no apparent age-associated mitochondrial density changes were observed as WT mice aged, it was found that there is a significant decrease in mitochondrial density as *16p11.2^{df/+}* mice aged from mature adult to middle-aged and old animals (Figures 5A, and D and Supplementary Figure 4D). Moreover, quantification of mitochondrial average area size showed no age-dependent changes (Figures 5A and E and Supplementary Figure 4E). Although no significant differences between genotypes were observed at 2-, 6-, and 18-month-old mice, a significant increase in the average area size of mitochondria was identified between *16p11.2^{df/+}* and WT at 12-month time point (Figures 5A, and E and Supplementary Figure 4E). Additionally, mitochondrial area fraction quantifications showed no apparent trends as WT mice aged (Figures 5A and F and Supplementary Figure 4F). However, the mitochondria area fraction was slightly increased in 6-month-old *16p11.2^{df/+}* and slightly decreased in 18-month-old in comparison to age-matched WT. Lastly, analysis of average mitochondrial maximal length showed no overall differences between mutant and WT across targeted mouse life stages (Figures 5A and G and Supplementary Figure 4G).

Next, we sought to further analyze NVU ageing by assessing basement membrane thickness. Quantification of basement membrane thickness showed an overall trend of increase as mice progressively aged. Despite lack of significant differences between WT and mutant at young and mature mice life stage, basement membrane size showed a significant increase in *16p11.2^{df/+}* middle-aged mice when compared to WT aged-matched mice (Figures 6A, and B and Supplementary Figure 4C). However, this

significant difference was eliminated at the 18-month time point, as basement membrane size increased in the WT while thickness remained relatively unchanged in the mutant.

3.2.Objective (2): Investigating molecular changes associated with aging between WT and 16p11.2^{df/+}

3.2.1. Objective 2A: Assessing Protein Levels (Western blot)

Previous studies found that protein levels of multiple tight junction markers, transport proteins, and angiogenic factors are differentially expressed in aged mice brains^{37,56}. Additionally, ASD has been found to be associated with dysregulation in multiple BBB proteins⁷³. Hence, to determine the differences in protein levels between the 16p11.2^{df/+} and WT at different time points in mice life cycle, western blots were performed to identify molecular changes associated with junctional markers in the hippocampus and PFC across life span of mice. Western blot analysis of Claudin-5 expression in the PFC revealed no significant differences between mutant and WT mice across ageing (Figures 7A and C and Supplementary Figure 5A). Interestingly, progressive aging was associated with a slight gradual increase in Claudin -5 expression in both groups. Particularly, Claudin-5 levels in 16p11.2^{df/+} mice significantly increased from 2-, and 6-month-old to 18-month-old mice in the PFC. A similar Claudin-5 expression profile was observed in the hippocampus, where genotype likewise had no significant effect, while aging resulted in a modest overall increase in expression (Figures 7A and C and Supplementary Figure 5A). Moreover, assessment of Occludin levels showed no significant effect of genotype and age in either the hippocampus or PFC (Figures 7B and D and Supplementary Figure 5B). Taken together, these data suggest that genotype does not affect expression levels of Claudin-5 and Occludin, and aging results in a slight increase in Claudin-5 levels in the hippocampus and prefrontal cortex.

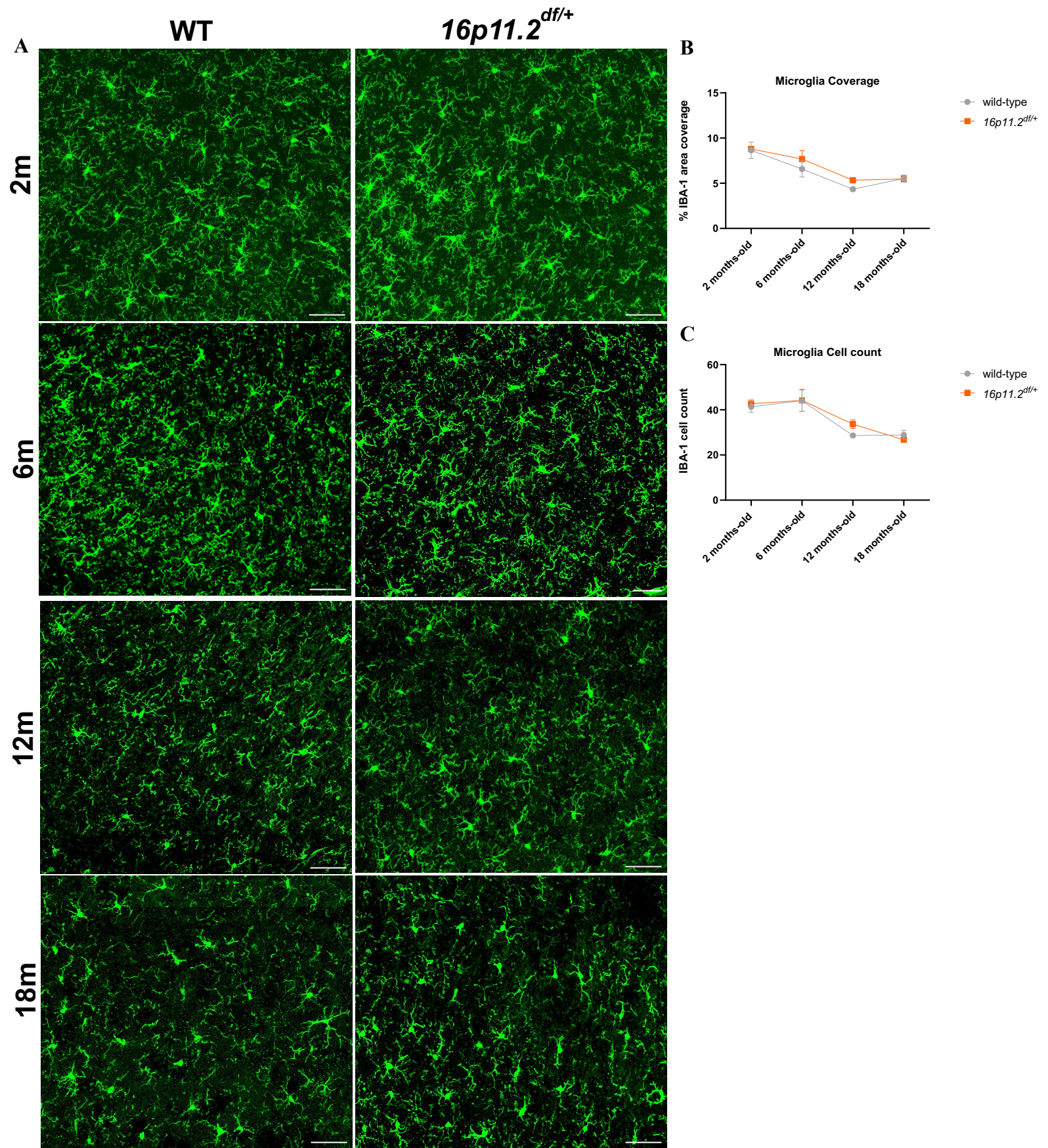


Figure 2: Baseline microglia levels in the parietal cortex of $16p11.2^{df/+}$ mice across lifespan.

A) IHC images of IBA-1 (green) in 2-, 6-, 12-, and 18-months-old/wild-type & $16p11.2^{df/+}$ mice in the parietal cortex. Quantification of B) IBA-1 percentage area coverage, and C) microglial cell number. Images were acquired at 20x magnification. 3-5 images per sample. All data are shown as the mean $\pm$ SEM. (n=4-5 animals per group). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (two-way repeated measure ANOVA and Tukey's post-hoc test).

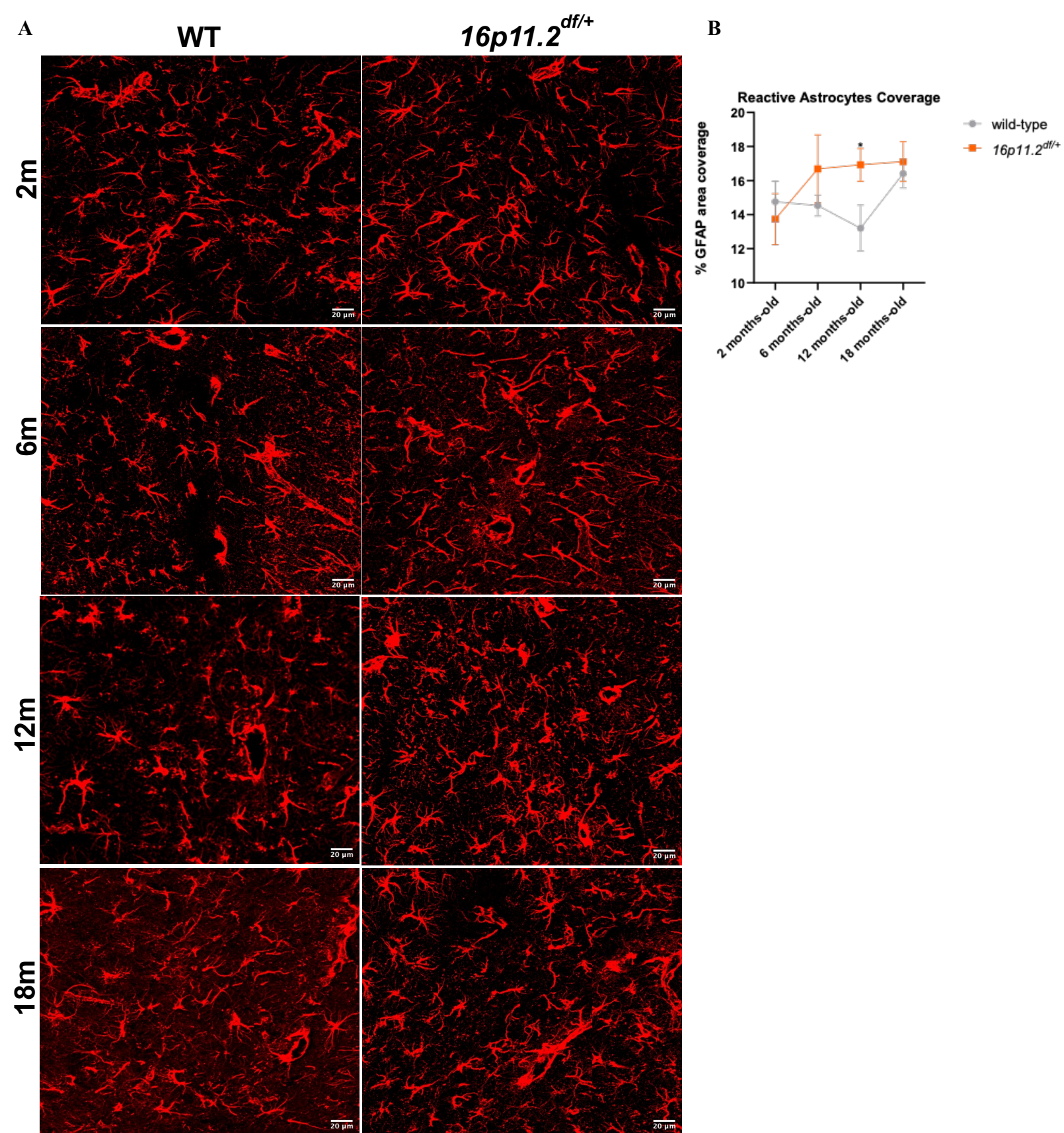


Figure 3: Reactive astrocytes coverage in the hippocampal region CA1 of 16p11.2^{df/+} mice.

*A) Immunofluorescence staining of GFAP⁺ (red) in 2-, 6-, 12-, and 18-months-old/wild-type & 16p11.2^{df/+} mice. B) Quantification of GFAP⁺ percentage area coverage. Images were acquired at 20x magnification. 3-5 images per sample. All data are shown as the mean $\pm$ SEM. (n=4-5 animals per group). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (two-way repeated measure ANOVA and Tukey's post-hoc test).*

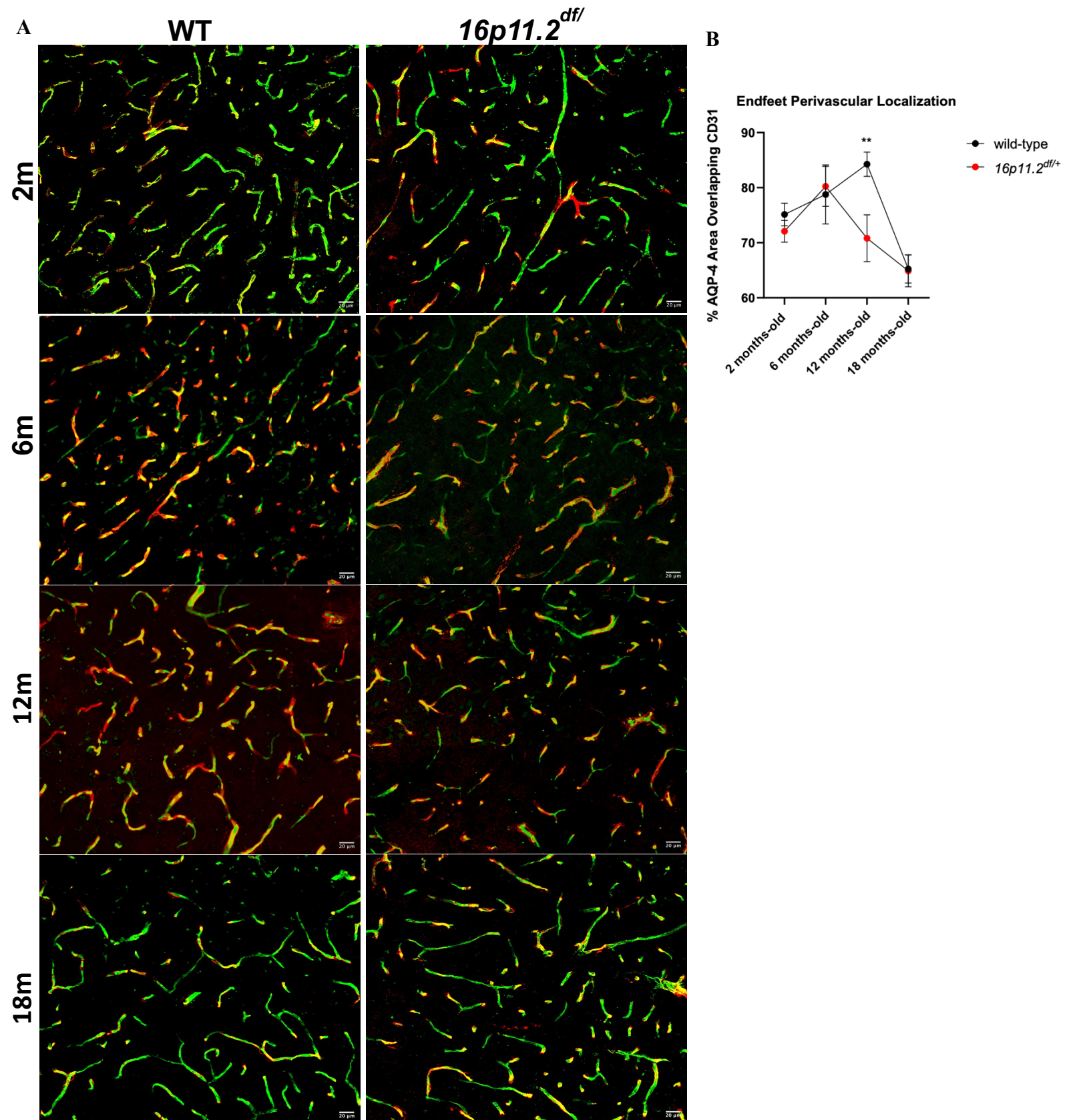
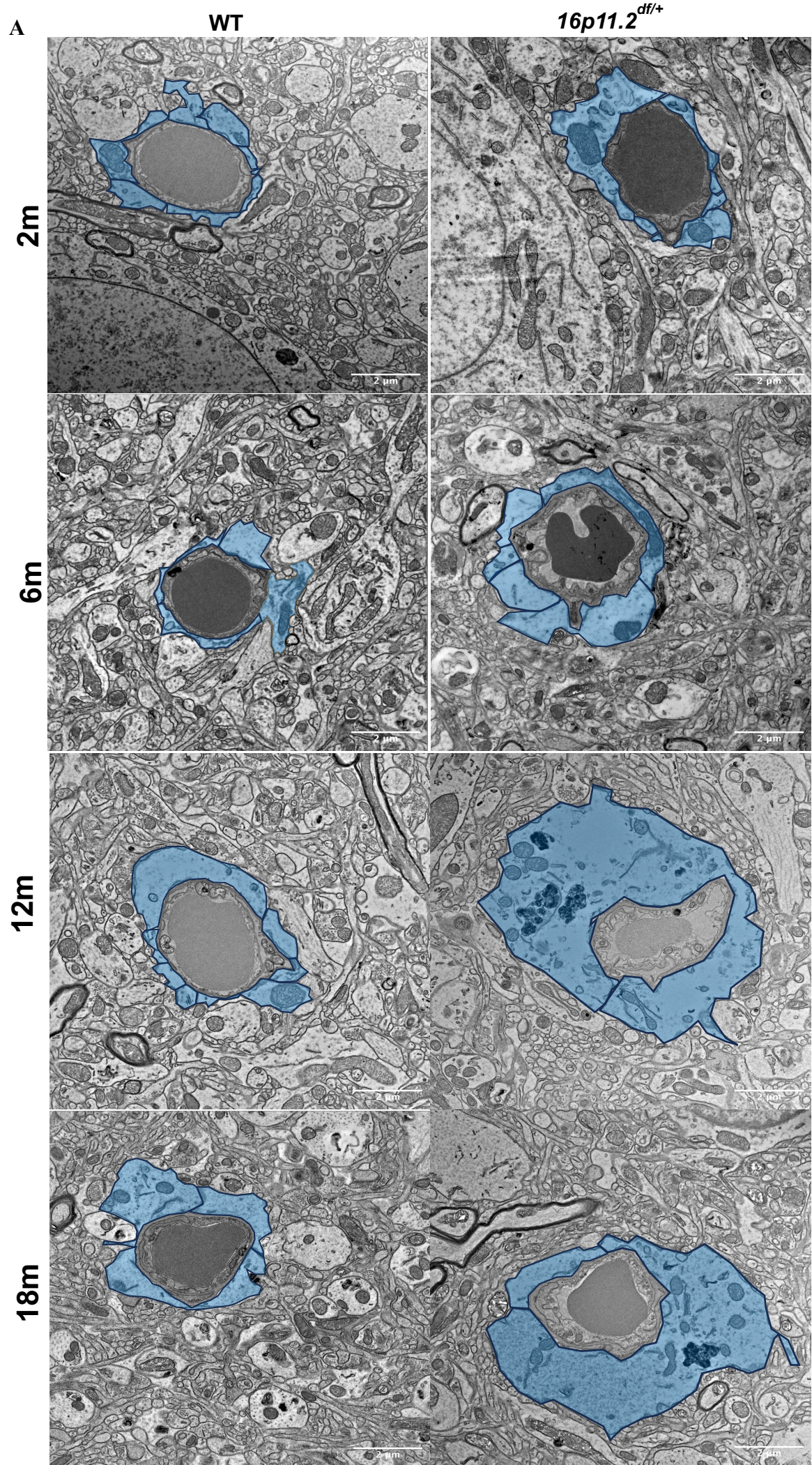


Figure 4: Astrocytic endfeet-vessel localization in the parietal cortex of $16p11.2^{df/+}$ mice.

A) IHC images of AQP-4 (red) and CD31 (green) in 2-, 6-, 12-, and 18-months-old/wild-type & $16p11.2^{df/+}$ mice.

B) Quantification of AQP-4 -CD31 co-localization. Images were acquired at 20x magnification. 3-5 images per sample. All data are shown as the mean $\pm$ SEM. (n=4-5 animals per group). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (two-way repeated measure ANOVA and Tukey's post-hoc test).



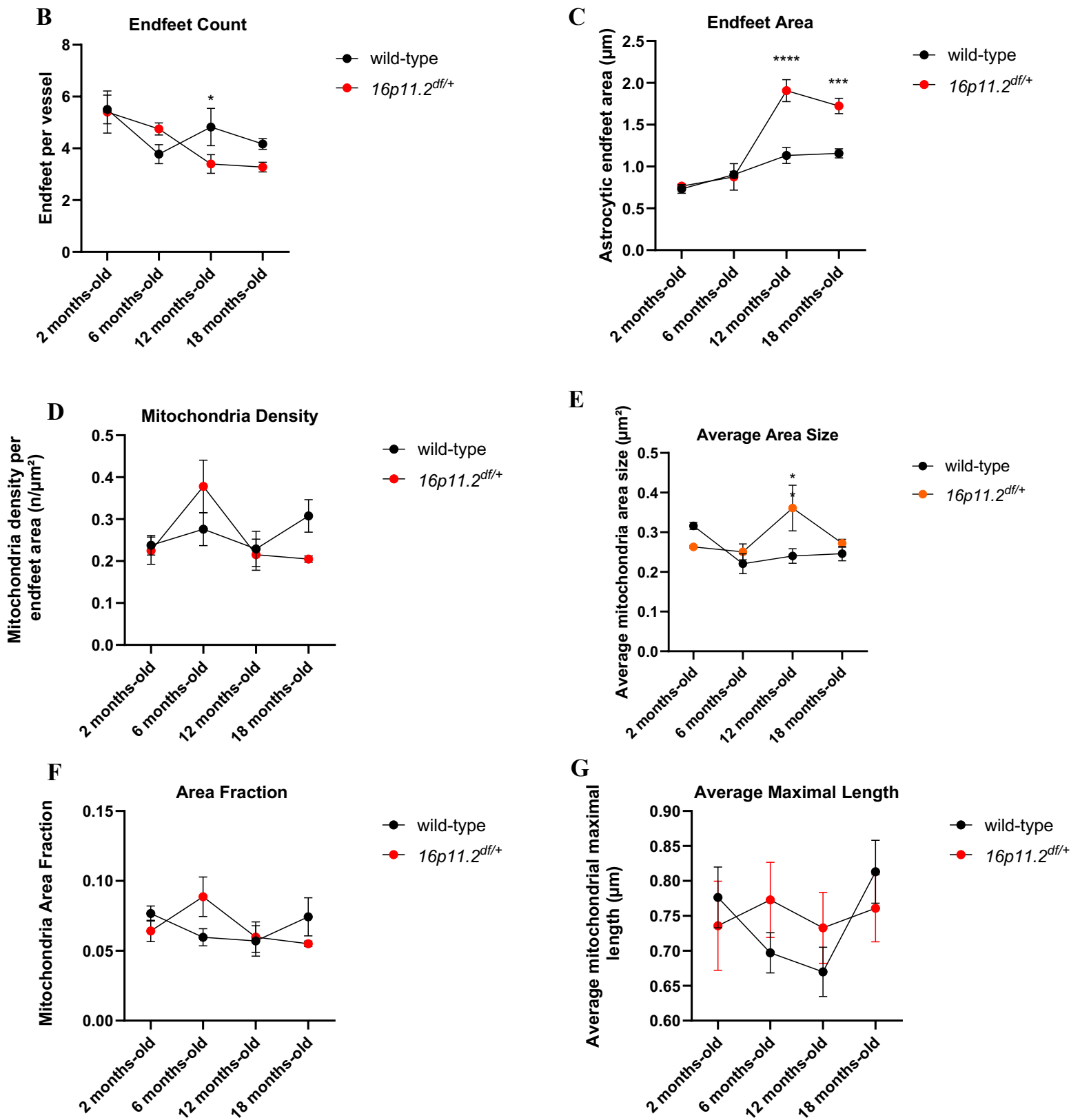


Figure 5: Assessment of NVU characteristics in parietal cortex capillaries of 16p11.2^{df/+} brain sections.

(A) Electron micrograph of 2-, 6-, 12-, and 18-months-old/wild-type & /16p11.2^{df/+} mice vessels. (B-I) Quantifications of multiple NVU features: B) Astrocytes endfeet count, C) astrocytes endfeet area, D) astrocytic endfeet mitochondrial density, E) astrocytic endfeet mitochondrial average area size, F) astrocytic endfeet mitochondrial area fraction, G) astrocytic mitochondrial average maximal length. Quantification was performed using 10 vessel images per mouse. All images scale bar (2 μm). Blue highlight; astrocytic endfeet. All data are shown as the mean ± SEM. (n=4-5 animals per group). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 (two-way repeated measure ANOVA and Tukey's post-hoc test).

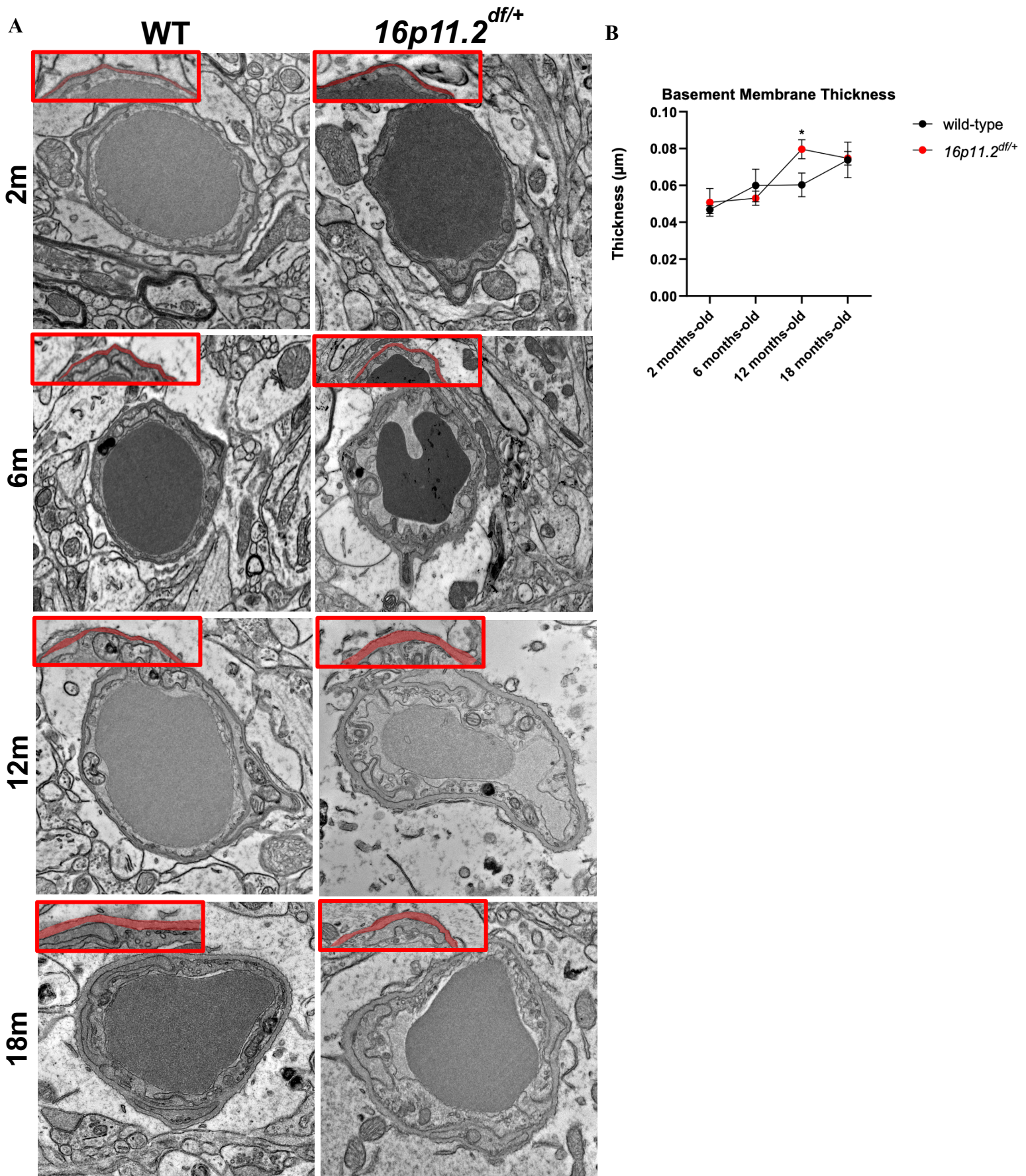


Figure 6: Assessment of basement membrane thickness in parietal cortex capillaries of 16p11.2^{df/+} brain sections.

(A) Electron micrograph of 2-, 6-, 12-, and 18-months-old/wild-type & /16p11.2^{df/+} mice vessels. (B) Quantifications of basement membrane thickness. Quantification was performed using 10 vessel images per mouse. All images scale bar (2 μm). Red box: zoomed-in image of a basement membrane section. Red highlight: basement membrane. All data are shown as the mean $\pm$ SEM. (n=4-5 animals per group). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ (two-way repeated measure ANOVA and Tukey's post-hoc test).

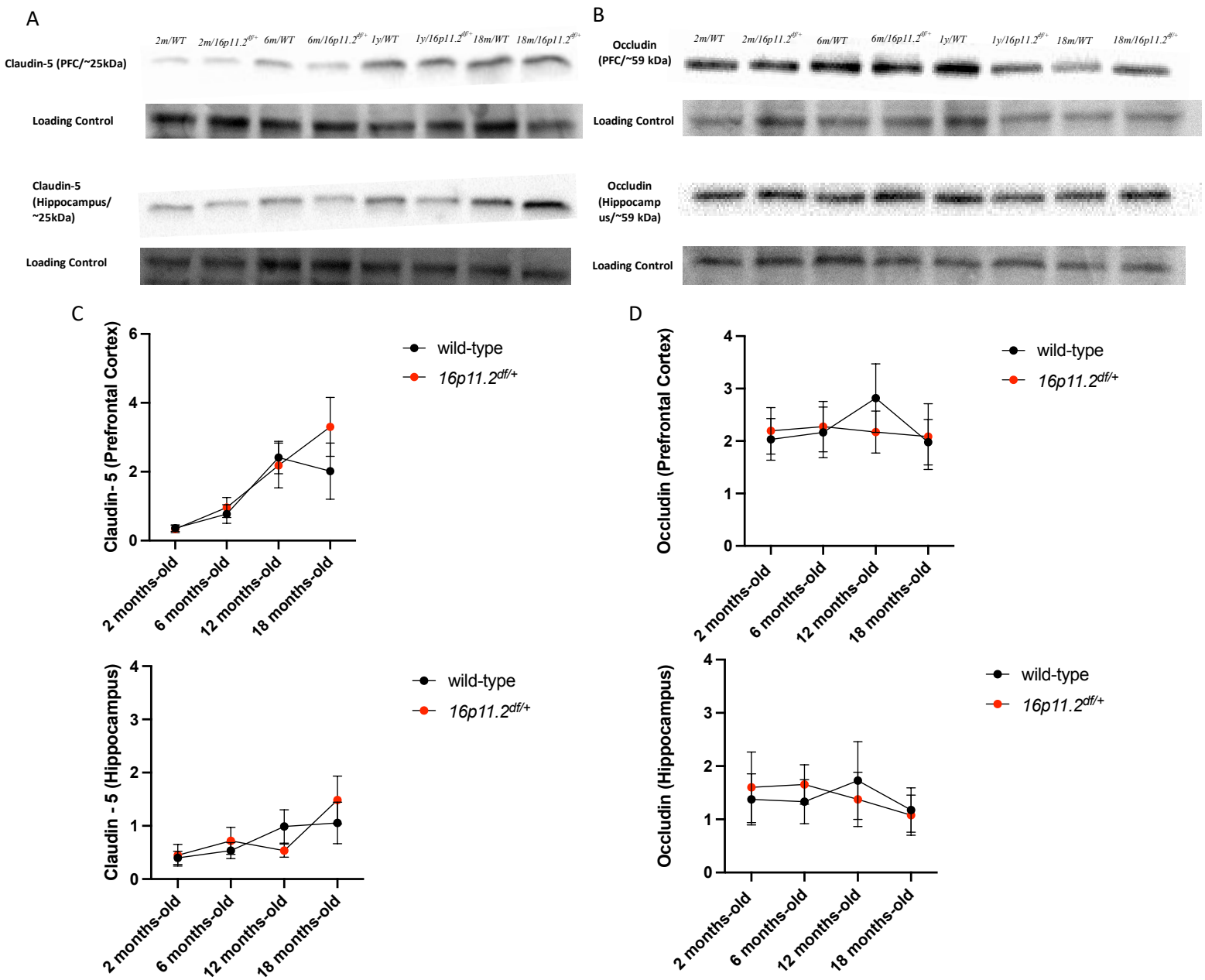


Figure 7: Western blots analysis of Claudin-5 and Occludin in prefrontal cortex (PFC) and hippocampus across different time points in mice life cycle.

A) Western blots of Claudin-5 expression in 2-, 6-, 12-, and 18-months-old/wild-type & 16p11.2^{df/+} mice. B) Immunoblots analysis of Occludin in 2-, 6-, 12-, and 18-months-old/wild-type & 16p11.2^{df/+} mice. Protein levels quantification of C) Claudin-5, and (D) Occludin. Protein expression was normalized to the loading control. All data are shown as the mean $\pm$ SEM. (n=4 animals per group). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (two-way repeated measure ANOVA and Tukey's post-hoc test).

4. Discussion

4.1. Summary

Ageing and neurodegeneration represent continuous progressive processes that result in gradual shifts in cellular homeostasis that precede the clinical manifestations. Given the brain's massive energy demand, paired with its sequestered microenvironment, the structural and functional health of the cerebrovasculature play a foundational role in maintaining homeostasis. Growing evidence link cerebrovascular dysfunction to neurodegeneration, yet the early origins of these vascular impairments remain unclear. Identifying early changes is crucial for designing effective and/or preventive therapeutic strategies. Therefore, the present study sought to determine the effects of early vascular deficits on the trajectory of NVU ageing in mouse model of ASD. Overall, male *16p11.2^{df/+}* ageing was associated with alterations across multiple characteristics of NVU, including cerebrovascular structural changes, gliovascular interactions, and neuroinflammation. Collectively, our findings suggest a modified trajectory of ageing in *16p11.2* deficient mouse model of ASD.

4.2. Age-associated changes in immunoreactivity in the *16p11.2^{df/+}* mice

Glial reactivity is a central feature of brain ageing and contributes to cerebral homeostasis and NVU health. The impact of *16p.11.2* haploinsufficiency on brain glial cells is unknown; thus, it brought us to investigate the contributions of microglia and astrocytes to *16p11.2^{df/+}* mice NVU ageing. To fill this knowledge gap, we assessed microgliosis and astrogliosis in both WT and mutant across different ages in mice life span. With regard to microglial ageing, our observation is in line with previous results showing no significant differences in microglia coverage and density in the cerebral cortex of *16p11.2^{df/+}* mice compared to WT at P14 and P50 ¹⁰⁵. However, in this thesis, we demonstrate that no genotype-dependant

significant differences in coverage and density were present as mice aged. Notably, in our study, both cohorts exhibited an age-associated decline in microglial area coverage and density. While microglial population exhibit a region-dependant age-related variations in proliferation, density and distribution, our results closely align with the observed reduction in microglia numbers in the cortex of aged mice^{112,113}. The observed decrease in microglial coverage in our study is possibly attributed to age-associated microglial morphological remodeling. In aged human and mouse samples, microglial cell processes were found to have reduced length, fewer branches, and an overall reduction in arborization^{114,115}. Additionally, cellular senescence has recently emerged as one of the primary factors underlying the age-associated reduction in microglial numbers. In rodent models, investigation of aged microglia telomeres revealed reduced length and activity¹¹⁶. Single-cell transcriptomic analysis showed an age-associated increase in microglia cellular senescence markers across various brain and spinal cord regions¹¹⁷. These findings suggest that ageing alone is insufficient to alter microglial behaviour in haploinsufficient 16p.11.2 mice compared to WT. While we report no age-associated shifts in microglial density and coverage in 16p.11.2 deletion syndrome, a definitive conclusion on the physiological state of microglia is constrained by the lack of morphological and functional analysis. Future studies are required to investigate microglia morphology and functional dynamics, which have been previously shown to be associated with ASD^{118,119}.

We further assessed age-associated changes in immunoreactivity in *16p11.2^{df/+}* mice by evaluating astrogliosis across mice lifespan. Our results revealed no significant genotype-dependent differences in reactive astrocyte coverage in the hippocampus, except in the middle-aged life cohort. At this specific timepoint, *16p11.2^{df/+}* mice exhibited significantly elevated reactive astrocyte coverage compared with age-matched WT mice. Importantly, the temporal trajectory of CA1 astrocytic reactivity was slightly altered between genotypes with advancing age. In our findings, WT mice displayed a late onset increase

in astrocyte coverage while 16p.11.2 deletion syndrome mice exhibited a modest progressive increase in GFAP coverage throughout their lifespan. Current literature establishes that WT age-associated astrocytic reactivity coverage and density are highly subregion-specific within the hippocampus^{120–122} Our results aligned with previous characterization showing a slight decline in reactive astrocytes coverage and density in 10-12-month-old WT mice (middle-aged) and a marked increase in GFAP coverage and expression at 18 month-old^{121,123,124}. The non-significant age-associated upward trend in reactive astrocytes coverage in the CA1 may be indicative of altered astrocytes reactivity in 16p.11.2 deletion syndrome. Earlier studies in ASD rodent model have consistently demonstrated an increase in hippocampal reactive astrocytes coverage and density^{125,126}. To our knowledge, reactive astrocyte coverage and density characterization within the hippocampus region CA1 of ASD individuals remains unexplored; however, a recent study documented an increase in both GFAP mRNA expression and reactive astrocyte density in the CA4 subregion of the hippocampus¹²⁷. Additionally, the observed *16p11.2^{df/+}* age-associated increase in astrogliosis may be indicative of early onset neuroinflammation. Evidence from human and rodent models documented increased levels of neuroinflammatory markers in the hippocampus and other brain regions in patients with ASD^{126,128}. Nevertheless, while this observed trend did not reach statistical significance, an expanded sample size might reveal a significant trend in GFAP coverage in the mutant. However, alternative methodologies such as evaluating other hippocampal subregions might also reveal distinct age-related region-dependent reactive astrocytes behaviour in the 16p.11.2 haploinsufficient mouse. Collectively, our results demonstrate an age-associated alteration of inflammatory trajectory in a mouse model of 16.11.2 deletion syndrome. Collectively, our results demonstrate an aged-associated alteration of inflammatory trajectory in mouse model of 16.11.2 deletion syndrome.

4.3. Age associated changes in gliovascular interaction in the $16p11.2^{df/+}$ mice

While age-associated changes in $16p11.2^{df/+}$ mice hippocampal astrocytes' reactivity may suggest an altered activation pattern and modified neuroinflammatory response, these findings do not reflect the integrity of gliovascular interaction. Given that previous research showed that normal ageing is associated with alterations in AQP4 vessel localization in human and mouse brains, this prompted us to evaluate the trajectory of this disruption in the $16p11.2^{df/+}$ mice across their life span^{109,129}. Consistent with earlier findings, we show a significant decrease in the perivascular localization of astrocytic endfeet in the aged WT mouse cortex. Notably, our findings revealed no significant changes to endfeet-vessel localization until middle-age life stage, followed by a significant decline in aged WT mice. While the mutant cohort followed a similar colocalization pattern, $16p11.2^{df/+}$ middle-aged mice showed an early onset decline in gliovascular interactions. Advanced age is widely associated with impaired glymphatic system and waste clearance. This is hypothesized to be largely due to the widespread reduction in perivascular AQP4 localization¹⁰⁹. Additionally, a previous report documented that individuals with ASD exhibit significant early-life impairment and dysfunction in the glymphatic exchange system¹³⁰. Therefore, a possible interpretation is that the impairment of the glymphatic system, coupled with the observed early-life sleep disturbances in the $16p11.2^{df/+}$ mice, may impose an early-life modification on gliovascular interaction¹³¹. Because most of the waste clearance processes occur during sleep, this early glymphatic system physiological stress may likely drive the early onset of the AQP4 perivascular localization loss. Nevertheless, functional waste clearance and cerebrospinal fluid (CSF) exchange studies are needed.

4.4. Age associated alterations in astrocytic endfeet in the *16p11.2^{df/+}* mice

Considering that it is thought that astrocytes change their morphology during ageing to adapt to the new cellular environment and maintain homeostasis and given that we observed age-related changes in astrogliosis and in gliovascular interaction in the *16p11.2* deletion syndrome mice, we questioned whether this suggests a possible alteration to astrocyte morphology around the vessel. Utilizing ultrastructural imaging, it was revealed that astrocytic endfeet undergo age-related alterations in number and size across genotypes in the parietal cortex. Although our findings displayed no significant differences in astrocytic endfeet numbers as WT mice aged, there was an overall downward trend. This aligned with a previous report showing a modest decline in cortical astrocytic endfeet density surrounding vessels as WT mice aged¹³². Nevertheless, this trend was significantly pronounced during mutant mice ageing. This observed age-associated reduction in endfeet numbers might be a result of an overall morphological change to astrocytes during ageing. This is supported by earlier evidence suggesting an age-related decrease in the number and length of astrocytes' processes¹³³. Interestingly, this decline in endfeet density was associated with age-related enlargement of astroglial endfeet in both groups. Notably, in our study, *16p11.2^{df/+}* mice endfeet exhibited a modified ageing trajectory, with a significant decline in count and a notable premature increase in area at the middle-age life stage. Astrocytic endfeet swelling has long been recognized as a phenotype of ischemic stroke pathology¹³⁴. However, recent publications showed a regional endfeet swelling in healthy aged rats and old mice^{132,135}. Physiologically, endfeet swelling is hypothesized to be a protective mechanism in which astrocytes swell and form a cuff around damaged vessels to limit brain hemorrhage and in response to BBB dysfunction during stroke¹³⁶. A possible interpretation of the early-onset loss of perivascular AQP4, combined with the premature reduction in astrocytic endfeet density in the *16p11.2^{df/+}* mice, might be that these events trigger compensatory enlargement of endfeet in an attempt to maintain coverage of the cerebral vessels. Additionally, one proposed mechanism to account for astrocytic endfoot

swelling is the exposure to higher amounts of glutamate and the dysregulation in K^+ gradient ¹³⁷. Interestingly, we recently found increased levels of glutamate products in isolated P8 male *16p11.2^{df/+}* astrocytes ¹³⁸. This suggests an early modification to glutamate clearance processes in the 16p.11.2 deletion syndrome. Perhaps this modification accelerates the impairment of age-associated astrocyte swelling, resulting in an earlier onset of the phenotype in *16p11.2^{df/+}* mice. These possible mechanisms, coupled with early-life sleep disturbances in the *16p11.2^{df/+}* and glymphatic system dysfunction observed in ASD, could provide insights on the mechanisms behind premature age-associated endfeet swelling ^{130,131}. Taken together, these findings illustrate an early onset of modification to astrocytic endfeet in the *16p11.2^{df/+}* middle-aged mice. Nonetheless, future studies are needed to elucidate whether endfoot functionality is also altered in the mutant during ageing compared to the WT. Additionally, metabolomics and other mechanistic studies are required to pinpoint the mechanism behind astrocytic endfeet swelling in both groups.

4.5. Age-associated alterations in endfeet mitochondrial morphology in the 16p11.2^{df/+} mice

Given the significant age-related changes characterized in astrocytic morphology, coupled with earlier evidence suggesting the appearance of mitochondrial malfunction in aged human cortical astrocytes ¹³⁹, this brought us to investigate the morphology of mitochondria within astrocytic endfeet using ultrastructural imaging. While our results revealed no age-associated trends in mitochondria density in the WT, *16p11.2^{df/+}* mice displayed a progressive age-related decline in mitochondria numbers. Although mitochondrial average area size showed no apparent age-associated pattern in both groups, mitochondrial area was larger in *16p11.2^{df/+}* endfeet at middle-age life stage. Furthermore, we report no age-dependant and genotype-dependant differences in mitochondrial area fraction and maximal length. To our knowledge, this is the first study to investigate age-associated changes in astrocytic endfeet mitochondrial density and morphology in a mouse model of ASD. Our results align with a recent report showing no significant

differences in cortical endfeet mitochondrial density in aged WT mice⁵³. We previously reported an early-life decrease in the numbers of mitochondria within endothelial cells at the young adult life stage of *16p11.2^{df/+}* mice¹⁴⁰. Within the same report, morphometric analysis revealed decreased mitochondrial area size in *16p11.2^{df/+}* endothelial cells. Interestingly, another study has reported unaffected cortical astrocytes' mitochondrial function under baseline conditions¹⁴¹. However, these astrocytes displayed context dependant mitochondrial respiration failure under challenged condition. Together, these findings illustrate an early life NVU mitochondrial dysfunction and suggest an overall modification to mitochondrial ageing in *16p11.2^{df/+}* mice. While we largely observed no age-associated morphometric alterations in astrocytic endfeet, it remains to be determined whether mitochondrial function is similarly maintained in astrocytes and NVU of *16p11.2^{df/+}* mice during ageing.

4.6. Age associated alterations in cerebrovascular structure in the 16p11.2^{df/+} mice

Given the age-related observed changes in astrogliosis, endfoot morphology and gliovascular interaction in *16p11.2^{df/+}* ageing, this led us to question other NVU features during ageing. The basement membrane is an integral component of the NVU and exerts many important structural functions, including support and protection¹⁴². Structural remodeling of the basement membrane is considered a leading factor in BBB disruption³⁸. In alignment with previous reports, our results displayed an age-associated increase in cortical basement membrane thickness in brain capillaries of both cohorts^{132,143}. In our study, however, we report an early onset of age-related basement membrane thickening in middle-aged *16p11.2^{df/+}* mice. While the exact mechanism of basement membrane thickening is unknown, previous reports suggest widespread expression changes in key basement membrane proteins including laminin, fibronectin, collagen IV, and perlecan^{144,145}. This is particularly interesting in relation to ASD, as previous reports displayed an altered gene expression and protein levels of multiple ECM proteins in the cortex and

hippocampus of rodent models of ASD ^{146,147}. Of note, basement membrane thickening has been previously associated with impaired glymphatic system drainage in aged mice ¹⁴⁴. Collectively, our results show an age-associated early onset increase in basement membrane thickness in *16p11.2^{df/+}* brain capillaries.

4.7. Age-associated BBB molecular modifications in 16p11.2^{df/+} mice

Given that we characterized several morphological alterations across multiple NVU compartments, this led us to next evaluate protein levels of key tight junction markers to assess the molecular integrity of the BBB in 16p.11.2 haploinsufficiency ageing. Our findings showed no genotype-dependant and age-associated trends in Occludin protein level expression in the hippocampus and PFC. These data run in parallel with work showing unaffected Occludin levels in P50 *16p11.2^{df/+}* mice (Pavel Kotchetkov, unpublished). In contrast to expectations, although no significant genotype differences in Claudin-5 expression levels were observed, our data displayed a trend toward a progressive increase in protein expression in the hippocampus and PFC of both groups. Interestingly, in our study, this age-associated progressive upward trend was particularly significant in the PFC of *16p11.2^{df/+}* mice. In two recently published single-cell sequencing datasets, Claudin-5 expression was found to be downregulated in brain endothelial cells during ageing ^{148,149}. However, in one of these datasets, they report that this downregulation became minimal when assessing whole brain tissue ¹⁴⁸. Of note, increasing levels of Claudin-5 in the bloodstream have been associated with ageing and neurodegenerative diseases. Also, increasing levels of Claudin-5 might be a compensatory mechanism in response to stress and neuroinflammation. Previous reports showed increased baseline levels of serum Claudin-5 in psychiatric disorder patients ¹⁵⁰. Within 6 weeks follow-up with patients after treatment, Claudin-5 levels were found to decrease to similar levels of healthy controls. It is hypothesised that the

purpose of this compensatory increase in Claudin-5 is to maintain BBB homeostasis and prevent the entry of harmful substances from the blood circulation during neuroinflammation ¹⁵¹. Therefore, these data suggest that the observed discrepancies in Claudin-5 levels may be due to a compensatory mechanism in our mouse model, or due to technical variations, such as the absence of transcardial perfusion during tissue processing in our sample. Alternatively, these contrast results may reflect a highly cell-type and region-specific expression pattern across the brain ¹⁴⁸.

5. Conclusion

Although brain aging has been extensively studied over the years, the specific involvement of the NVU in this process is still largely underexplored. In this thesis, we investigated early-life cerebrovascular deficits contributions to brain ageing in mouse model ASD. Among the NVU cellular components examined, astrocytes emerged as the most morphologically altered cell-type in male *16p11.2^{df/+}* ageing. This was primarily evident in the significant premature increase in reactive astrocytes coverage, decreased astrocytic endfeet count and hypertrophy, and altered gliovascular interactions. This in addition to the early onset of cerebrovascular structural remodeling and modification to BBB molecular integrity, highlights a modified NVU ageing trajectory is in the 16p.11.2 deletion syndrome mouse model of ASD. This study points to glymphatic system and waste clearance impairment and suggest altered NVU functions during ASD brain ageing. Nevertheless, further studies are required to determine whether these NVU modifications are accompanied by functional alterations and to decipher the mechanism behind NVU ageing in ASD. In addition, expanding future cohorts to include female mice will be critical to identify potential sex-specific differences in these NVU trajectories. This project provides insights on factors that are correlated with the increased rate of mortality among ASD individuals. Importantly, this thesis contributes to a broader understanding that early-life cerebrovascular dysfunction may result in altering the trajectory of NVU ageing. Given that ageing is a major risk factor for neurodegenerative diseases, delineating the trajectory of NVU decline fills a critical knowledge gap and importantly, allows for designing effective and preventive therapeutic strategies.

6. References

1. Gaviano, L. *et al.* Definitions of Ageing According to the Perspective of the Psychology of Ageing: A Scoping Review. *Geriatrics* **9**, 107 (2024).
2. Teo, N., Gao, Q., Nyunt, M. S. Z., Wee, S. L. & Ng, T.-P. Social Frailty and Functional Disability: Findings From the Singapore Longitudinal Ageing Studies. *J. Am. Med. Dir. Assoc.* **18**, 637.e13-637.e19 (2017).
3. Gilbert, S. F. Aging: The Biology of Senescence. in *Developmental Biology*. 6th edition (Sinauer Associates, 2000).
4. Li, Y. *et al.* Molecular mechanisms of aging and anti-aging strategies. *Cell Commun. Signal. CCS* **22**, 285 (2024).
5. Government of Canada, S. C. A portrait of Canada's growing population aged 85 and older from the 2021 Census. <https://www12.statcan.gc.ca/census-recensement/2021/as-sa/98-200-x/2021004/98-200-x2021004-eng.cfm> (2022).
6. Canada, P. H. A. of. Aging and chronic diseases: A profile of Canadian seniors. <https://www.canada.ca/en/public-health/services/publications/diseases-conditions/aging-chronic-diseases-profile-canadian-seniors-report.html> (2021).
7. Li, A. *et al.* Vascular Aging: Assessment and Intervention. *Clin. Interv. Aging* **18**, 1373–1395 (2023).
8. Ya, J. & Bayraktutan, U. Vascular Ageing: Mechanisms, Risk Factors, and Treatment Strategies. *Int. J. Mol. Sci.* **24**, 11538 (2023).
9. Zhao, Y., Vanhoutte, P. M. & Leung, S. W. S. Vascular nitric oxide: Beyond eNOS. *J. Pharmacol. Sci.* **129**, 83–94 (2015).

10. Cai, H. & Harrison, D. G. Endothelial Dysfunction in Cardiovascular Diseases: The Role of Oxidant Stress. *Circ. Res.* **87**, 840–844 (2000).
11. Zeng, Y., Buonfiglio, F., Li, J., Pfeiffer, N. & Gericke, A. Mechanisms Underlying Vascular Inflammaging: Current Insights and Potential Treatment Approaches. *Aging Dis.* **16**, 1889–1917 (2025).
12. Yasmin *et al.* C-Reactive Protein Is Associated With Arterial Stiffness in Apparently Healthy Individuals. *Arterioscler. Thromb. Vasc. Biol.* **24**, 969–974 (2004).
13. Lin, P. K. & Davis, G. E. Extracellular matrix remodeling in vascular disease: Defining its regulators and pathological influence. *Arterioscler. Thromb. Vasc. Biol.* **43**, 1599–1616 (2023).
14. Brito, D. V. C. *et al.* Assessing cognitive decline in the aging brain: lessons from rodent and human studies. *Npj Aging* **9**, 23 (2023).
15. Idowu, M. I. & Szameitat, A. J. Executive function abilities in cognitively healthy young and older adults—A cross-sectional study. *Front. Aging Neurosci.* **15**, 976915 (2023).
16. Miner, B. & Kryger, M. H. Sleep in the Aging Population. *Sleep Med. Clin.* **12**, 31–38 (2017).
17. Wennberg, A. M. V., Wu, M. N., Rosenberg, P. B. & Spira, A. P. Sleep Disturbance, Cognitive Decline, and Dementia: A Review. *Semin. Neurol.* **37**, 395–406 (2017).
18. Pfefferbaum, A. *et al.* Variation in longitudinal trajectories of regional brain volumes of healthy men and women (ages 10 to 85 years) measured with atlas-based parcellation of MRI. *NeuroImage* **65**, 176–193 (2013).
19. O’Shea, A., Cohen, R. A., Porges, E. C., Nissim, N. R. & Woods, A. J. Cognitive Aging and the Hippocampus in Older Adults. *Front. Aging Neurosci.* **8**, 298 (2016).

20. Jost, Z. & Kujach, S. Understanding Cognitive Decline in Aging: Mechanisms and Mitigation Strategies – A Narrative Review. *Clin. Interv. Aging* **20**, 459–469 (2025).
21. Ranasinghe, K. G. *et al.* Neurophysiological signatures of ageing: compensatory and compromised neural mechanisms. *Brain Commun.* **7**, fcaf131 (2025).
22. Dickstein, D. L., Weaver, C. M., Luebke, J. I. & Hof, P. R. Dendritic spine changes associated with normal aging. *Neuroscience* **251**, 21–32 (2013).
23. Navakkode, S. & Kennedy, B. K. Neural ageing and synaptic plasticity: prioritizing brain health in healthy longevity. *Front. Aging Neurosci.* **16**, (2024).
24. Liu, P., Smith, P. F. & Darlington, C. L. Glutamate receptor subunits expression in memory-associated brain structures: Regional variations and effects of aging. *Synapse* **62**, 834–841 (2008).
25. Doehner, J. & Knuesel, I. Reelin-mediated Signaling during Normal and Pathological Forms of Aging. *Aging Dis.* **1**, 12–29 (2010).
26. López-Otín, C., Blasco, M. A., Partridge, L., Serrano, M. & Kroemer, G. The Hallmarks of Aging. *Cell* **153**, 1194–1217 (2013).
27. Na, D. *et al.* Energy Metabolism and Brain Aging: Strategies to Delay Neuronal Degeneration. *Cell. Mol. Neurobiol.* **45**, 38 (2025).
28. Kakizawa, S. Involvement of ROS signal in aging and regulation of brain functions. *J. Physiol. Sci. JPS* **75**, 100003 (2024).
29. Deery, H. A., Di Paolo, R., Moran, C., Egan, G. F. & Jamadar, S. D. Lower brain glucose metabolism in normal ageing is predominantly frontal and temporal: A systematic review and pooled effect size and activation likelihood estimates meta-analyses. *Hum. Brain Mapp.* **44**, 1251–1277 (2022).

30. Padamsey, Z. & Rochefort, N. L. Paying the brain's energy bill. *Curr. Opin. Neurobiol.* **78**, 102668 (2023).
31. Mergenthaler, P., Lindauer, U., Dienel, G. A. & Meisel, A. Sugar for the brain: the role of glucose in physiological and pathological brain function. *Trends Neurosci.* **36**, 587–597 (2013).
32. Garry, P. S., Ezra, M., Rowland, M. J., Westbrook, J. & Pattinson, K. T. S. The role of the nitric oxide pathway in brain injury and its treatment — From bench to bedside. *Exp. Neurol.* **263**, 235–243 (2015).
33. Zhang, X. *et al.* The neurovascular coupling response of the aged brain is brain-state dependent. 2024.07.09.602636 Preprint at <https://doi.org/10.1101/2024.07.09.602636> (2024).
34. Bennett, H. C. *et al.* Aging drives cerebrovascular network remodeling and functional changes in the mouse brain. *Nat. Commun.* **15**, 6398 (2024).
35. Kang, H.-M., Sohn, I., Jung, J., Jeong, J.-W. & Park, C. Age-related changes in pial arterial structure and blood flow in mice. *Neurobiol. Aging* **37**, 161–170 (2016).
36. Ungvari, Z. *et al.* Endothelial dysfunction and angiogenesis impairment in the ageing vasculature. *Nat. Rev. Cardiol.* **15**, 555–565 (2018).
37. Sadanandan, J., Sathyanesan, M. & Newton, S. S. Aging alters the expression of trophic factors and tight junction proteins in the mouse choroid plexus. *Fluids Barriers CNS* **21**, 77 (2024).
38. Sá-Pereira, I., Brites, D. & Brito, M. A. Neurovascular Unit: a Focus on Pericytes. *Mol. Neurobiol.* **45**, 327–347 (2012).

39. Hariharan, A., Robertson, C. D., Garcia, D. C. G. & Longden, T. A. Brain capillary pericytes are metabolic sentinels that control blood flow through a KATP channel-dependent energy switch. *Cell Rep.* **41**, (2022).
40. Berthiaume, A.-A. *et al.* Dynamic Remodeling of Pericytes In Vivo Maintains Capillary Coverage in the Adult Mouse Brain. *Cell Rep.* **22**, 8–16 (2018).
41. Berthiaume, A.-A. *et al.* Pericyte remodeling is deficient in the aged brain and contributes to impaired capillary flow and structure. *Nat. Commun.* **13**, 5912 (2022).
42. Sparkman, N. L. & Johnson, R. W. Neuroinflammation Associated with Aging Sensitizes the Brain to the Effects of Infection or Stress. *Neuroimmunomodulation* **15**, 323–330 (2008).
43. Ruiz-Rodríguez, V. M., Cuellar-Santoyo, A. O. & Estrada-Sánchez, A. M. Advances in how the peripheral immune system interacts with the brain in health and disease. *Neuroscience* **596**, 24–35 (2026).
44. Colonna, M. & Butovsky, O. Microglia Function in the Central Nervous System During Health and Neurodegeneration. *Annu. Rev. Immunol.* **35**, 441–468 (2017).
45. Damani, M. R. *et al.* Age-related Alterations in the Dynamic Behavior of Microglia. *Aging Cell* **10**, 263–276 (2011).
46. Škandík, M. *et al.* Age-associated microglial transcriptome leads to diminished immunogenicity and dysregulation of MCT4 and P2RY12/P2RY13 related functions. *Cell Death Discov.* **11**, 16 (2025).
47. Long, J. M. *et al.* Stereological analysis of astrocyte and microglia in aging mouse hippocampus. *Neurobiol. Aging* **19**, 497–503 (1998).

48. Zöller, T., Attaai, A., Potru, P. S., Ruß, T. & Spittau, B. Aged Mouse Cortical Microglia Display an Activation Profile Suggesting Immunotolerogenic Functions. *Int. J. Mol. Sci.* **19**, 706 (2018).
49. Clarke, L. E. *et al.* Normal aging induces A1-like astrocyte reactivity. *Proc. Natl. Acad. Sci.* **115**, E1896–E1905 (2018).
50. Elahy, M. *et al.* Blood–brain barrier dysfunction developed during normal aging is associated with inflammation and loss of tight junctions but not with leukocyte recruitment. *Immun. Ageing* **12**, 2 (2015).
51. Hayakawa, N., Kato, H. & Araki, T. Age-related changes of astorocytes, oligodendrocytes and microglia in the mouse hippocampal CA1 sector. *Mech. Ageing Dev.* **128**, 311–316 (2007).
52. Díaz-Castro, B., Robel, S. & Mishra, A. Astrocyte Endfeet in Brain Function and Pathology: Open Questions. *Annu. Rev. Neurosci.* **46**, 101–121 (2023).
53. Bravo-Ferrer, I. *et al.* Multiregional blood-brain barrier phenotyping identifies the prefrontal cortex as the most vulnerable region to ageing in mice. 2025.03.09.642233 Preprint at <https://doi.org/10.1101/2025.03.09.642233> (2025).
54. Zeppenfeld, D. M. *et al.* Association of Perivascular Localization of Aquaporin-4 With Cognition and Alzheimer Disease in Aging Brains. *JAMA Neurol.* **74**, 91–99 (2017).
55. Stamatovic, S. M., Johnson, A. M., Keep, R. F. & Andjelkovic, A. V. Junctional proteins of the blood-brain barrier: New insights into function and dysfunction. *Tissue Barriers* **4**, e1154641 (2016).

56. Chandra, P. K. *et al.* Effects of aging on protein expression in mice brain microvessels: ROS scavengers, mRNA/protein stability, glycolytic enzymes, mitochondrial complexes, and basement membrane components. *GeroScience* **44**, 371–388 (2021).
57. Verheggen, I. C. M. *et al.* Increase in blood–brain barrier leakage in healthy, older adults. *GeroScience* **42**, 1183–1193 (2020).
58. Cummins, M. J., Cresswell, E. T., Bevege, R. J. & Smith, D. W. Aging disrupts blood–brain and blood-spinal cord barrier homeostasis, but does not increase paracellular permeability. *GeroScience* <https://doi.org/10.1007/s11357-024-01404-9> (2024) doi:10.1007/s11357-024-01404-9.
59. Vacca, M. *et al.* Definitions of “Mental Disorder” from DSM-III to DSM-5. *Behav. Sci.* **15**, 830 (2025).
60. Wertz, J. *et al.* Association of History of Psychopathology With Accelerated Aging at Midlife. *JAMA Psychiatry* **78**, 530–539 (2021).
61. Mason, D. *et al.* Autistic traits are associated with faster pace of aging: Evidence from the Dunedin Study at age 45. *Autism Res. Off. J. Int. Soc. Autism Res.* **14**, 1684–1694 (2021).
62. Sacchet, M. D., Camacho, M. C., Livermore, E. E., Thomas, E. A. C. & Gotlib, I. H. Accelerated aging of the putamen in patients with major depressive disorder. *J. Psychiatry Neurosci.* **42**, 164–171 (2017).
63. Cai, L. & Huang, J. Schizophrenia and risk of dementia: a meta-analysis study. *Neuropsychiatr. Dis. Treat.* **14**, 2047–2055 (2018).
64. Gimson, A., Schlosser, M., Huntley, J. D. & Marchant, N. L. Support for midlife anxiety diagnosis as an independent risk factor for dementia: a systematic review. *BMJ Open* **8**, e019399 (2018).

65. Ayora, M. *et al.* Leukocyte telomere length in patients with schizophrenia and related disorders: a meta-analysis of case-control studies. *Mol. Psychiatry* **27**, 2968–2975 (2022).
66. Smeland, O. B. *et al.* Genome-wide Association Analysis of Parkinson’s Disease and Schizophrenia Reveals Shared Genetic Architecture and Identifies Novel Risk Loci. *Biol. Psychiatry* **89**, 227–235 (2021).
67. Ouellette, J. & Lacoste, B. From Neurodevelopmental to Neurodegenerative Disorders: The Vascular Continuum. *Front. Aging Neurosci.* **13**, 749026 (2021).
68. Stolp, H. B. & Solito, E. Developmental priming of early cerebrovascular ageing: Implications across a lifetime. *Int. J. Geriatr. Psychiatry* **39**, e6090 (2024).
69. Chen, J. J., Rosas, H. D. & Salat, D. H. Age-Associated Reductions in Cerebral Blood Flow Are Independent from Regional Atrophy. *NeuroImage* **55**, 468–478 (2011).
70. Ge, Y. *et al.* Dynamic Susceptibility Contrast Perfusion MR Imaging of Multiple Sclerosis Lesions: Characterizing Hemodynamic Impairment and Inflammatory Activity. *Am. J. Neuroradiol.* **26**, 1539–1547 (2005).
71. Kamagata, K. *et al.* Posterior hypoperfusion in parkinson’s disease With and without dementia measured with arterial spin labeling MRI. *J. Magn. Reson. Imaging* **33**, 803–807 (2011).
72. Ohnishi, T. *et al.* Abnormal regional cerebral blood flow in childhood autism. *Brain* **123**, 1838–1844 (2000).
73. Fiorentino, M. *et al.* Blood–brain barrier and intestinal epithelial barrier alterations in autism spectrum disorders. *Mol. Autism* **7**, 49 (2016).
74. Kook, S.-Y. *et al.* A β 1–42-RAGE Interaction Disrupts Tight Junctions of the Blood–Brain Barrier Via Ca²⁺-Calcineurin Signaling. *J. Neurosci.* **32**, 8845–8854 (2012).

75. Nishiura, K. *et al.* PKA activation and endothelial claudin-5 breakdown in the schizophrenic prefrontal cortex. *Oncotarget* **8**, 93382–93391 (2017).
76. Crockett, A. M. *et al.* Disruption of the blood–brain barrier in 22q11.2 deletion syndrome. *Brain* **144**, 1351–1360 (2021).
77. Shao, X. *et al.* Age-Related Decline in Blood-Brain Barrier Function is More Pronounced in Males than Females in Parietal and Temporal Regions. *eLife* **13**, (2024).
78. Tai, L. M., Holloway, K. A., Male, D. K., Loughlin, A. J. & Romero, I. A. Amyloid- β -induced occludin down-regulation and increased permeability in human brain endothelial cells is mediated by MAPK activation. *J. Cell. Mol. Med.* **14**, 1101–1112 (2010).
79. Canada, P. H. A. of. Autism Spectrum Disorder among Children and Youth in Canada 2018. <https://www.canada.ca/en/public-health/services/publications/diseases-conditions/autism-spectrum-disorder-children-youth-canada-2018.html> (2018).
80. Autism Spectrum Disorder: Highlights from the 2019 Canadian Health Survey on Children and Youth. *Health Promot. Chronic Dis. Prev. Can. Res. Policy Pract.* **42**, 122 (2022).
81. CDC. Clinical Testing and Diagnosis for Autism Spectrum Disorder. *Autism Spectrum Disorder (ASD)* <https://www.cdc.gov/autism/hcp/diagnosis/index.html> (2025).
82. Baron-Cohen, S. The extreme male brain theory of autism. (2002).
83. Leow, K. Q., Tonta, M. A., Lu, J., Coleman, H. A. & Parkinson, H. C. Towards understanding sex differences in autism spectrum disorders. *Brain Res.* **1833**, 148877 (2024).
84. Hull, L., Petrides, K. V. & Mandy, W. The Female Autism Phenotype and Camouflaging: a Narrative Review. *Rev. J. Autism Dev. Disord.* **7**, 306–317 (2020).

85. Andersen, R. E. *et al.* Transcriptomic Convergence and the Female Protective Effect in Autism. 2025.01.20.634000 Preprint at <https://doi.org/10.1101/2025.01.20.634000> (2025).
86. Alinejad-Rokny, H., Heng, J. I. T. & Forrest, A. R. R. Brain-Enriched Coding and Long Non-coding RNA Genes Are Overrepresented in Recurrent Neurodevelopmental Disorder CNVs. *Cell Rep.* **33**, 108307 (2020).
87. Rylaarsdam, L. & Guemez-Gamboa, A. Genetic Causes and Modifiers of Autism Spectrum Disorder. *Front. Cell. Neurosci.* **13**, 385 (2019).
88. Sebat, J., Muotri, A. R. & Iakoucheva, L. M. Getting to the cores of autism. *Cell* **178**, 1287–1298 (2019).
89. Hirvikoski, T. *et al.* Premature mortality in autism spectrum disorder. *Br. J. Psychiatry* **208**, 232–238 (2016).
90. O’Nions, E. *et al.* Estimating life expectancy and years of life lost for autistic people in the UK: a matched cohort study. *Lancet Reg. Health - Eur.* **36**, 100776 (2024).
91. Griesi-Oliveira, K. & Sertié, A. L. Autism spectrum disorders: an updated guide for genetic counseling. *Einstein São Paulo* **15**, 233–238 (2017).
92. Tamada, K. & Takumi, T. Neurodevelopmental impact of CNV models in ASD: Recent advances and future directions. *Curr. Opin. Neurobiol.* **92**, 103001 (2025).
93. Auwerx, C., Kutalik, Z. & Reymond, A. The pleiotropic spectrum of proximal 16p11.2 CNVs. *Am. J. Hum. Genet.* **0**, (2024).
94. Chung, W. K., Roberts, T. P., Sherr, E. H., Snyder, L. G. & Spiro, J. E. 16p11.2 deletion syndrome. *Curr. Opin. Genet. Dev.* **68**, 49–56 (2021).

95. Ouellette, J., Crouch, E. E., Morel, J.-L., Coelho-Santos, V. & Lacoste, B. A Vascular-Centric Approach to Autism Spectrum Disorders. *Neurosci. Insights* **19**, 26331055241235921 (2024).
96. Hanson, E. *et al.* Cognitive and Behavioral Characterization of 16p11.2 Deletion Syndrome. *J. Dev. Behav. Pediatr.* **31**, 649 (2010).
97. Kim, S. H. *et al.* Language characterization in 16p11.2 deletion and duplication syndromes. *Am. J. Med. Genet. B Neuropsychiatr. Genet.* **183**, 380–391 (2020).
98. Soliman, P., Goin-Kochel, R. P. & Alexander, G. Motor Impairment in Children with 16p11.2-Deletion and -Duplication Syndromes. *J. Autism Dev. Disord.* <https://doi.org/10.1007/s10803-025-06874-0> (2025) doi:10.1007/s10803-025-06874-0.
99. Hanson, E. *et al.* The cognitive and behavioral phenotype of the 16p11.2 deletion in a clinically ascertained population. *Biol. Psychiatry* **77**, 785–793 (2015).
100. Kamara, D., De Boeck, P., Lecavalier, L., Neuhaus, E. & Beauchaine, T. P. Characterizing Sleep Problems in 16p11.2 Deletion and Duplication. *J. Autism Dev. Disord.* **53**, 1462–1475 (2023).
101. Moufawad El Achkar, C. *et al.* Clinical Characteristics of Seizures and Epilepsy in Individuals With Recurrent Deletions and Duplications in the 16p11.2 Region. *Neurol. Genet.* **8**, e200018 (2022).
102. Shinawi, M. *et al.* Recurrent reciprocal 16p11.2 rearrangements associated with global developmental delay, behavioural problems, dysmorphism, epilepsy, and abnormal head size. *J. Med. Genet.* **47**, 332–341 (2010).
103. Owen, J. P. *et al.* Aberrant White Matter Microstructure in Children with 16p11.2 Deletions. *J. Neurosci.* **34**, 6214–6223 (2014).

104. Taylor, C. M. *et al.* 16p11.2 Recurrent Deletion. in *GeneReviews*® (eds Adam, M. P. et al.) (University of Washington, Seattle, Seattle (WA), 1993).
105. Ouellette, J. *et al.* Vascular contributions to 16p11.2 deletion autism syndrome modeled in mice. *Nat. Neurosci.* **23**, 1090–1101 (2020).
106. Béland-Millar, A. *et al.* 16p11.2 haploinsufficiency reduces mitochondrial biogenesis in brain endothelial cells and alters brain metabolism in adult mice. *Cell Rep.* **42**, 112485 (2023).
107. Blakeley, N. *et al.* Metabolic, epigenetic and transcriptomic alterations in postnatal 16p11.2-deficient murine astrocytes. 2025.12.16.694710 Preprint at <https://doi.org/10.64898/2025.12.16.694710> (2025).
108. Sandhir, R., Onyszchuk, G. & Berman, N. E. J. Exacerbated Glial Response in the Aged Mouse Hippocampus Following Controlled Cortical Impact Injury. *Exp. Neurol.* **213**, 372–380 (2008).
109. Kress, B. T. *et al.* Impairment of paravascular clearance pathways in the aging brain. *Ann. Neurol.* **76**, 845–861 (2014).
110. Shao, X. *et al.* Age-Related Decline in Blood-Brain Barrier Function is More Pronounced in Males than Females in Parietal and Temporal Regions. *bioRxiv* 2024.01.12.575463 (2024) doi:10.1101/2024.01.12.575463.
111. Pop, V. *et al.* Early brain injury alters the blood–brain barrier phenotype in parallel with β -amyloid and cognitive changes in adulthood. *J. Cereb. Blood Flow Metab.* **33**, 205–214 (2013).
112. Moca, E. N. *et al.* Microglia Drive Pockets of Neuroinflammation in Middle Age. *J. Neurosci.* **42**, 3896–3918 (2022).

113. Zöller, T., Attaai, A., Potru, P. S., Ruß, T. & Spittau, B. Aged Mouse Cortical Microglia Display an Activation Profile Suggesting Immunotolerogenic Functions. *Int. J. Mol. Sci.* **19**, 706 (2018).
114. Davies, D. S., Ma, J., Jegathees, T. & Goldsbury, C. Microglia show altered morphology and reduced arborization in human brain during aging and Alzheimer's disease. *Brain Pathol.* **27**, 795–808 (2016).
115. Tieu, T., Cruz, A.-J. N., Weinstein, J. R., Shih, A. Y. & Coelho-Santos, V. Physiological and injury-induced microglial dynamics across the lifespan. *Cell Rep.* **44**, 115991 (2025).
116. Flanary, B. E., Sammons, N. W., Nguyen, C., Walker, D. & Streit, W. J. Evidence That Aging And Amyloid Promote Microglial Cell Senescence. *Rejuvenation Res.* **10**, 61–74 (2007).
117. Matsudaira, T. *et al.* Cellular senescence in white matter microglia is induced during ageing in mice and exacerbates the neuroinflammatory phenotype. *Commun. Biol.* **6**, 665 (2023).
118. Morgan, J. T. *et al.* Microglial Activation and Increased Microglial Density Observed in the Dorsolateral Prefrontal Cortex in Autism. *Biol. Psychiatry* **68**, 368–376 (2010).
119. Yu, C., Zhang, X.-P. & Wang, W. Dysfunction of microglia-mediated synaptic pruning in Autism spectrum disorder. 2025.07.20.665801 Preprint at <https://doi.org/10.1101/2025.07.20.665801> (2025).
120. Bondi, H., Bortolotto, V., Canonico, P. L. & Grilli, M. Complex and regional-specific changes in the morphological complexity of GFAP⁺ astrocytes in middle-aged mice. *Neurobiol. Aging* **100**, 59–71 (2021).

121. Hayakawa, N., Kato, H. & Araki, T. Age-related changes of astrocytes, oligodendrocytes and microglia in the mouse hippocampal CA1 sector. *Mech. Ageing Dev.* **128**, 311–316 (2007).
122. Wu, Y., Zhang, A.-Q. & Yew, D. T. Age related changes of various markers of astrocytes in senescence-accelerated mice hippocampus. *Neurochem. Int.* **46**, 565–574 (2005).
123. Tanaka, A., Watanabe, Y., Kato, H. & Araki, T. Immunohistochemical changes related to ageing in the mouse hippocampus and subventricular zone. *Mech. Ageing Dev.* **128**, 303–310 (2007).
124. Kohama, S. G., Goss, J. R., Finch, C. E. & McNeill, T. H. Increases of glial fibrillary acidic protein in the aging female mouse brain. *Neurobiol. Aging* **16**, 59–67 (1995).
125. Choi, J. *et al.* Pathophysiological and neurobehavioral characteristics of a propionic acid-mediated autism-like rat model. *PLOS ONE* **13**, e0192925 (2018).
126. Codagnone, M. G., Podestá, M. F., Uccelli, N. A. & Reínés, A. Differential Local Connectivity and Neuroinflammation Profiles in the Medial Prefrontal Cortex and Hippocampus in the Valproic Acid Rat Model of Autism. *Dev. Neurosci.* **37**, 215–231 (2015).
127. Rexrode, L. E. *et al.* Molecular profiling of the hippocampus of children with autism spectrum disorder. *Mol. Psychiatry* **29**, 1968–1979 (2024).
128. Vargas, D. L., Nascimbene, C., Krishnan, C., Zimmerman, A. W. & Pardo, C. A. Neuroglial activation and neuroinflammation in the brain of patients with autism. *Ann. Neurol.* **57**, 67–81 (2005).
129. Zeppenfeld, D. M. *et al.* Association of Perivascular Localization of Aquaporin-4 With Cognition and Alzheimer Disease in Aging Brains. *JAMA Neurol.* **74**, 91–99 (2017).

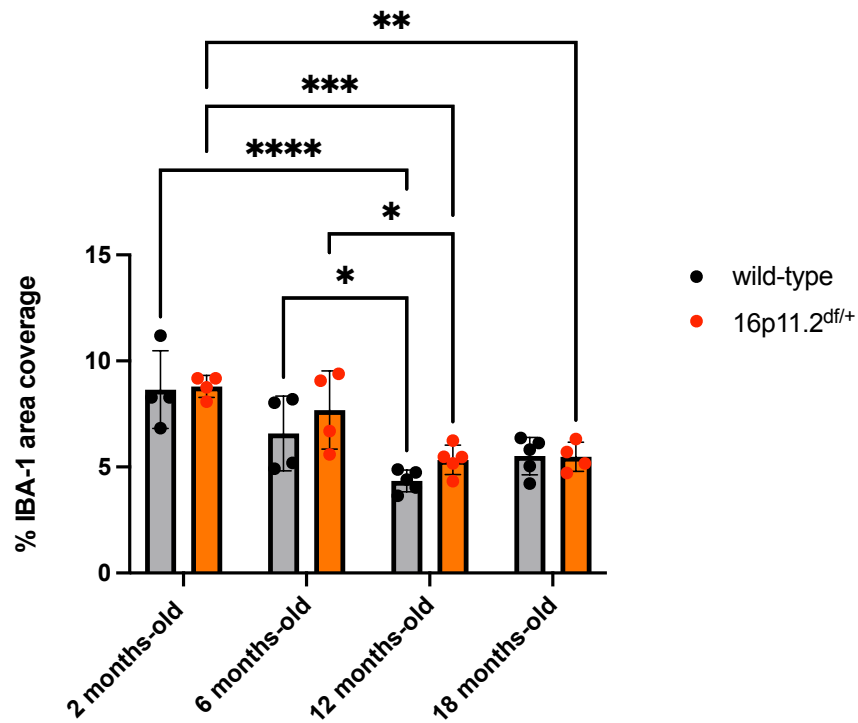
130. Li, X. *et al.* Children with autism spectrum disorder present glymphatic system dysfunction evidenced by diffusion tensor imaging along the perivascular space. *Medicine (Baltimore)* **101**, e32061 (2022).
131. Angelakos, C. C. *et al.* Hyperactivity and Male-Specific Sleep Deficits in the 16p11.2 Deletion Mouse Model of Autism. *Autism Res. Off. J. Int. Soc. Autism Res.* **10**, 572–584 (2017).
132. Bravo-Ferrer, I. *et al.* Multiregional blood-brain barrier phenotyping identifies the prefrontal cortex as the most vulnerable region to ageing in mice. *Brain Commun.* **7**, fcac332 (2025).
133. Popov, A. *et al.* Astrocyte dystrophy in ageing brain parallels impaired synaptic plasticity. *Aging Cell* **20**, e13334 (2021).
134. Ito, U., Hakamata, Y., Kawakami, E. & Oyanagi, K. Temporary focal cerebral ischemia results in swollen astrocytic end-feet that compress microvessels and lead to focal cortical infarction. *J. Cereb. Blood Flow Metab.* **31**, 328–338 (2011).
135. Bors, L. *et al.* Age-dependent changes at the blood-brain barrier. A Comparative structural and functional study in young adult and middle aged rats. *Brain Res. Bull.* **139**, 269–277 (2018).
136. Xiang, J. *et al.* Mechanisms Underlying Astrocyte Endfeet Swelling in Stroke. in *Brain Edema XVI* (eds Applegate, R. L., Chen, G., Feng, H. & Zhang, J. H.) vol. 121 19–22 (Springer International Publishing, Cham, 2016).
137. Bender, A. S., Schousboe, A., Reichelt, W. & Norenberg, M. D. Ionic mechanisms in glutamate-induced astrocyte swelling: Role of K⁺ influx. *J. Neurosci. Res.* **52**, 307–321 (1998).

138. Blakeley, N. *et al.* Metabolic, epigenetic and transcriptomic alterations in postnatal 16p11.2-deficient murine astrocytes. 2025.12.16.694710 Preprint at <https://doi.org/10.64898/2025.12.16.694710> (2025).
139. Popov, A. *et al.* Mitochondrial malfunction and atrophy of astrocytes in the aged human cerebral cortex. *Nat. Commun.* **14**, 8380 (2023).
140. Béland-Millar, A. *et al.* 16p11.2 haploinsufficiency reduces mitochondrial biogenesis in brain endothelial cells and alters brain metabolism in adult mice. *Cell Rep.* **42**, (2023).
141. Blakeley, N. *et al.* Metabolic, epigenetic and transcriptomic alterations in postnatal 16p11.2-deficient murine astrocytes. 2025.12.16.694710 Preprint at <https://doi.org/10.64898/2025.12.16.694710> (2025).
142. Can, B. & Alataş, İ. Ö. The blood-brain barrier: a focus on neurovascular unit components. *Eur. J. Life Sci.* **3**, 127–135 (2024).
143. Ceafalan, L. C. *et al.* Age-related ultrastructural changes of the basement membrane in the mouse blood-brain barrier. *J. Cell. Mol. Med.* **23**, 819–827 (2019).
144. Hawkes, C. A. *et al.* Perivascular drainage of solutes is impaired in the ageing mouse brain and in the presence of cerebral amyloid angiopathy. *Acta Neuropathol. (Berl.)* **121**, 431–443 (2011).
145. Hawkes, C. A. *et al.* Regional differences in the morphological and functional effects of aging on cerebral basement membranes and perivascular drainage of amyloid- β from the mouse brain. *Aging Cell* **12**, 224–236 (2013).
146. Ioannidis, V. *et al.* Disrupted extracellular matrix and cell cycle genes in autism-associated Shank3 deficiency are targeted by lithium. *Mol. Psychiatry* **29**, 704–717 (2024).

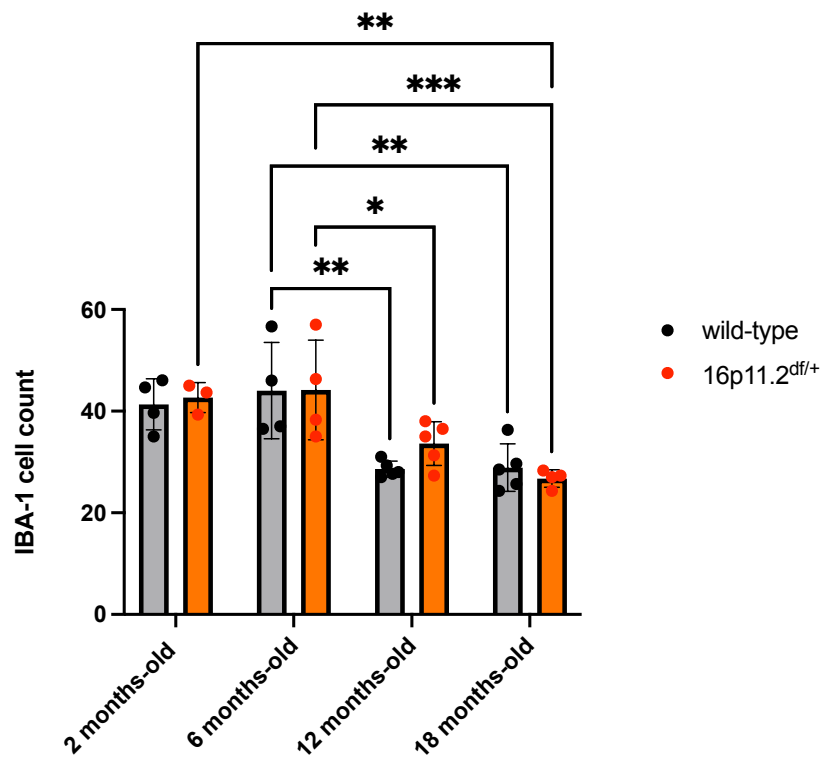
147. Loohuis, N. F. O. *et al.* Altered Expression of Circadian Rhythm and Extracellular Matrix Genes in the Medial Prefrontal Cortex of a Valproic Acid Rat Model of Autism. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **77**, 128–132 (2017).
148. Ximerakis, M. *et al.* Single-cell transcriptomic profiling of the aging mouse brain. *Nat. Neurosci.* **22**, 1696–1708 (2019).
149. Zhao, L. *et al.* Pharmacologically reversible zonation-dependent endothelial cell transcriptomic changes with neurodegenerative disease associations in the aged brain. *Nat. Commun.* **11**, 4413 (2020).
150. Örü̇m, D., Çatak, Z., Tanrı̇kulu, A. B. & Atmaca, M. Claudin-5 and inflammatory biomarkers in panic disorder: a 6-week follow-up study. *Front. Psychiatry* **16**, (2025).
151. Aydoğ̇an Avş̇ar, P. *et al.* Serum zonulin and claudin-5 levels in children with attention-deficit/hyperactivity disorder. *Int. J. Psychiatry Clin. Pract.* **25**, 49–55 (2021).

7. Supplementary Figures

A

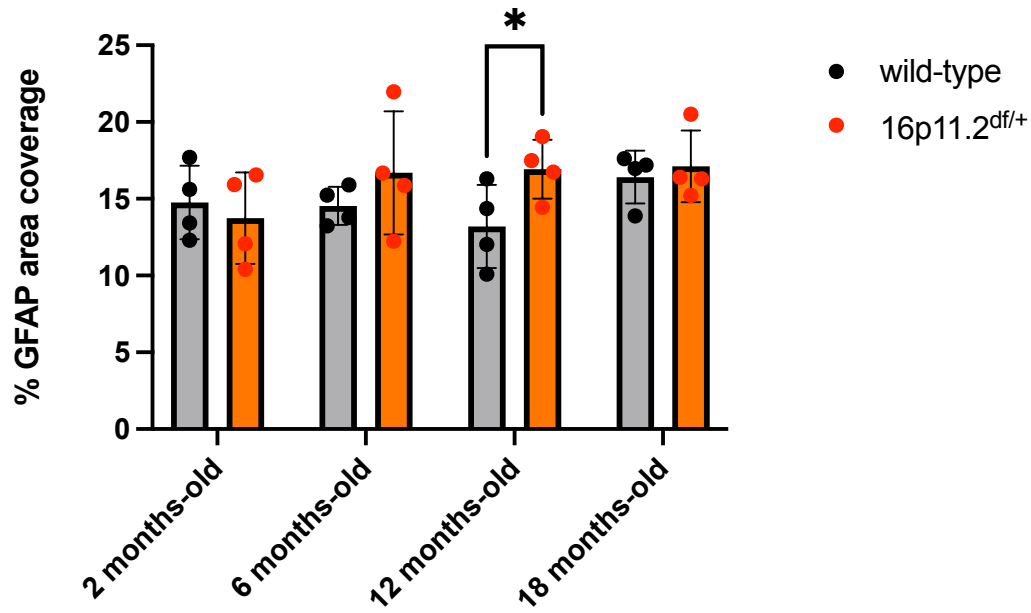


B



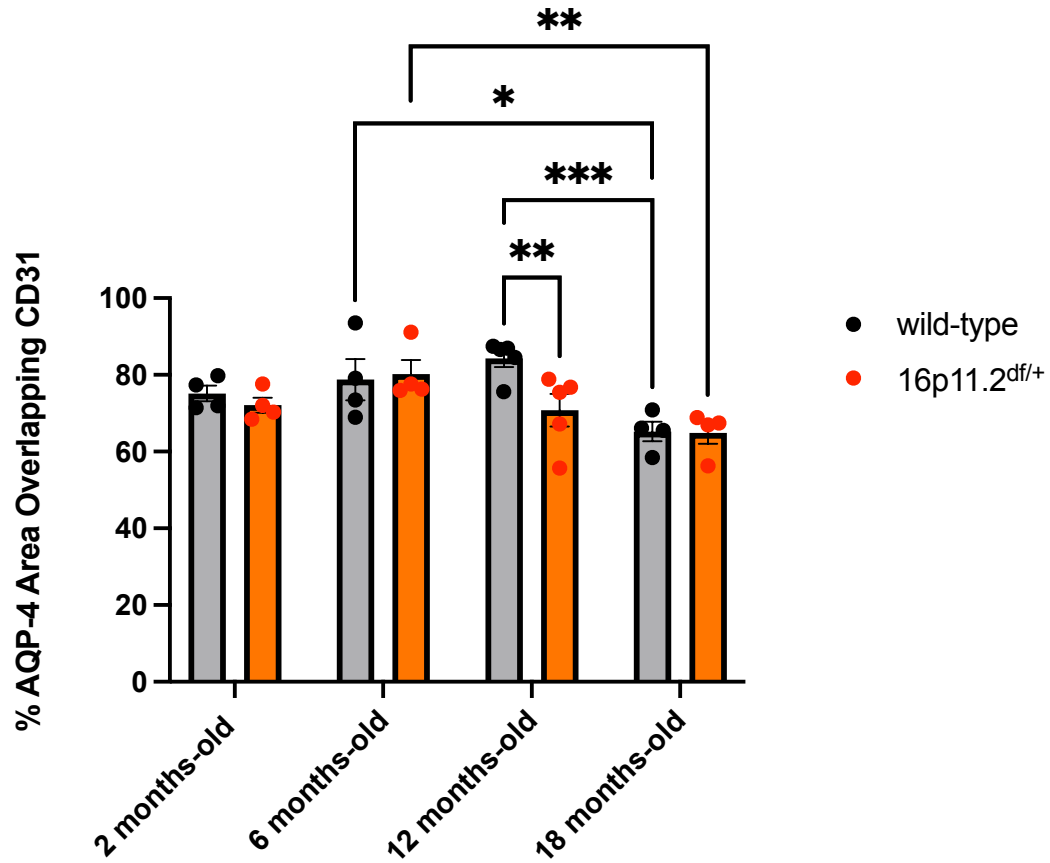
Supplementary Figure 1: **Bar graph representation of microglia levels in the parietal cortex of 16p11.2^{df/+}.** A) Quantification of IBA-1 percentage area coverage in 2-, 6-, 12-, and 18-months-old/wild-type & 16p11.2^{df/+} mice in the parietal cortex, and B) microglial cell number. 3-5 images per sample. All data are shown as the mean $\pm$ SEM. (n=4-5 animals per group). *P<0.05, **P<0.01, ***P<0.001 (two-way repeated measure ANOVA and Tukey's post-hoc test).

A



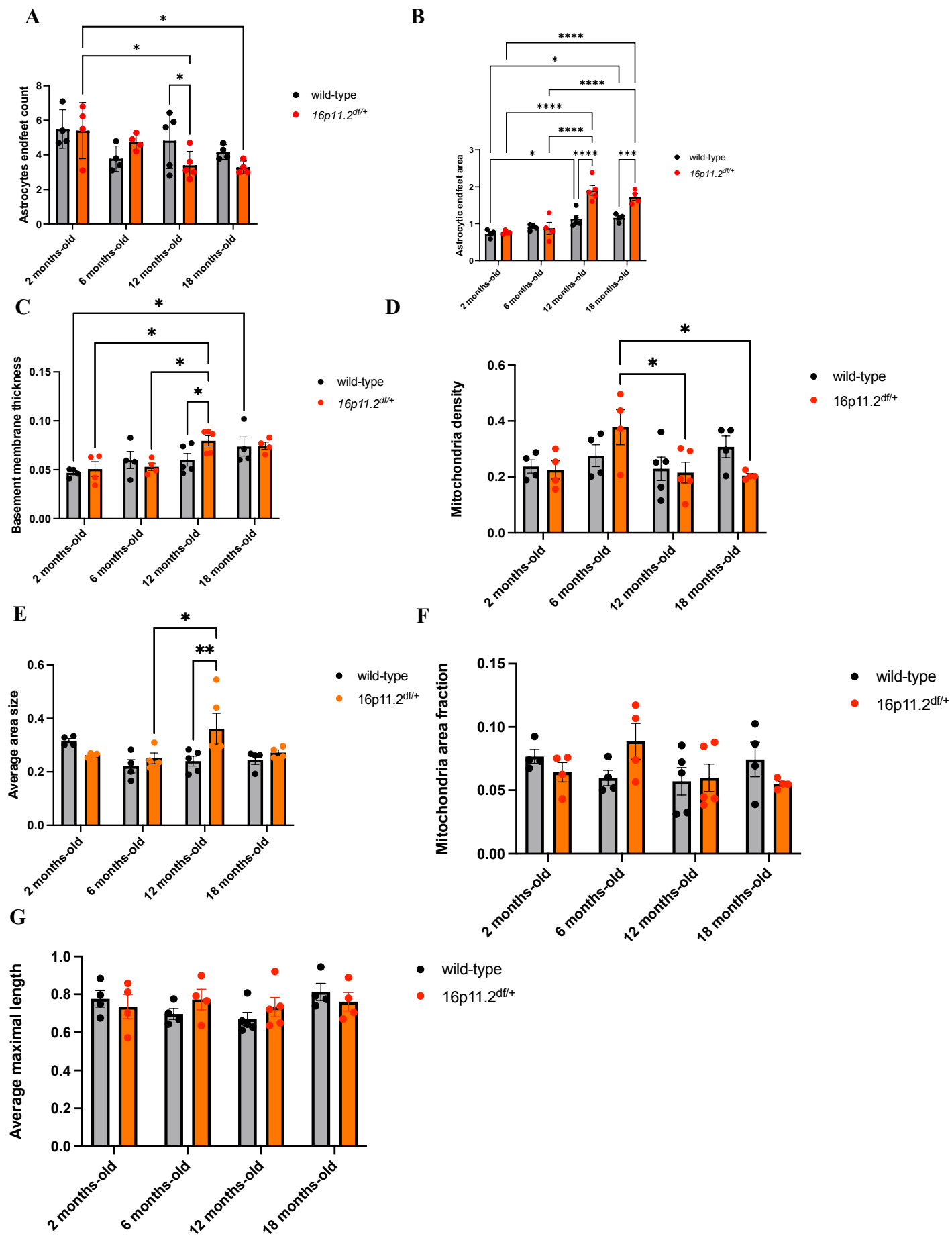
Supplementary Figure 2: **Bar graph representations of astrogliosis.** Reactive astrocytes coverage in the hippocampal region CA1 of 16p11.2^{df/+} mice. A) Quantification of GFAP⁺ percentage area coverage) in 2-, 6-, 12-, and 18-months-old/wild-type & 16p11.2^{df/+} mice. 3-5 images per sample. All data are shown as the mean ± SEM. (n=4-5 animals per group). *P<0.05, **P<0.01, ***P<0.001 (two-way repeated measure ANOVA and Tukey's post-hoc test).

A



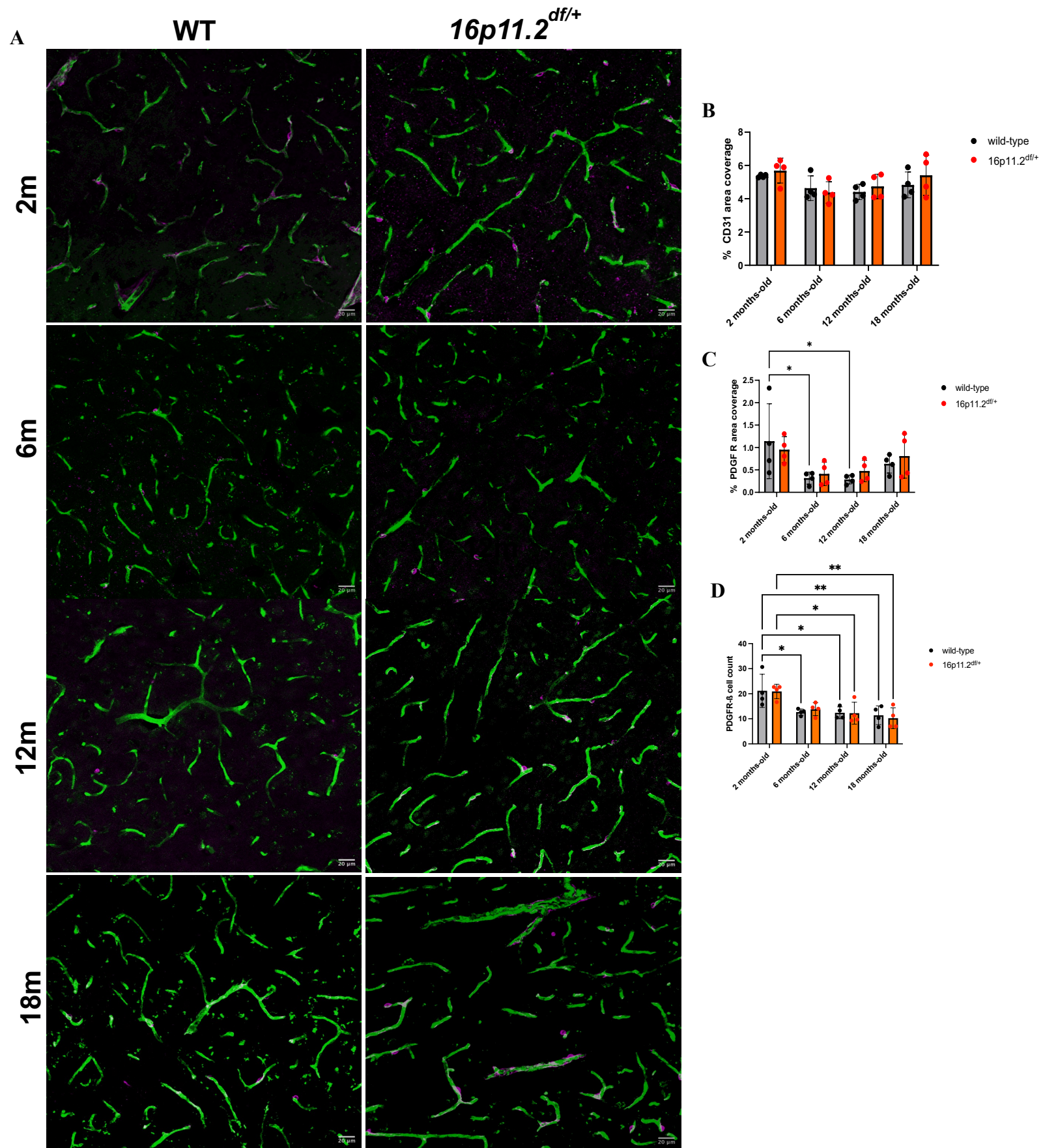
Supplementary Figure 3: **Bar graph Astrocytic endfeet-vessel localization.**

A) Quantification of AQP-4-CD31 co-localization in 2-, 6-, 12-, and 18-months-old/wild-type & 16p11.2^{df/+} mice in the parietal cortex. Images were acquired at 20x magnification. 3-5 images per sample. All data are shown as the mean $\pm$ SEM. (n=4-5 animals per group). *P<0.05, **P<0.01, ***P<0.001 (two-way repeated measure ANOVA and Tukey's post-hoc test).



Supplementary Figure 4: **Bar graph representation of NVU characteristics.**

(A-G) Quantifications of multiple NVU features in parietal cortex capillaries of 2-, 6-, 12-, and 18-months-old/wild-type & /16p11.2^{df/+} mice vessels: A) Astrocytes endfeet count, B) astrocytes endfeet area, C) Basement membrane thickness, D) astrocytic endfeet mitochondrial density E) astrocytic endfeet mitochondrial average area size, F) astrocytic endfeet mitochondrial area fraction, G) astrocytic mitochondrial average maximal length. Quantification was performed using 10 vessel images per mouse. All images scale bar (2 μ m). Blue highlight; astrocytic endfeet. All data are shown as the mean $\pm$ SEM. (n=4-5 animals per group). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ (two-way repeated measure ANOVA and Tukey's post-hoc test).



Supplementary Figure 5: Cortical CD31 and PDGFR β levels.

*A) Immunofluorescence staining of CD31 (green) and PDGFR β (magenta) in 2-, 6-, 12-, and 18-months-old/wild-type & 16p11.2^{df/+} mice in the parietal cortex. Quantification of B) CD31 percentage area coverage, and C) PDGFR β percentage area coverage, D) PDGFR β cell count. n=4 analyzed per group. 3-5 images per sample. Images were acquired at 20x magnification. 3-5 images per sample. All data are shown as the mean $\pm$ SEM. (n=4-5 animals per group). *P<0.05, **P<0.01, ***P<0.001 (two-way repeated measure ANOVA and Tukey's post-hoc test).*