MICROGLIA IN THE CEREBRAL AND CEREBELLAR CORTICES IN INDIVIDUALS WITH AUTISM

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Nicole A. Tetreault

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ABSTRACT

In this thesis, we explore the density of the microglia in the cerebral and cerebellar cortices of individuals with autism to investigate the hypothesis that neuroinflammation is involved in autism. We describe in our findings an increase in microglial density in two disparate cortical regions, frontal insular cortex and visual cortex, in individuals with autism (Tetreault et al., 2012). Our results imply that there is a global increase in the microglial density and neuroinflammation in the cerebral cortex of individuals with autism.

We expanded our cerebellar study to additional neurodevelopmental disorders that exhibit similar behaviors to autism spectrum disorder and have known cerebellar pathology. We subsequently found a more than threefold increase in the microglial density specific to the molecular layer of the cerebellum, which is the region of the Purkinje and parallel fiber synapses, in individuals with autism and Rett syndrome. Moreover, we report that not only is there an increase in microglia density in the molecular layer, the microglial cell bodies are significantly larger in perimeter and area in individuals with autism spectrum disorder and Rett syndrome compared to controls that implies that the microglia are activated. Additionally, an individual with Angelman syndrome and the sibling of an individual with autism have microglial densities similar to the individuals with autism and Rett syndrome. By contrast, an individual with Joubert syndrome, which is a developmental hypoplasia of the cerebellar vermis, had a normal density of microglia, indicating the specific pathology in the cerebellum does not

necessarily result in increased microglial densities. We found a significant decrease in Purkinje cells specific to the cerebellar vermis in individuals with autism.

These findings indicate the importance for investigation of the Purkinje synapses in autism and that the relationship between the microglia and the synapses is of great utility in understanding the pathology in autism. Together, these data provide further evidence for the neuroinflammation hypothesis in autism and a basis for future investigation of neuroinflammation in autism. In particular, investigating the function of microglia in modifying synaptic connectivity in the cerebellum may provide key insights into developing therapeutics in autism spectrum disorder.

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CHAPTER 1: INTRODUCTION

Autism Spectrum Disorder

Autism spectrum disorder is heterogenous clinically and etiologically where the diagnosis is entirely based on the behavioral phenotype (Miles et al., 2011). It is a neurodevelopmental disorder described by two core features: a lack in social communication and restricted repetitive behaviors, often diagnosed by age three. (American Psychiatric Association, DSM 5, 2013). The diagnosis of the disorder is at present entirely clinical, based on differing aspects of behavior and its developmental time course. This includes a number of specifiers (extensions to a diagnosis that further clarify its course, severity, or special features). Diagnosis in the clinic can include additional disorders such as attention deficit hyperactivity disorder, Rett syndrome, seizures, other disorders and neurological symptoms that are comorbid with autism (American Psychiatric Association, DSM 5, 2013). Since autism spectrum disorder is a heterogeneous disorder, it may involve additional symptoms including seizures, sensory abnormalities such as hypersensitivity, motor deficits, and gastrointestinal alterations (Danielsson et al., 2005; Leekam et al., 2007; Vilensky et al., 1981; D'Eufemia et al., 1996; Horvath et al., 1999; De Magistris et al., 2010). Seizures occur in 38% of individuals with autism, and the frequency of seizures has great impact on the individuals' lives (Danielsson et al., 2005). Sensory abnormalities have been often described in autism spectrum disorder. These include pain insensitivity and hypersensitivity to tactile, auditory, and visual stimuli, which can be debilitating (Bemporad et al., 1979; Grandin & Scariano, 1986; Cesaroni & Garber, 1991). Over 90% of individuals with autism have sensory abnormalities in multiple domains, and that the

sensory disabilities are extensive, multimodal and continuous across age and ability in individuals with autism (Leekam et al., 2007). In addition, individuals with autism experience a number of motor deficits, which may include gait disturbances, various postural instabilities, greater clumsiness, and altered motor coordination (Vilensky et al., 1981; Bauman & Kemper 2005; Molloy et al., 2003). A number of individuals with autism experience gastrointestinal disturbances and 43% of individuals with autism in one sample had altered intestinal permeability compared to neurotypicals, who had none (D'Efufemia et al., 1996). Of these gastrointestinal disturbances, the most severe symptoms include constipation and diarrhea, and constipation is associated with severity of the language and social impairment in individuals with autism (Gorrindo et al., 2012; Chandler et al., 2013). Emerging evidence of increased autoantibodies may indicate an inflammatory state of the intestines and may alter the mucosal barrier and integrity, which may lead to gastrointestinal issues in individuals with autism (Coury et al., 2012).

As of 2012, about 1 in 50 children in the United States have been diagnosed with autism or autism spectrum disorder (up from 11 per 1,000 in 2008) (Blumberg et al., 2013; Rutter et al., 2005). This increase in prevalence has raised tremendous concern among parents, clinicians and the scientific community, leading to the suggestion that environmental factors may contribute to the epidemic of autism (Miles et al., 2011). Currently, the cause of autism remains unknown. Considerable advances in the genetic causes of autism can be attributed to identifying known genetic mutations and disorders that predispose to the development of autism (Miles et al., 2011). Approximately 20-25% of autism spectrum disorder can be identified by a genetic cause, leaving 75-85% as presently unknown causes (Miles et al., 2011). Moreover there are a number of

environmental exposures during fetal development that increase the risk of autism that include maternal infection and immune activation, elevated metabolic conditions such as diabetes and obesity and elevated levels of C reactive protein in the mother's serum (Brown et al., 2004; Patterson et al., 2011; Krakowiak et al, 2012; Brown et al., 2013). It is possible that an interaction of genes and environmental exposures contribute to the increased incidence in autism. Insights from three areas of study are invaluable for understanding the underlying mechanisms of autism: first, examination of autopsy tissue of individuals with autism for pathological and genetic analysis; second, patient studies investigating behavior and genetics in living individuals with autism; and third, investigations using mouse models of autism for both genetic causes of autism and environmental contributions to developing autism. Integrating these research areas provides promise for better diagnostics, behavioral treatments and therapeutics for individuals with autism.

Genetic Studies of Autism

There is now strong evidence that genetic alterations can contribute strongly to the autism phenotype. First, there are data on overall heritability. If one of a pair of monozygotic twins has autism, there is an 88% likelihood that the other will have a form of autism, whereas if the twins are dizygotic, the chances are 31% that the other twin will have autism (Rosenberg et al., 2009). A more recent study by Hallmayer and colleagues (2011) describes a 77% concordance for monozygotic male twins with autism spectrum disorder and a 31% concordance among male dizygotic pairs, as well as a 50% concordance for female monozygotic pairs, and 36% concordance for female dizygotic

pairs, and attributes the variance in liability to shared environmental factors and a moderate genetic heritability (Hallmayer et al., 2011). Second, single gene disorders, referred to as "syndromic autism," present autism-like features; these include fragile X, Rett syndrome, tuberous sclerosis complex, and Timothy syndrome (Miles et al., 2011). The genetic causes of autism account for 20-25% of autism cases based on data from genome-wide association studies of autism, single gene mutations in autism, and copy number variations (CNVs), *i.e.*, chromosomal deletions or duplications (Miles et al., 2011). The most common chromosomal abnormalities in autism are found on the maternally-derived 15q duplications of the Prader Willi/Angelman region, which accounts for 1-3% of cases with autism (Wang et al., 2013). The most commonly implicated gene in this region is UBE3A. Clinically-relevant CNVs were found in eight out of 29 individuals with autism (27.5%) using a 1Mb genome-wide array (Jacquemont et al., 2006). Sebat and colleagues (2007) described *de novo* copy number variations in 10% of children in which they are the sole family member with autism, 2% from autism in multiple family members compared to 1% of neurotypicals, implicating *de novo* germline mutation as a substantial risk factor for autism (Sebat et al., 2007). Genes encoding neuronal cell-adhesion molecules, including NRXN1, NLGN1 ASTN2, CNTN4, were enriched with CNVs in autism, and genes involved in the ubiquitin pathways, including UBE3A, PARK2, RFWD2 and FBXO40, were affected by CNVs not observed in controls using a whole genome CNV study of 550,000 single nucleotide polymorphism markers in 859 individuals with autism and 1,409 neurotypicals (Glessner et al., 2009). In some monozygotic twins who are discordant for autism, the UBE3A gene is methylated in the autistic twin, which suppresses the expression of the gene (Wang et

al., 2013). To date, the SFARI website (https://gene.sfari.org/autdb/) reports 252 annotations for CNVs and 582 genes thought to be associated with autism (Basu et al., 2007) which is 2% of the entire genome. Thus, multi-genetic alterations, as well as genetic and environmental interactions during critical periods of development, may contribute to autism.

Additionally, investigators are studying the genetics in autism by analyzing the transcriptome, the set of all mRNA molecules. The number of transcripts and transcript abundance for each gene is measured in various tissues and cells to determine genes and possible networks of genes that may be dysregulated in autism. Microarray analysis of cerebellar tissue, prefrontal cortex and caudate-putamen reveal an increase in transcripts related to AMPA-type glutamate receptors in individuals with autism compared to neurotypicals (Purcell et al., 2001). Lymphoblastoid cell lines from five male twins discordant for autism based on language impairment revealed an increase in transcripts for the pro-inflammatory cytokines and a decrease in transcripts for those that are involved in brain development, neuronal differentiation and axon guidance in the individuals with autism (Hu et al., 2006).

Lymphoblastoid cell lines genomic profiling in individuals with autism compared to controls reveals a small number of genes expressed predominately in natural killer cell mediated cytotoxicity (Gregg et al., 2008). These findings indicate that a number of genes involved in natural killer cell proliferation are elevated in individuals with autism that can contribute to the autism phenotype. Gene expression profile analysis of superior temporal gyrus, auditory cortex, shows an increased transcript level of many immune system-related genes specifically related to T cell mediated acquired immune mechanisms

individuals with autism compared to controls (Garbett et al., 2008). Additionally, there is a decrease in genes involved in neuronal development and neurite outgrowth in autistic cases compared to neurotypicals (Garbett et al., 2008). The expression patterns appear to be associated with late recovery of an autoimmune disorder, rather than an innate immune response (Garbett et al., 2008). Importantly, this study was limited to a small number of individuals with autism (N=6); thus, replication with a larger sample size would be useful would be useful for testing the auto-immune hypothesis.

In a more recent study using microarray analysis of gene expression in individuals with autism compared to controls, Voineagu et al. (2011) describe a module of elevated immune and microglial genes and deduce that the increased gene expression of the immune and microglia genes is a non-genetic etiology since these genes have not been found in genome-wide association studies, which points to the genes being causally related to autism. This implies that the elevated immune and microglial genes are possibly a result of internal or external environmental influences on inflammation in autism (Voineagu et al., 2011). These data describe alterations in the expression of genes involved in synapse formation and inflammation in the developing brain of individuals with autism. Since only 20-25% of the incidence of autism is accounted for by genetics, and the etiology is largely unknown, environmental factors likely warrant further exploration. In particular, it is not clear how inflammation alters synaptic development and how disrupted synaptic development of neural network formations can influence inflammatory genes. One explanation advanced by Paul Ashwood and colleagues (2006) is that "immune dysregulation could result in the generation of localized or systemic inflammation and/or the release of immunomodulatory molecules that could influence,

alter, or modify neurodevelopment and/or neuronal function, especially at critical times of development." Specifically, there could be inflammatory events at critical time periods during fetal and postnatal development that could alter proper network formation and maintenance dysregulating the normal brain development. Since then, substantial work has been done that addresses this general set of possibilities as discussed below.

Neuroinflammation in Autism

There have been numerous reports implicating neuroinflammation or an increase in production of microglial cells in the central nervous system in autism spectrum disorder (Vargas et al., 2005; Pardo et al., 2005; Zimmerman et al., 2005; Voineagu et al., 2011; Chez & Guido-Estrada, 2010; Wei et al., 2011; Morgan et al., 2010; Tetreault et al., 2012; Suzuki et al., 2013). Neuroinflammation is best described as activated microglia and astrocytes and increased production of cytokines such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-alpha), which are all measures of an inflammatory state (Monnet-Tschudi et al., 2011).

Vargas and colleagues' (2005) innovative study of human autopsy tissue reported an increase in cytokines in the brains of individuals with autism compared to neurotypicals. In addition, individuals with autism have a significant increase in cytokines in the cerebral spinal fluid and in the frontal cortex compared to neurotypicals (Zimmerman et al., 2005; Vargas et al., 2005; Li et al., 2009). Wei and colleagues (2011) found an increase in IL-6 in the cerebellum of individuals with autism and hypothesized that the microglia are altering the cerebellar granule cell excitatory synapses (Wei et al., 2011). There is a significant increase in the area fraction scored positive for an immuno

stain for microglia of human leukocyte antigen receptor (HLA-DR) in the cerebellum of individuals with autism compared to controls (Vargas et al., 2005). However, this method did not measure the microglial cell densities, sizes and shapes. Thus, quantification of microglia and shape analysis are essential to studying the state of microglia activation in the brains of individuals with autism. Morgan and colleagues (2010) reported an increase in microglia in dorsal lateral prefrontal cortex in individuals with autism and observed microglial processes retracting and thickening suggesting the microglia were in an activated state. Similarly, we reported an increase in the microglial density in fronto-insular cortex and visual cortex in individuals with autism. Since these are two disparate cortical areas, this indicates a global increase in the microglial density in individuals with autism (Tetreault et al., 2012). These data support the hypothesis that neuroinflammation, specifically, increased density of microglia, is involved in autism spectrum disorder. Our results for molecular and granule cell layers of cerebellar cortex for individuals with autism and related disorders compared to neurotyical subjects are reported in Chapter 3 of this thesis.

Microglia

Microglia reside throughout the brain and have long been thought to mainly comprise the brain's immune system, which is distinctly separated from the body's immune system by the blood-brain barrier. In the quiescent state, microglia actively survey their environment and make contact with synapses (Wake et al., 2009). Microglia phagocytose invading microorganisms and debris to defend against infection, clear damaged tissue, and dispose of metabolic waste (Graeber & Streit 1990, 2010).

Moreover, microglia are essential for normal brain development and are critical for synaptic plasticity (Paolicelli et al., 2011; Schafer et al., 2012).

Microglia arise from cells in the yolk sac in embryogenesis and later in life from cells in the bone marrow. Microglia originate in the yolk sac during embryogenesis and can be detected in the brain rudiment from embryonic day eight in mice where microglia continually rise steadily during the first two postnatal weeks, when the majority of microglia are born (Alliot et al., 1999). More recently, using a tamoxifen-inducible Cre recombinase under the control of Runx-1, a hematopoietic transcription factor, confirmed that microglia originate from embryonic Runx-1-expressing yolk sac hematopoietic progenitors between 7.25 and 7.5 days post-conception (Ginhoux et al., 2010). Moreover, the authors reported that in the healthy mouse brain that peripheral myeloid cells do not contribute to new microglia to achieve maintenance numbers in the adult brain (Ginhoux et al., 2010). Additionally, Kierdorf described the yolk sac progenitors that give rise to microglia and identified them as early 8 days post conception (Kierdorf et al., 2013). Mouse microglia originated from primitive c-kit^{+} yolk sac precursors developing into CD45^{+} c-kit^{lo} CX3CR1^{-} cells prior to maturation and migrate into the embryonic maturing brain as CD45^{+} c-kit^{-} CX3CR1^{+} cells and proliferate into the microglia (Kierdorf et al., 2013). These studies provide evidence that the majority of adult microglia in the health brain arise during early development.

To defend against infection, microglia phagocytose invaders, damaged tissue and metabolic waste (Graeber & Streit 1990, 2010). It is conceivable that maternal inflammation elicits an activation of microglia, which can be a risk factor for autism (Patterson, 2009, 2011). In a mouse model of peripheral organ inflammation, cerebral

microglia recruit monocytes into the brain through the release of TNF-alpha (D'Mello et al., 2009). When microglia are activated by an infection, traumatic brain injury, stroke, or neurodegenerative disease, they enter the phagocytic phase, engulfing and removing debris in the brain in contrast to the quiescent stage where microglia are primarily engaged in surveying their environment (Lin & Bergles, 2004; Wake et al., 2009).

Microglia appear to have functions separate from immune activity. Increasing evidence points to a key role in neurodevelopment and neural plasticity through the mechanism of synaptic pruning (Paolicelli et al., 2011; Schafer et al., 2012). Visualization of fluorescently labeled microglia in the mouse brain using two-photon imaging shows that microglia actively survey their environments by retracting and expanding their processes in response to neuronal stimuli (Nimmerjahn et al., 2005; Davalous et al., 2005 and Wake et al., 2009). A mouse model of neurodegeneration resulting from brain ischemia shows microglia increase contact with synapses that have reduced activity (Wake et al., 2009).

During the peak critical period in postnatal development of the visual system, upon visual stimulation microglia contact dendritic spines, synaptic terminals, and clefts in layer II and IV of the visual cortex (Tremblay et al., 2010). In the developing visual system, microglia are necessary for phagocytic synapse elimination in the mouse retino-geniculate pathway for normal development, which is dependent on the microglia phagocytic signaling pathway of the complement receptor CR/C3, a property of the innate immune system (Schaffer et al., 2012). Together these data exhibit that microglial phagocytosis is crucial for normal brain development and circuit formation. As a result of these studies, a number of questions arise: Is the neuroinflammation an indicator of

dysregulated synaptic connectivity? Or is the neuroinflammation in response to systemic infection? Or could it be both? Since autism is a heterogenous disorder, could the neuroinflammation represent a dysregualtion in the synaptic connectivity and a systemic infection? Or could it be due to a history of systemic infections?

Maternal Infection in Autism

There is evidence that maternal viral infection in the first trimester and maternal bacterial infection in the second trimester have significant association with autism (Atladóttir et al., 2010). Individuals with autism have elevated levels of a number of inflammatory cytokines in amniotic fluid compared to neurotypicals (Abdallah et al., 2011). There is also a significant association between elevated levels of C-reactive protein (indicative of the inflammatory response) in maternal serum and the likelihood that her child was autistic (Brown et al., 2013). In addition, a subset of people with autism have a continuous pro-inflammatory pathology in the brain and cerebral spinal fluid, and it is hypothesized that maternal infection or a systemic infection may lead to inflammation and autism spectrum disorder (Chez & Guido-Estrada, 2010). Prenatal infection and immune dysfunction are biologically plausible potential causes of autism (Patterson et al., 2011). Using a mouse model of maternal immune activation (MIA), a single injection of interleukin-6 (IL-6) during mouse pregnancy results in offspring with behavioral deficits similar to those in autism, including deficiencies in prepulse inhibition (PPI) and latent inhibition (LI), but these deficits do not occur when IL-6 is eliminated from the MIA model using genetic methods or blocking antibodies (Smith et al., 2007). Simultaneous administration of a poly(I:C) and an anti-IL-6 antibody inhibits the PPI, LI,

and exploratory and social deficits as well as the alterations in gene expression in the brains MIA offspring (Smith et al., 2007). Similarly, MIA in IL-6 knock-out mice do not exhibit behavioral deficits as seen in the MIA wild-type offspring (Smith et al., 2007). Together these data indicate that the cytokine IL-6 crucial for moderating behavioral and transcriptional changes in the MIA mouse model.

In a mouse model of MIA, polyinosine:cytosine (poly(I:C)) injection elicits a viral infection response, where the offspring demonstrate behaviors similar to autism, including deficits in social communication and interaction, reduction in ultrasonic vocalizations (USVs), and increased stereotyped/repetitive behaviors such as excessive marble burying and self-grooming (Malkova et al., 2012; Hsiao et al., 2011). Additionally, the MIA offspring exhibit a reduction in the linear density of Purkinje cells specific to lobule VII of the cerebellum, which is a hallmark of autism neuropathology, indicating that the alteration of the Purkinje cells is induced by maternal activation of the immune system occurring during embryonic development (Shi et al., 2009). The MIA offspring after a poly(I:C) injection exhibit changes in a number of cytokines in the brains and sera in a region and age-specific manner (Garay et al., 2012). The MIA-induced cytokines do not breach the blood brain barrier, permit immune cell infiltration or lead to an increase in microglia (Garay et al., 2012). Although these results argue that there is an increased level of cytokines in brain regions similar to those reported in autism, there is no elevation in the microglia in this mouse model.

In another model of maternal inflammation, administering a lipopolysaccharaide (LPS) injection and a concurrent single treatment of an IL-1 receptor antagonist results in

normal brain development in the mouse, providing promise for treatments that are protective for normal fetal brain development (Girard et al., 2010).

Gastrointestinal Abnormalities in Autism

Gastrointestinal disturbances occur in individuals with autism including inflammatory bowel syndrome, reflux esophagitis, chronic gastritis, chronic duodenitisdisaccharide malabsorptionmay (Horvath et al., 1999). Again, the question arises as to whether it is causative or correlative, relative to the behavioral phenotype that defines autism. Based on parent questionnaires and evaluations by pediatric gastroenterologists, diagnosis of autism was concordant with a clinical diagnosis of any (95%) gastrointestinal disorder, most commonly (85%) constipation, which was associated with younger age of onset and increased social impairment (Gorrindo et al., 2012). Using parent reports, individuals with autism have increased history of vomiting and diarrhea and nearly half of the individuals with autism described having an individual lifetime gastrointestinal symptom (Chandler et al., 2013). Altered intestinal permeability was reported in nine of the 21 (43%) children with autism compared to none of the 40 neurotypicals, indicating an increased passage of peptides through the gut mucosa in individuals with autism (D'Eufemia et al., 1996). It has been hypothesized that individuals with autism have a "leaky gut," or high intestinal permeability, that is caused by a disruption in the tight junctions of the gut that have their origin during infancy (D'Eufemia et al., 1996; De Magistris et al., 2010). The gut, like the blood-brain barrier, has tight junctions, which when disrupted could allow for absorption of substances that could adversely affect brain function (Theoharides & Doyle, 2008). A subset of

individuals with autism (36.7%) and their first degree relatives (21.2%) show increased intestinal permeability (De Magistris et al., 2010). In addition, individuals with autism that have gastrointestinal problems exhibit elevated levels of autoantibodies that bind to the intestinal mucosa on the basement membranes of epithelial cells (Torrente et al., 2004). Autoantibodies may indicate an inflammatory state of the intestines and could in turn alter the mucosal barrier and integrity leading to gastrointestinal problems in individuals with autism (Coury et al., 2012). This could be another line of evidence for systemic infection and chronic inflammation in individuals with autism, where the gut and brain together are susceptible to systemic inflammation. Specifically, Buie and colleagues (2010) describe a set of beneficial diagnostics for individuals with autism spectrum disorders, including guidelines for routine pediatric testing of abdominal pain, chronic constipation, and gastroesophageal reflux disease. Coury and colleagues (2012) describe the importance of ascertaining whether individuals with autism differ from controls in several gastrointestinal features such as the microbiome, metabolites, inflammation, neurotransmitters, immune response and mucosal integrity, which may lead to possible biomarkers for individuals that may be a risk for developing autism.

Rett Syndrome, Angelman Syndrome and Fragile X Syndrome

Syndromic autism is described as a single gene disorder that presents autism-like features. Ubiquitin-protein ligase E3A (UBE3A), methyl CpG binding protein 2 (MECP2), and fragile X (FMR1) are genes associated with syndromes in which individuals present the core behaviors of autism (Jellinger et al., 1988; Bonati et al., 2007; Farzin et al., 2006).

Rett syndrome is caused by a mutation of the methyl CpG binding protein 2 (MECP2) and is almost exclusively found in females. Since MECP2 is found on the X chromosome, females have a normal copy of the gene; by contrast, the condition in males is generally fatal early in life. It accounts for 1% of children diagnosed with autism (Lintas & Persico, 2009). Individuals with Rett syndrome present a lack of communication, increased stereotyped behaviors, severe mental retardation, and gastrointestinal disorders. It has been reported that individuals with Rett syndrome have neuropathology involving increased numbers of astrocytes in the cortex, a decrease in dendritic spine density in the cerebral cortex, and a decrease in the number of cerebellar Purkinje cells (Armstrong et al., 2005). MECP2 knockout mice exhibit features of Rett syndrome, including a decreased life span, reduced body weight, reduced brain weight, and behaviors of Rett syndrome which can be assayed in mice using behavioral tests to exhibit the extent of the disease (Chen et al., 2011; Guy et al., 2001).

Angelman syndrome is caused by a deletion on chromosome 15 Q, 2Q 13, which most often is the ubiquitin E3 ligase (UBE3A) gene (Wang et al., 2013). UBE3A is an imprinted gene in which only the maternal copy is expressed in the brain (Smith et al, 2011). Angelman syndrome is a neurodevelopmental disorder characterized by ataxia, severe learning impairments, and epilepsy. Individuals with Angelman syndrome commonly meet the diagnositic criteria for autism based on the autism diagnostic observation scale (ADOS) (Bonati et al., 2007). The UBE3A maternal knockout mouse model involves deficits in reversal learning, impairments in motor function, hypoactivity, and reduced rearing and marble burying (Huang et al., 2013). By increasing the UBE3A gene in the mouse threefold, Smith and colleagues have developed a mouse model that

exhibits core autism behaviors including defective social interaction, impaired communication and increased stereotypic behaviors as well as altered gluatamatergic synaptic transmission, resulting in reduced excitatory transmission (Smith et al., 2011). Thus, increasing or decreasing the expression of UBE3A has important behavioral consequences relevant to autism (McNamara & Isles, 2013). These mouse models have promise for studying the behavioral features in autism, allowing histological comparative analysis, and genetic manipulations, thus providing insights for the genetic and environmental factors contributing to autism.

Fragile X syndrome is caused by mutations in the FMR1 gene and accounts for 1-3% of the cases of autism. The mutation is an expansion in of the CGG trinucleotide on the FMR1gene to a full mutation size of 200 or more repeats, children with autism have been reported to have 55-200 CGG repeats (Reddy et al., 2005). Fragile X- associated tremor/ataxia syndrome (FXTAS) is progressive degenerative movement disorder with the features of tremors, cerebellar gait ataxia, parkinsonism and cognitive decline with an onset at age 50 (Hall & O'Keefe, 2012). Often, these individuals have family members with fragile X and it occurs in both males and females and often the female carriers have 45-54 CGG (Hall & O'Keefe, 2012). Individuals with fragile X syndrome exhibit a marked Purkinje cell loss, axonal swelling and glial cell loss in the cerebellum (Tassone et al., 2004). The female CGG knock-in mice, which are fragile X carriers exhibit deficits for learning a skilled forelimb reaching task compared to wild-type littermates, and that these deficits worsen with increasing CGG repeat lengths (Diep et al., 2012). The FMR1 knockout mice exhibit features similar to fragile X and specifically represent behaviors of

autism such as impaired social interaction and repetitive behavior (Bernardet et al., 2006).

Genetics and Inflammation in Mental Illness: Schizophrenia, Sickness Behavior, and Depression

A recent study of genome-wide single-nucleotide polymorphism (SNP) found shared SNPs for five major neuropsychiatric disorders including schizophrenia, bipolar disorder, autism, major depression and attention deficit hyperactivity disorder, based on genetic data from 60,000 subjects worldwide (Cross-Disorder Group of the Psychiatric Genomics Consortium et al., 2013). The authors report that SNPs within two L-type voltage-gated calcium channel subunits, CACNA1C and CACNB2, had genome-wide significance, a model selected analysis supported these loci in several disorders, and pathway analysis supported a role for calcium channel signaling genes for all five disorders (Cross-Disorder Group of the Psychiatric Genomics Consortium et al., 2013). This finding provides promise for a greater understanding of the disease process in mental illness and particularly for investigation for therapeutics in the diseases. It provides a common genetic link across the disorders, highlighting the complexity of differentiating the pathologies across the different mental illnesses. Moreover, each of these diseases has a neuroinflammatory component similar to autism, so understanding the genetics and neuroinflammation in these neurodevelopmental and psychiatric disorders is essential for developing treatments.

A genome-wide microarray study of left superior temporal cortex in schizophrenic individuals shows a downregulation of immune genes, suggesting that the

altered genes related to the immune system may compromise synaptic functioning in the region (Schmitt et al., 2011). Moreover, individuals with schizophrenia exhibit executive dysfunction, and it is proposed that schizophrenia has a strong neurodevelopmental component (Brown et al., 2007). An epidemiological study of maternal infection in schizophrenia revealed that mid-gestational influenza is associated with schizophrenia (Brown et al., 2000; Brown et al., 2004). A study of autopsy tissue found increased activation of microglia labeled by HLA-DR in a subset of cases with schizophrenia (Bayer et al., 1999). Microglia analysis in postmortem tissue in the frontal and temporal lobes of individuals with schizophrenia shows evidence for both microglial activation and degeneration (Wierzba-Bobrowicz et al., 2004). In addition, Wierzba-Bobrowicz and colleagues (2005) found evidence of degeneration in microglia cells from schizophrenic individuals, including reduction of the cytoplasm, mitochondrial damage, fragmenting, and thinner and shortened microglial processes (Wierzba-Bobrowicz et al., 2005). A number of postmortem studies show greater evidence of microglia activation and infiltration by other immune cells in the brain in individuals with schizophrenia (Radewicz et al., 2000; Steiner et al., 2006; Drexhage et al., 2011). In schizophrenia, inflammation and possible maternal immune activation plays a key role in the neuroinflammation in the brain.

Another result of a systemic infection is “sickness behavior” which drives an increase in signals to the brain causing changes in metabolism, social withdrawal, appetite suppression and a general ill feeling. The main pro-inflammatory cytokines involved are interleukin-1 beta (IL1B) and tumor necrosis factor alpha (TNF-alpha) (Exton et al., 1997; Hart et al., 1998; Perry et. al., 2010). Sickness behavior is another

example of how systemic infection and inflammation can alter both the inflammatory response and the brain and behavior. There are a number of parallels between sickness behavior and major depression, such as the somatic features of fatigue, loss of appetite, sleep disturbances, and depressed mood (Dantzer et al., 2009). Elderly individuals with depression exhibit elevated levels of inflammatory cytokines, including IL-6, in the blood (Trzonkowski et al., 2004). In a postmortem study of microglia in depression there is evidence for microglial activation in patients with depression who completed suicide, compared to patients who died via other methods and healthy controls (Steiner et al., 2008).

It is important to note that each of these mental illnesses is associated with neuroinflammation and immune dysregulation, which is a common pathology in autism spectrum disorder. Understanding the connection of the neuroinflammation in mental illness could advance the therapies for these diseases.

Conclusion

In this thesis, we explore the density of the microglia in the cerebral and cerebellar cortices of individuals with autism to investigate the hypothesis that neuroinflammation is involved in autism. We chose fronto-insular cortex (FI) because multiple lines of evidence have previously implicated FI in autism (Allman et al., 2005, Di Martino et al., 2009, Santos et al., 2011). Vargas et al. (2005) reported that inflammation was widespread throughout the brains of individuals with autism and found an increase in inflammatory cytokines in the cerebellum and cerebral cortex in cases with autism (Vargas et al., 2005).

In our cerebellar study we included additional neurodevelopmental disorders that exhibit similar behaviors to autism spectrum disorder and have known cerebellar pathology. We found increased microglial density in two disparate cortical regions, frontal insular cortex and visual cortex, in individuals with autism (Tetreault et al., 2012). Our results imply that there is a global increase in the microglial density and neuroinflammation in the cerebral cortex of individuals with autism. We subsequently found a more than threefold increase in the microglial density specific to the molecular layer of the cerebellum, which is the region of the Purkinje and parallel fiber synapses, in individuals with autism and Rett syndrome. Moreover, we report that not only is there an increase in the microglia density in the molecular layer, but also, the microglial cell bodies are significantly larger in perimeter and area in individuals with autism spectrum disorder and Rett syndrome compared to controls, which implies that the microglia are in the activated state.

In addition, we report that an individual with Angelman syndrome and the sibling of an individual with autism have microglial densities similar to the individuals with autism and Rett syndrome. By contrast, an individual with Joubert syndrome, which is a developmental hypoplasia of the cerebellar vermis, had a normal density of microglia, indicating the specific pathology in the cerebellum does not necessarily result in increased microglial densities. Finally, we found a significant decrease in Purkinje cells specific to the cerebellar vermis in individuals with autism thus providing direct quantitative evidence for abnormal cerebellar circuitry in this conditions.

These findings indicate the importance of investigation of the Purkinje synapses in autism and that the relationship between the microglia and the synapses is of great

utility in understanding the pathology in autism. Together, these data provide further evidence for the neuroinflammation hypothesis in autism and a basis for future investigation of neuroinflammation in autism. In particular, investigating the function of microglia in modifying synaptic connectivity in the cerebellum may provide key insights into developing therapeutics in autism spectrum disorder.

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Chapter 2: Microglia in the Cerebral Cortex in Autism

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ORIGINAL PAPER

Microglia in the Cerebral Cortex in Autism

Nicole A. Tetreault · Atiya Y. Hakeem · Sue Jiang · Brian A. Williams · Elizabeth Allman · Barbara J. Wold · John M. Allman

Abstract We immunocytochemically identified microglia in fronto-insular (FI) and visual cortex (VC) in autopsy brains of well-phenotyped subjects with autism and matched controls, and stereologically quantified the microglial densities. Densities were determined blind to phenotype using an optical fractionator probe. In FI, individuals with autism had significantly more microglia compared to controls ($p = 0.02$). One such subject had a microglial density in FI within the control range and was also an outlier behaviorally with respect to other subjects with autism. In VC, microglial densities were also significantly greater in individuals with autism versus controls ($p = 0.0002$). Since we observed increased densities of microglia in two functionally and anatomically disparate cortical areas, we suggest that these immune cells are probably denser throughout cerebral cortex in brains of people with autism.

Keywords Microglia · Autism · Fronto-insular cortex · Visual cortex

Introduction

The brain is substantially isolated from the body's immune system by the blood–brain barrier, which restricts the passage of most immune cell types and proteins from capillaries into brain tissue. The brain has its own immune system based on microglia, which are derived from the macrophage lineage and reside throughout the brain, where they mount defenses against invading microorganisms and clear damaged tissue and metabolic waste (Graeber and Streit 1990, 2010). This is achieved through phagocytosis, in which the microglia ingest these substances.

Nimmerjahn et al. (2005), Davalos et al. (2005) and Wake et al. (2009) directly observed the activity of microglia in intact living mouse brains using two-photon microscopy in animals that express green fluorescent protein specifically in microglia. Their experiments showed that microglial cell bodies are relatively stationary, but their fine processes are in constant motion on a minute-to-minute basis. They observed that the microglial processes continually probe the immediate area, so that the population conducts a complete surveillance coverage of brain tissue every few hours. When the microglial processes encounter damaged tissue, metabolic byproducts such as oxidized lipoproteins, or invading microorganisms, they respond by expanding and engulfing these substances and transporting them back to the microglial cell body where they are stored for an indeterminate period of time. The microglia contact other types of glia and neurons as part of their constant surveillance, but when they encounter other microglia there is mutual repulsion of their processes, which may account for their relatively uniform spacing. There is also evidence that microglial processes can strip synapses away from their dendrites, suggesting that microglia may have another role in modifying neuronal connections in development and plasticity (Blinzinger and Kreutzberg 1968; Graeber et al. 1993; Kreutzberg 1996; Paolicelli et al. 2011). Wake et al. (2009) reported through in vivo imaging that microglia make transient direct contact with synapses, and that the frequency of this contact is

N. A. Tetreault (✉) · A. Y. Hakeem · S. Jiang · B. A. Williams · B. J. Wold · J. M. Allman
Division of Biology, California Institute of Technology, 1200 East California Blvd., MC216-76, Pasadena, CA 91125, USA
e-mail: nicolet@caltech.edu

E. Allman
Comprehensive Autism Center, 2604 B El Camino Real #214, Carlsbad, CA 92009, USA

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dependent on neural activity. In experiments in visual cortex, microglia contact frequency was decreased by silencing the visual input by injecting tetrodotoxin into the eyes (Wake et al. 2009). Inducing neural degeneration by transient ischemia increased the duration of microglial contact with synapses which was followed by synapse elimination (Wake et al. 2009).

Microglia are closely related developmentally and functionally to macrophages. Both originate from the monocyte lineage in the bone marrow. Microglia first appear in small numbers in the brain during embryogenesis, but they emerge prominently during the early postnatal period when they enter the brain from the bloodstream to form what has been called the fountain of microglia, in which they migrate along the course of the fibers of the corpus callosum to all parts of the brain (Imamoto and Leblond 1978). The initial population of microglia can be augmented by subsequent invasion into the brain of circulating macrophages, which apparently assume the microglial phenotype after entering neural tissue (Schmid et al. 2009). In a preliminary study of gene expression, we observed in some of our autistic cases increased expression of a network of genes centered on interleukin-6 (Tetreault et al. 2009). Interleukin-6, together with several other genes in the network, is characteristic of activated versus quiescent microglia (Thomas et al. 2006). People with autism have significantly increased cytokines in frontal cortex and elevated levels of cytokines in the cerebrospinal fluid compared to control subjects (Li et al. 2009; Zimmerman et al. 2005) and there is evidence for immune system dysfunction in the development of autistic children (Ashwood et al. 2006; Chez and Guido-Estrada 2010). These observations motivated us to conduct a quantitative study of the density of microglia in brains of individuals with autism compared to controls. Our goal in this work is to quantify microglial differences between subjects with autism and age-matched controls in two cortical areas, fronto-insular cortex (FI) and primary visual cortex (VC). Multiple lines of evidence have previously implicated FI in autism (Allman et al. 2005; Di Martino et al. 2009; Santos et al. 2011); VC was selected because of its functional difference and anatomical distance from FI, in an effort to span the diversity within neocortex.

Methods

Tissue Samples

Formaldehyde-fixed (8 % solution) human right FI and right VC tissue from subjects with autism and controls was obtained from the NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland-Baltimore, as shown in Table 1. FI was identified based on criteria such as the presence of the Von Economo neurons and sulcal location (Allman et al. 2010) and corresponds to the posterior part of Brodmann's area 47. VC was identified by using the calcarine sulcus as a landmark; the dissections involved the sulcal lip corresponding to Brodmann's areas 17 and 18. The NICHD Brain and Tissue Bank for Developmental Disorders provided detailed clinical records, with personal identification removed, for each individual with autism whose brain we studied, as summarized in the phenotypic descriptions in Table 2. To confirm the diagnosis of autism, the medical records of each person with autism were reviewed in depth by a clinical psychologist (EA) who specializes in autism. In each case we have at least one thorough clinical description of the subject by either a psychologist or psychiatrist. Ten of our eleven subjects with autism had the autistic diagnostic interview-revised (ADI-R), which is the result of a structured interview with a parent of the individual with autism. Three of the individuals with autism had ADIR records, but the actual scores were not in the file. One individual with autism had a childhood autism rating scale and met the criteria for an autism diagnosis. The records additionally include measures of behavioral development such as the Bayley tests, as well as a history of medications and other health issues reported by physicians and clinical psychologists, described in Table 2.

Sectioning and Immunocytochemistry

Samples were sectioned in the coronal plane at 50 μm on a microtome with a vibrating blade (Microm HM 650 V) in 0.1 M phosphate buffer solution (PBS) and stored in well dishes with PBS and sodium azide. The microglia were immunocytochemically stained with an antibody to IBA1 (ionizing calcium adaptor molecule-1), the gene product of the Aif1 gene (allograft inflammatory factor 1), raised against the C-terminus of IBA1, which labels microglia and monocytes. We used the IBA1 antibody because it yields excellent and selective staining of microglia in formaldehyde-fixed human archival brain tissue (Streit et al. 2009). The utility of IBA1 for the study of microglia has also been shown through expression of the IBA1 gene coupled with enhanced green fluorescent protein in experiments employing 2-photon microscopy to image the development and motility of this class of cells in the brains of living mice (Hirasawa et al. 2005; Wake et al. 2009). We used a concentration of 1:1,000 of IBA1 antibody (Wako, Code No. 019-19741). Four batches of immunostaining were performed including duplicate sections from both FI and VC of each of the subjects, and each of the staining procedures showed consistent and robust immunostaining across the sections. Free-floating sections were rinsed with

Table 1 Autistic and neurotypical control subjects used for microglial density measurements

ID	GUID	Age	Sex	Cause of death	Brain weight (g)	PMI (h)	FI	VC
Autistic								
M4021	NDAR_INVUX206VRV	3a	M	Drowning	1,330	15	X	X
M4029	NDAR_INVRX268EH4	3b	M	Drowning	1,130	13	X	X
UMB4671	Not provided	4	F	Fall from 9th story	1,320	13	X	X
UMB797	NDARYX624FEY	9	M	Drowning	1,175	12	X	
M2004	NDAR_INVAK979XTP	10	M	Drowning	1,400	25	X	X
UMB4305	NDARWL137ER1	12	M	Serotonin syndrome	1,360	13	X	
UMB4315	NDAR_INVHD069UM7	14a	M	Natural	1,590	22	X	X
UMB4899	NDAR_INVGW538MM3	14b	M	Drowning	1,450	9	X	X
UMB5278	NDARYH540PL4	15	F	Drowning with seizure	1,417	13	X	X
UMB4999	Not provided	20	M	Cardiac arrhythmia	1,427	14		X
UMB5176[1]	NDARHU383HFF	22	M	Subdural hemorrhage	1,525	18	X	X
Control								
UMB5282	Not provided	2	M	Asphyxia	1,345	16	X	X
UMB1185	Not provided	4a	M	Drowning	1,450	17	X	X
UMB4670	Not provided	4b	M	Commoti cordis	1,470	17	X	X
UMB5387	Not provided	12	M	Drowning	1,750	13	X	X
UMB4925	Not provided	13a	M	Natural	1,650	16	X	
UMB917	Not provided	13b	M	Accident, multiple injuries	1,450	10	X	X
UMB4591	Not provided	16	F	Multiple injuries	1,330	14	X	X
UMB1712	Not provided	20a	M	Gunshot to chest	1,500	8	X	X
UMB4727	Not provided	20b	M	Multiple injuries	Not available	5	X	X
UMB1542	Not provided	22a	M	Multiple injuries	1,510	4	X	X
UMB4542	Not provided	22b	M	Multiple injuries	1,460	8	X	X
UMB1713	Not provided	23	M	Head and neck injuries	1,600	8	X	X

The tissue source is NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, MD. The letters in the age column are for the purpose of differentiating subjects of the same age in the graphs in Figs. 2 and 3 *PMI* post-mortem interval, *X* microglia density measurements were made for this structure

PBS and then incubated with 1 % citrate buffer (Chemicon, cat # 21545) for 30 min at 37 °C for antigen retrieval. Sections were rinsed with PBS, treated to remove endogenous peroxidase activity with 0.75 % hydrogen peroxide and methanol for 20 min, and then rinsed with PBS. The blocking step, to eliminate random antibody binding, used 0.1 % Triton X-100, 4 % normal goat serum (NGS), 1 % BSA, and 3 % dry milk in PBS for 3 h. Primary antibody was incubated for 38 h at 4 °C in a PBS solution that included 0.1 % Triton X-100, 2 % NGS and 1:1000 anti-IBA1. Sections were then rinsed with PBS, incubated with biotinylated anti-rabbit (BA-1000,Vector Laboratories) at 1:200 for 2 h, and then rinsed again with PBS. A Vectastain Elite ABC kit (pk-6100, Vector Laboratories) was used for the avidin–biotin-peroxidase method, then sections were incubated for 30 min. After sections were once again rinsed with PBS, immunoreactivity was visualized by using a chromagen, 3′-diaminobenzidine and nickel (SK-4100, Vector Kit). Null control sections were incubated without primary antibody and incubated with goat IgG at the same concentration as the primary antibody. No immunostaining was observed in these control sections.

Quantification of Microglial Densities

Microglial density in FI and VC was measured blind to phenotype and quantified using the program Stereo Investigator (MBF Bioscience, Williston, VT) with a Reichert Polyvar microscope equipped with a motorized stage and a camera for visualization. All sections were quantified in at least two separate replications with different regions of interest, and some sections were quantified up to four times with both different and identical regions of interest. For all of the samples, duplicate sections of FI and VC were classified and quantified for reproducibility. Independent raters quantified and classified blind random sections to replicate the method. The represented density measure is an average of the blind replicated runs. Quantification was

Table 2 Below is a phenotypic description of the autistic subjects including age, gender, seizure status, medications, medical history, cause of death, PMI

Patient ID	Age and gender	PMI (hours)	Cause of death	Seizure disorder	Medication	Additional medical hist	ADI-R[a]	Neuropath report or autopsy	Patient summary	Respirator or traumatic death state
M4021	3 Years Male	15	Drowning	No	None reported	None reported	Completed not in file A: B: C: D:	None provided	Rigid routine, many repetitive[f] and aggressive behaviors. No gestures for communication, auditory sensitivity	None reported
M4029	3 Years Male	13	Drowning	No	None reported	None reported	Completed not in file A: B: C: D:	None provided	Autistic regression[d] at 24 months, aggressive behaviors, negative response to several sensory stimuli and ran from sounds	Found in a canal and resuscitated with CPR. Five hours on respirator
UMB4671	4 Years Female	13	Fall 9 stories	No	None	Diaper rash	A: 26 B:13 C: 3 D: 5	Autopsy; noted normal brain	Lacked body self awareness and awareness of others[b] (observed by mother); could not identify body parts when tested by a psychologist. Normal hearing. Cognition delayed; no socialization	None reported
UMB797	9 Years Male	22	Drowning	No	Desipramine	ADHD; seizure associated with medication, migraines	A: 24 B: 20 C: 6 D: NA	Neuropath; noted normal brain	No hypersensitivity to sensory stimuli, 8 cortical regions stained with H&E, no microgliosis in cortex, VC had unusually large Meynert cells and an irregular shaped claustrum	Overdose of desipramine a week prior to death; no revival
M2004	10 Years Male	25	Drowning	No	None reported	Hyperactivity	CARS:39 A: B: C: D:	None provided; normal brain noted macro-scopically	Good visual and fine motor skills, ritualistic behaviors and inflexibility, severe language delay	None reported
UMB4305	12 Years Male	13	Serotonin syndrome	Yes	Clonazepam Depakote Olazapine Quetiapine	Pervasive development disorder NOS psychosis NOS ADHD	A: 25 B: 15 C: 8 D: 4	Neuropath; necrosis effects; large macrophage and astrocytes in RH	Very aggressive, destructive and abusive behavior without provocation; special education, lived in group home, unmanageable behavior. Lacked bladder control	Large contusion in right frontal lobe; cystic necrosis
UMB4899	14 Years Male	9	Drowning	Yes	Clonidine Trileptal Zoloft	None reported	A: 22 B: 14 C: 8 D: 4	Neuropath; normal brain and cortex	Loss of verbal skills at 1 year; high levels of sensory interest, compulsions and stereotypy[f] Autistic regression[d]	Found in bottom of pool, CPR revived, on life support for 24 h

Table 2 continued

Patient ID	Age and gender	PMI (hours)	Cause of death	Seizure disorder	Medication	Additional medical hist	ADI-R[a]	Neuropath report or autopsy	Patient summary	Respirator or traumatic death state
UMB4315	14 Years Male	22	Natural	Yes	None reported	None reported	A: 26 B: 16 C: 2 D: 12	Neuropath; brain edema	Prior to death patient had a seizure a week	Brain edema
UMB5278	15 Years Female	13	Drowning	Yes	Depakote Keppra Prozac	None reported	A: 22 B: 11 C: 5 D: 5	None provided; noted normal brain	Noted to have mild autism, high functioning and at grade level, seizures from infancy, second cousin has autism, had spontaneous speech and aggressive behavior	None reported
UMB4999	20 Years Male	14	Cardiac arrhythmia	No	Amoxicillin Depakote Naltrexone Risperdal	Severe mental retardation, aggression, compulsion	Not in file A: B: C: D:	Neuropath; normal brain, increase in leptomengial cells of meninges and gliosis	Self injurious behavior including severe head banging[c], treated with Naltrexone,[e] stereotypy[f], sensitive GI tract and no communication	None reported
UMB5176	22 Years Male	18	Subdural hemorrhage	No	Risperdal	Medical exams reported good health	A: 25 B: 13 C: 7 D: 5	Neuropath; brain intact, deep grey matter structures intact	Nonverbal; makes gestures. Nervousness, behavioral and emotional problems	50 cc left subdural hematoma consistent with head trauma

[a] ADI-R (autism diagnostic interview-revised) test description and cutoffs: qualitative abnormalities in social interaction (A = 10), qualitative abnormalities in communication (B = 7), stereotyped patterns (C = 3), abnormal development (D = 1). All subjects have a psychological evaluation and a Bayley or a developmental test

[b] Minio-Paluello et al. (2009)

[c] Matson and Lovullo (2008)

[d] Goldberg et al. (2003)

[e] Walters et al. (1990)

[f] MacDonald et al. (2007)

performed within a region of interest that spanned layers two to six; microglial distribution appeared relatively homogenous throughout the layers in our samples. Estimated cell counts were performed using the optical dissector probe at 40× magnification (oil immersion NA = 1.0) with a dissector height of 16 µm (flanked by 2.0 µm guard zones), a counting frame of 260 µm × 160 µm and a grid size of 425 µm × 425 µm. To avoid oversampling, we used the Gunderson counting rule such that cells intersecting only 3 of the 6 surfaces of the dissector cube were counted. Microglial density per mm^3 was calculated by dividing the optical fractionator estimate of the number of cells present in the full thickness of the section within the region of interest by the area of the region of interest and the thickness at which the section was cut to account for any tissue shrinkage.

Statistical Analysis

Densities for the subjects with autism and control populations were compared using the Mann–Whitney test with two-tailed p value. Correlation levels between replications were measured using Pearson's r-squared. Possible confounds in the subjects with autism that could alter microglial densities were examined. Binary confounds, including whether death was by drowning and whether seizures were present, were tested using the Mann–Whitney test; a possible confounding correlation with postmortem interval was tested using Pearson's r-squared.

Results

Figure 1 depicts the stereological method and photomicrograph of the microglial quantification method in a brain of an individual with autism. We found significantly higher density in the individuals with autism than in the controls in both FI ($p = 0.0206$, see Fig. 2) and VC ($p = 0.0002$, see Fig. 3). The numbers are represented as the average of the microglial densities for the multiple replications performed in each individual. Comparisons were made using Mann–Whitney tests with two-tailed p values. The repeated quantifications in the same structure are highly significantly correlated: for FI, and VC, $r^2 = 0.6480$, $p < 0.0001$ (Fig. 4) when the blind replications are from the exact ROI the correlation is $r^2 = 0.\ 9780$, $p < 0.0001$ for the intrarater reliability. Notably, the individuals with autism cluster together in FI and VC, except for a single outlier subject with autism, while the controls all cluster together in both FI and VC.

Figure 2 shows the microglial cell densities in FI of autistic subjects and controls for the combined and averaged data for both microglial quantifications. Individuals

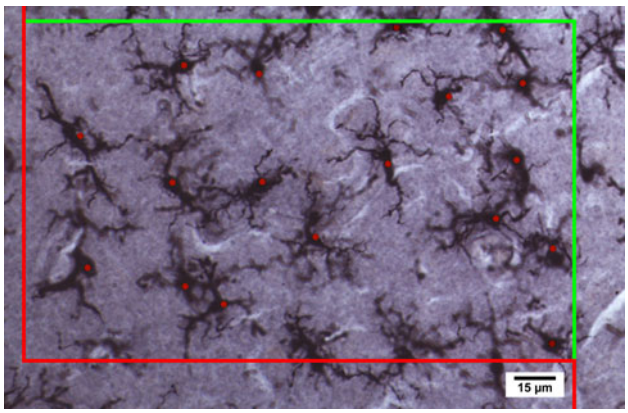

Fig. 1 Stereological procedure for quantifying and identifying microglia in control and the brains of individuals with autism; the *red* and *green* frame defines the borders of the region of interest for counting microglial cells according to the Gundersen et al. (1988) procedure. A microglial cell was included if it was in the counting frame or if the soma crossed the green line and was excluded from the counting when the cell soma crossed the *red line* to avoid oversampling. We used an optical dissector height 16 µm (flanked by 2.0 µm guard zones) and dissector probe at 40× magnification (oil immersion NA = 1.0). Some of the cells are out of focus in the photomicrograph, which is caused by the high numerical aperture of the lens which creates many depth planes through the tissue which is necessary for quantifying cells in three dimensions. Immunocytochemical labeling with Iba1 (1:1000, Wako), a specific marker for microglia and macrophages (Sasaki et al. 2001), in FI of the 14 year old male with autism (UMB4315) (Color figure online)

with autism (n = 10) had significantly higher microglial density ($p = 0.02060$) than control subjects (n = 12) (Mann–Whitney test with two-tailed p value).

The 12-year-old male UMB4305 was a unique case in this group of people with autism because there was no increase in microglial density compared with controls. Although the ADI-R scores for this case are in the autistic range, he was diagnosed as having pervasive developmental disorder not otherwise specified (PDD-NOS), and, in addition, with psychosis NOS, and ADHD. UMB4305 was the only one among all subjects tested who was treated for psychosis, including administration of the drugs quetiapine, olzapine, and risperdal (Table 2). For these reasons, we think this individual may have suffered from a condition distinct from the other individuals who had the autism diagnosis. According to the neuropathology report for UMB4305, "there were three small foci of yellow discoloration noted in the leptomeninges overlying the right antero-inferior frontal pole, right gyrus rectus and left gyrus rectus which measured 0.2 × 0.2 × 0.2 cm. Well-circumscribed regions of shrinkage and slight yellow discoloration were present in the cortical ribbon underlying the discolored leptomeninges. … There was necrosis around the small area of the contusions that included the entire cortical ribbon through layer one. The small frontal lobe contusions had visible macrophages surrounded by

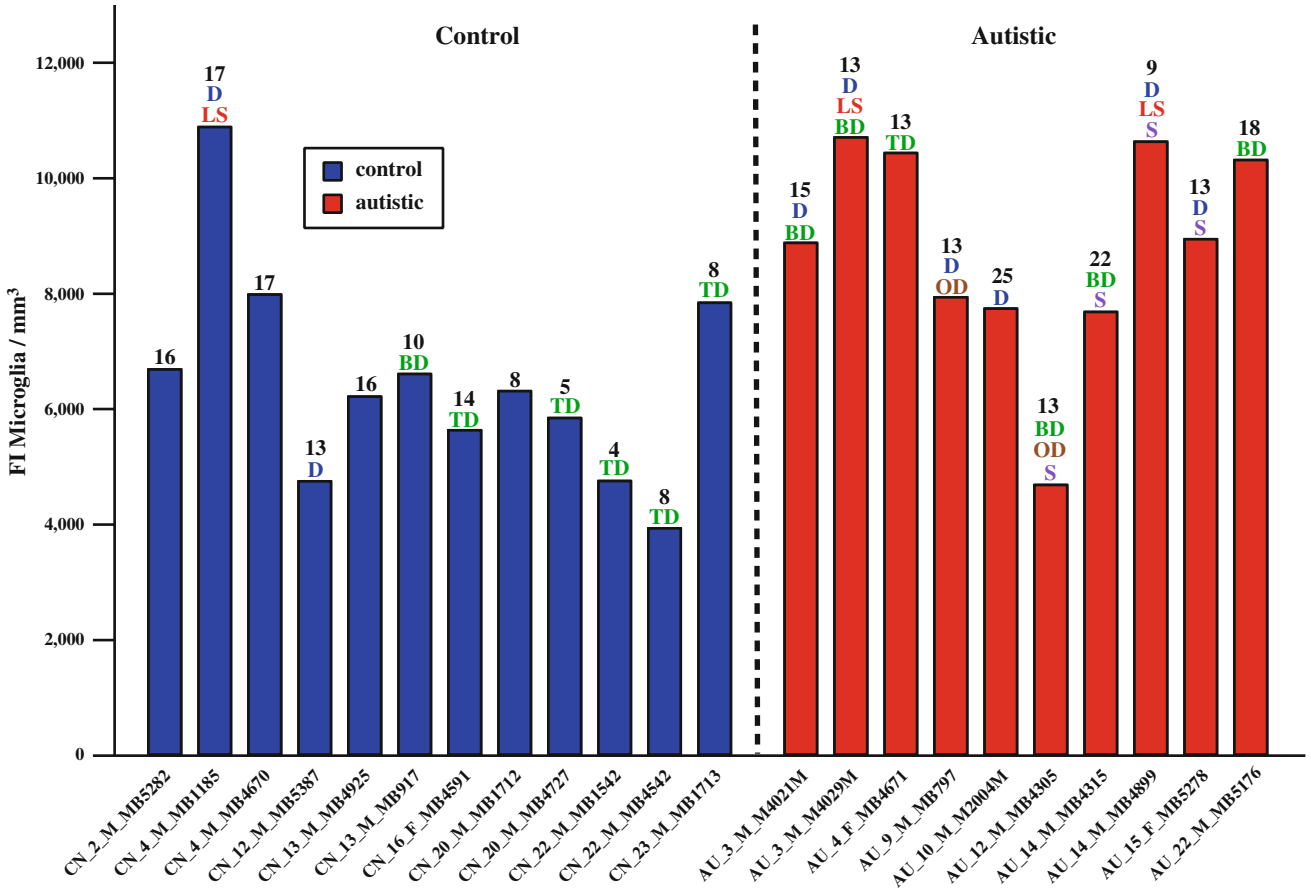

Fig. 2 Microglial densities in FI in subjects with autism and neurotypical brains are represented as the average for the replicated runs. Individuals with autism (n = 10) have a significantly greater density of microglia, the key cellular participants in the inflammatory response in the brain, compared to controls (n = 12) $p = 0.0206$ (Mann–Whitney). LS: known to have spent time on life support. D: cause of death was drowning. BD: brain damage, brain contusion, hemorhage, or edema. TD: traumatic death (MVA, fall) with possible head injury, not explicitly mentioned. OD: drug overdose (not necessarily cause of death). S: seizures (not necessarily cause of death). Numbers in *black* are post-mortem interval in hours

reactive astrocytes observable with a hematoylin-eosin stained sections." The report noted that beyond these local contusions, the cortical layers were normal and the neurons in the cerebral cortex of the fronto-parietal lobe, hippocampus, basal ganglia, and cerebellum were unremarkable.

Figure 3 presents similar data for primary visual cortex (VC). Total microglial densities were significantly greater in VC for the individuals with autism (n = 9) versus the control (n = 11) subjects ($p = 0.0002$ Mann–Whitney test with two-tailed p value). The increase in microglial density is present throughout almost our entire sample of subjects with autism, with ages ranging from 3 years of age to 22. We address the two exceptions to this broad finding, UMB 1185 and UMB 1713, below.

After measuring microglial densities, we consulted Lyck et al. (2009) in which the number of microglia in the cortex of three well-documented neurotypical brains was carefully and comprehensively quantified using a CD45 antibody with unbiased stereology. They reported an average of 3.48 billion CD45 positive cells in the entire human neocortex. Using the estimated value for human neocortical grey volume from Frahm et al. (1982), which is 584,706 mm^3, one can then estimate the density of microglia in the neurotypical human cortex by dividing by the total number of microglia, which is approximately 5,951 (CD45 positive cells) per mm^3 in the total human neocortex (Fig. 5a, b).[1] This is close to our estimated microglial densities for

[1] In Table 5 of Lyck et al. (2009) the column headed "total neocortex" refers to the neocortical gray matter only. In their methods Section 2.2.7, "Estimation of Cell Numbers," they describe their selection of the region of interest, saying, "… followed by delineation the border between white matter and neocortex at 210× magnification (10 × lens) marking the white matter as 'exclusive region'," indicating that their cell number estimates were made from a region that excluded white matter. Further, Fig. 2b from this paper indicates that the brain slices were segmented into "frontal neocortex," "temporal neocortex," "parietal neocortex," "occipital neocortex," and "white matter," implying that the various neocortex segments do *not* include white matter. Thus, in Table 5 the column heads "frontal cortex," "temporal cortex," etc. presumably refer specifically to the gray matter portions of those regions, and "total neocortex" (which is a sum of the other four columns) also includes only gray matter.

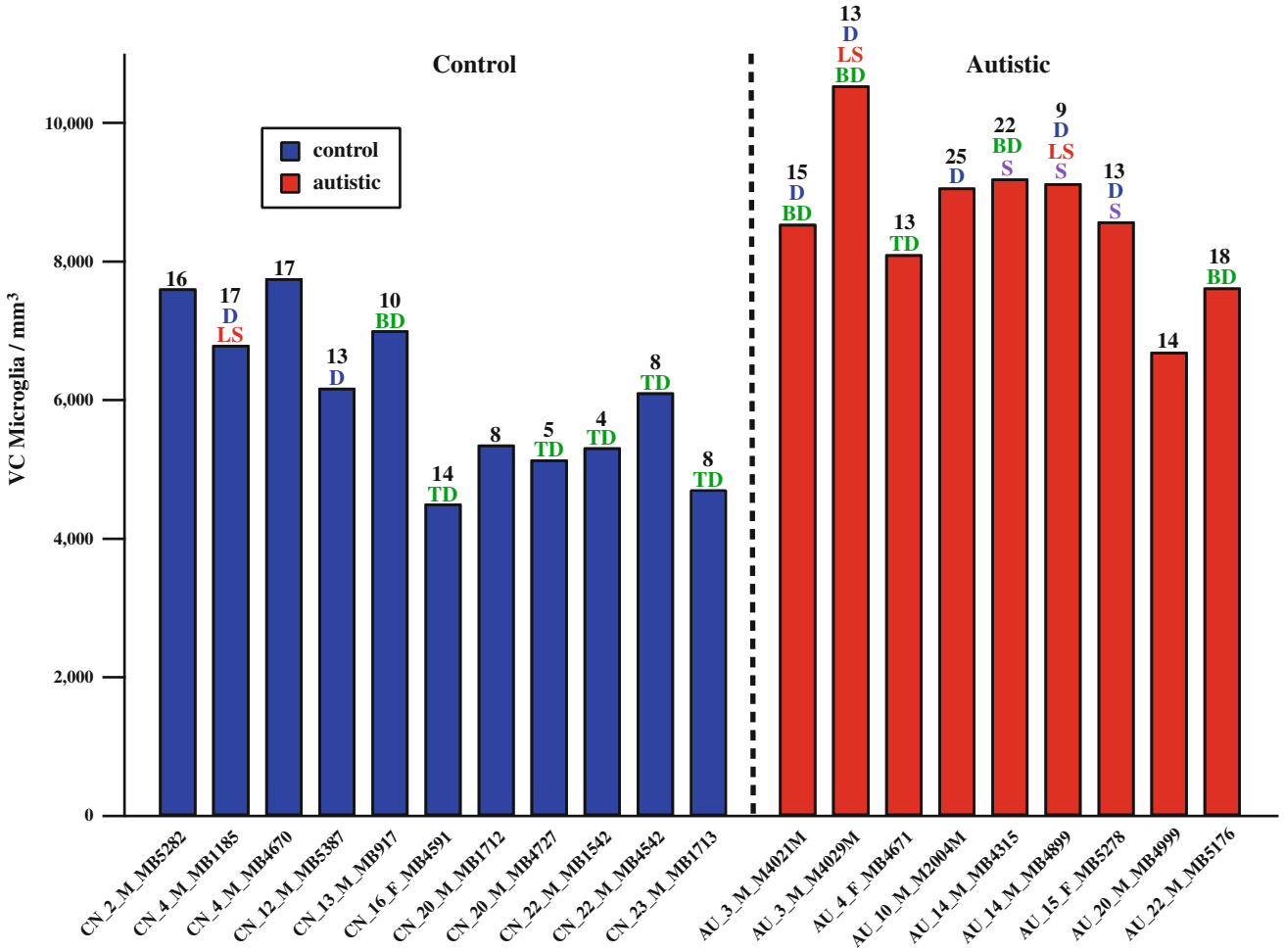

Fig. 3 Microglial densities in visual cortex in autistic and neurotypical brains are represented for the average of the replicated runs. Individuals with autism (n = 9) have a significantly greater density of total microglia, the key cellular participants in the inflammatory response in the brain, compared to controls (n = 11) $p = 0.0002$ (Mann–Whitney)

control FI (6,479 microglia per mm^3) and control VC (6,048 microglial cells per mm^3). In FI, individuals with autism had an 18 % higher microglial density compared to our neurotypical cases, and in VC 21 % higher microglial density compared to our neurotypical cases.

These findings demonstrate that, at the time of death, there were significantly higher microglial densities in the subjects with autism compared to the control subjects, and that this change in microglial density is widespread throughout the cerebral cortex in autism. The microglial densities in FI and VC in the same subject were significantly correlated (both measures were available in 10 controls and 8 autistic subjects for a total of 18 subjects) with Pearson's $r^2 = 0.4285$, $p = 0.0024$ (Fig. 6). This indicates that the elevation in density is consistent between these areas, and probably throughout the cortex, in both subjects with autism and controls.

We tested several confounding variables that could alter microglial densities in FI and VC of the subjects with autism and found no statistically significant relationship between microglial density and drowning versus other causes of death; traumatic versus other causes of death; having been on life support or not; having a recorded drug overdose or not; or having had seizures or not (Table 3). There was no significant difference between the subjects with autism and controls with respect to age of the subjects or post-mortem interval (PMI). However, the controls had significantly greater ($p = 0.0328$) brain weight (1,501 g) versus the subjects with autism (1,374 g) (Mann–Whitney test). This difference was driven mainly by one control subject (M5387) with very high brain weight (1,750 g). This is 310 g greater than the average brain weight (1,440 g) for a 12 year old male (Dekaban 1978), and when the outlier is removed there is no significant difference in brain weight between the subjects with autism and the control subjects. The differences in microglial density between individuals with autism and controls remain significant when the one outlier was removed for density

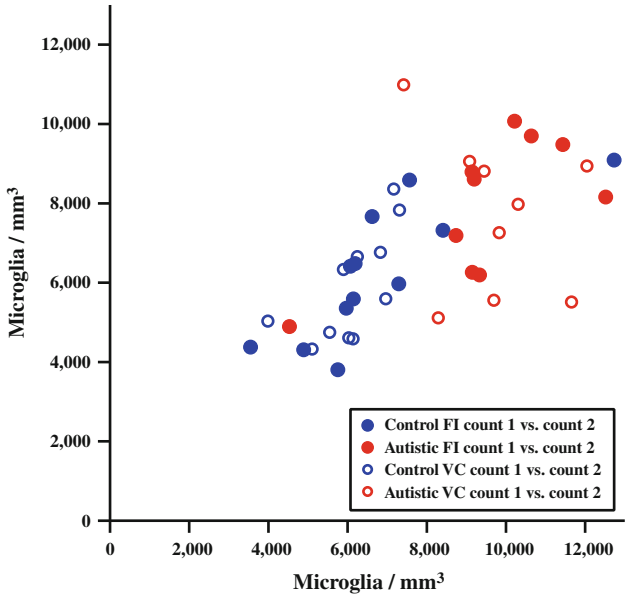

Fig. 4 The repeated blind density measurements are strongly correlated. Density measurement count one in FI versus density measurement count two in FI and density measurement count one in VC versus density measurement count two in VC (Pearson's $r^2 = 0.6480$, $p < 0.0001$) for two different regions of interest (ROI). When the blind replications are from the exact ROI the correlation is $r^2 = 0.9780$, $p < 0.0001$. Notably, the subjects with autism (FI *solid red circles* and VC outlined *red circles*) and controls (FI *solid blue circles* and VC outlined *blue circles*) cluster in FI and VC, except for one autistic outlier in FI (Color figure online)

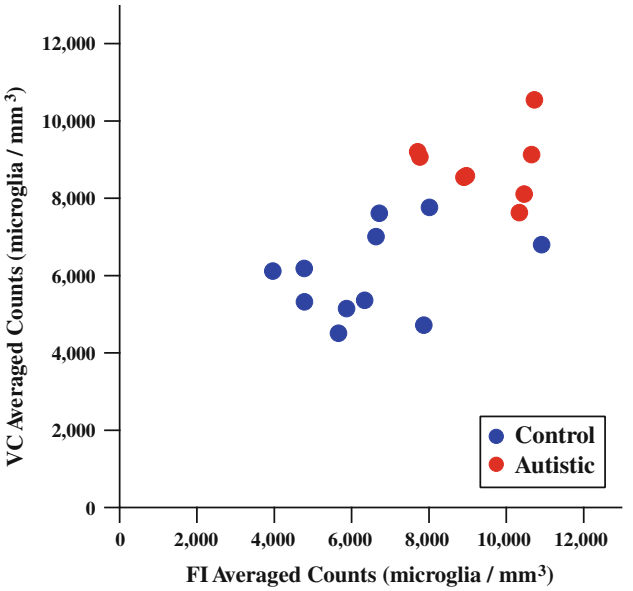

Fig. 6 Microglial densities in FI and VC are significantly correlated. Pearson's $r^2 = 0.4285$, $p = 0.0024$, for the sample of 10 controls and 8 individuals with autism in which measurements were available for both structures. Note that for both structures the individuals with autism (*red*) cluster, as do the controls (*blue*) (Color figure online)

measurements (FI, $p = 0.0257$ and VC, $p = 0.0001$, Mann–Whitney tests). In addition, brain weight and microglial density were not significantly correlated in individuals with autism compared to control cases for FI and VC (Table 3). Morgan et al. (2010) found brain weight was negatively correlated with microglial density in the grey matter, but that the microglial differences between subjects with autism and control subjects persisted when they statistically controlled for brain weight.

Microglial densities were negatively correlated with age in VC in our controls (Pearson's $r^2 = 0.6833$, $p = 0.0017$) and barely missed statistical significance in FI (Table 3). Microglial densities thus tend to decrease with age in controls, while in people with autism the microglial densities remain relatively high and constant with age in both FI and in VC. Finally, microglial densities in VC in controls were negatively correlated with PMI (Pearson's $r^2 = 0.3952$, $p = 0.0383$) but there was no significant correlation in VC for individuals with autism, or in FI for either group (Table 3). Morgan et al. (2010) found that microglial densities were negatively correlated with PMI across their subjects as a whole population but not for controls or people with autism as subgroups.

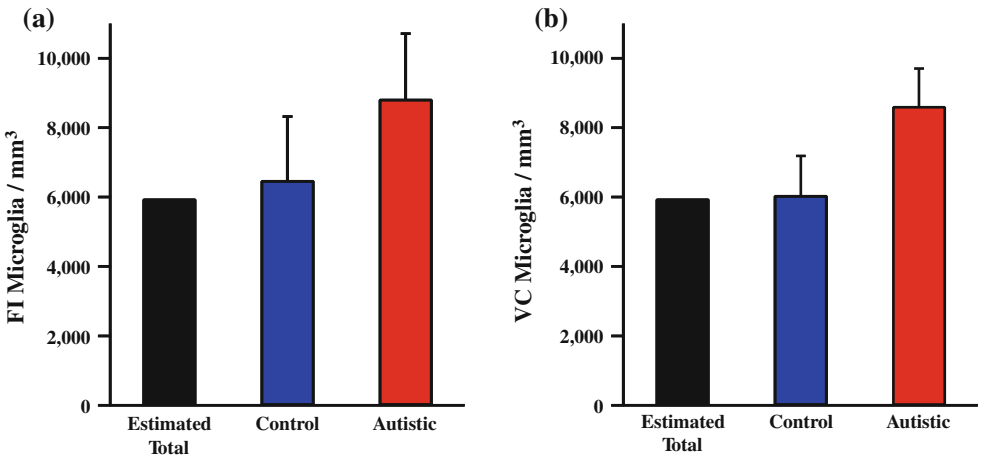

Fig. 5 **a** Average microglial densities for subjects with autism (*red*) and control subjects (*blue*) in FI in comparison to total microglial density (*black*) estimated from data in Frahm et al. (1982) and Lyck et al. (2009). **b** Average microglial densities in VC. *Error bars* represent the standard deviation (Color figure online)

Table 3 Confound statistics for the autistic cases

Confound	FI	VC
Drowning	FI autistics (6 drowning, 5 non-drowning): total density drowning versus other COD, $p = 0.7619$ (ns)	VC autistics (5 drowning, 4 non-drowning): total density drowning versus other COD, $p = 0.2857$ (ns)
Seizures	FI autistics (4 seizures, 6 no seizures): total density seizures versus no seizures, $p = 0.2571$ (ns)	VC autistics (3 seizures, 6 no seizures): total density seizures versus no seizures, $p = 0.7143$ (ns)
PMI	FI autistics, total density versus PMI, N = 10, Pearson's $r^2 = 0.0658$, $p = 0.4743$ (ns)	VC autistics, total density versus PMI, N = 9, Pearson's $r^2 = 0.00159$, $p = 0.9189$ (ns)
	FI controls, total density versus PMI, N = 12, Pearson's $r^2 = 0.2628$, $p = 0.0883$ (ns)	VC controls, total density versus PMI, N = 11, Pearson's $r^2 = 0.3952$, $p = 0.0383$ (significant)
Brian weight	FI autistics, total density versus brain weight, N = 10, Pearson's $r^2 = 0.0077$, $p = 0.8095$ (ns)	VC autistics, total density versus brain weight, N = 9, Pearson's $r^2 = 0.1311$, $p = 0.3384$ (ns)
	FI controls, total density versus brain weight, N = 11, Pearson's $r^2 = 0.00296$, $p = 0.6126$ (ns)	VC controls, total density versus brain weight, N = 10, Pearson's $r^2 = 0.0295$, $p = 0.6348$ (ns)
Age	FI autistics, total density versus age, N = 10, Pearson's $r^2 = 0.0080$, $p = 0.8054$ (ns)	VC autistics, total density versus age, N = 9, Pearson's $r^2 = 0.3477$, $p = 0.0947$ (ns)
	FI controls, total density versus age, N = 12, Pearson's $r^2 = 0.3159$, $p = 0.0572$ (ns)	VC controls, total density versus age, N = 11, Pearson's $r^2 = 0.6833$, $p = 0.0017$ (significant)

Drowning, seizures, PMI age and brain weight do not account for the increase in microglial density for autistics compared to the controls. The controls had significantly greater ($p = 0.0302$) brain weight (1,501.4 g) verses the autistics (1,356.7 g). This difference was driven mainly by one control subject (M5387) with very high brain weight (1,750 g) which is 310 g greater than the average brain weight (1,440 g) for a 12 year old male (Dekaban 1978) and when the outlier is removed there is no significant difference (ns) for brain weight comparing the autistic and control cases. The controls have a significant correlation for microglial density with age in VC ($r^2 = 0.6833$ and $p = 0.0017$), where over time the microglia decrease with age and a similar trend occurs in FI but does not reach statistical significance

We found that FI of two control subjects had unusually high microglial densities compared to the other controls. They were UMB1185, the 4-year-old control case, and the 23-year-old control UMB1713, who had suffered from head and neck injuries. The injuries sustained by UMB1713 are such that could cause an increase in microglial density if death was not immediate (Engel et al. 2000, Loane and Byrnes 2010). Both of these individuals showed increases in microglial densities in FI, but not in VC. By contrast, our subjects with autism had global increases in microglial densities, shown both in FI and in VC. This regional difference suggests the possibility of injury-related pathology in these two controls.

Discussion

We observed increased densities of microglia in two disparate cortical areas. One possibility is that these increased densities reflect abnormalities specific to these particular cortical areas, since there is evidence that each is involved in autism, or alternatively these results may reflect a widespread difference that occurs throughout the cortex or even much of the brain. Consistent with the possibility that the effect is pan-cortical, Morgan et al. (2010) reported an increase in microglia in subjects with autism in dorsal lateral prefrontal cortex (dlPFC) compared to controls, and found an increase in somal size in microglia in subjects with autism in grey and white matter. Five of Morgan et al.'s cases with autism overlap with those used in our study (Table 4). We found that the subjects with autism we had in common with Morgan et al. showed an increase in microglial density in both FI (five subjects in common) and VC (four subjects in common), which is consistent with Morgan et al.'s findings in dlPFC. In addition, Morgan found five of the thirteen individuals with autism had an increase in microglial activation (Morgan et al. 2010). Precedent for Morgan's and our microglial observations comes from Vargas et al. (2005) who found significantly more microglial activation in the cerebellum of autistic brains versus controls and a trend toward more microglial activation in the middle frontal and anterior cingulate cortices, although the cortical results were not statistically significant. One of our individuals with autism was used in the Vargas study (Table 4) (Vargas et al. 2005).

Our methodologies differed, however, in several respects from those of Morgan et al. (2010). We quantified microglia in two cortical regions, FI and VC, consistently in the right hemisphere, whereas Morgan quantified a single region, dlPFC, using either the right or left hemisphere. The reports of increased microglial densities are consistent, but there are differences in density measurements in Morgan's and our studies. The differences in density measurements for the individuals with autism and controls can be attributed to our differing calculations and consideration of the shrinkage factor within the tissue. To account

Table 4 Autistic cases used in the Vargas, Morgan and our study for microglial densities

ID	GUID	Vargas et al. (2005)	Morgan et al. (2010)	Tetreault et al. (this study)
UMB797	NDARYX624FEY	X	X	X
M4021M	NDAR_INVUX206VRV		X	X
M4029M	NDAR_INVRX268EH4		X	X
M2004M	NDAR_INVAK979XTP		X	X
UMB4899	NDAR_INVGW538MM3		X	X
UMB4671	Not provided			X
UMB4305	NDARWL137ER1			X
UMB4315	NDAR_INVHD069UM7			X
UMB5278	NDARYH540PL4			X
UMB4999	Not provided			X
UMB5176	NDARHU383HFF			X

For this study we quantified two regions in cortex, FI and VC which have not previously been quantified and showed that six additional autistic cases have increased microglial density measures. An X indicates that the subject was evaluated in the study

for shrinkage, we calculated the microglial density per mm^3 by dividing the optical fractionator estimate of the number of cells present in the full thickness of the section within the region of interest by the area of the region of interest and the thickness at which the section was cut. Our results for control samples are very close to values calculated for microglia based on the total number of microglia in the entire neurotypical cortex determined through stereology (Lyck et al. 2009) and cortical volumes (Frahm et al. 1982) (see Fig. 5a, b).

The differences between our study and Vargas et al. (2005) are that they stained microglia with an antibody to HLA-DR and used an area fraction quantification method based on the Delesse sampling procedure (Gundersen et al. 1988). That method gives an estimate of the fractional area of the region of interest covered by the cell type being measured. The Delesse method does not, however, produce cell numbers or three-dimensional densities. By contrast, we stained with an antibody to IBA1 and measured microglial density in our tissue. Though the specifics of antibody and methods differed, our data taken together with Vargas et al. (2005) and Morgan et al. (2010) point to elevated microglial density in autism, possibly involving the entire cerebral and cerebellar cortices. This argues that further investigation of microglial abnormalities and the microglial pathways in people with autism may be important for understanding the cellular basis of the autism phenotype.

There are also some caveats. We cannot be sure that IBA1 stains all microglia, and there is evidence for microglial heterogeneity (Carson et al. 2007; Mittelbronn et al. 2001; Schmid et al. 2009). However, the spacing of the stained microglia we have observed is consistent with complete coverage of a relatively regular array of microglia in the cortex. In addition, we found microglial densities in control FI and VC that are near the expected densities calculated from Lyck et al. (2009) and Frahm et al. (1982). On average the individuals with autism had 18–21 % higher microglial density in FI and VC compared to neurotypical subjects. How and when does the increased density of autistic microglial arrays arise, and how is it maintained? Of course we have no data prior to the time of death, but the consistency of results among 10 subjects with autism of differing ages argues that people with autism have developed a remarkably stable steady-state microglial density. Given the age range, this is probably established before age three. It is not clear how long the increase in microglia persisted in each of the subjects with autism, but our results show that control subjects have a significant negative correlation between microglial density in VC and age, indicating that microglial densities normally decrease throughout childhood and early adulthood in neurotypical subjects. However, in people with autism, there is a relatively steady condition of increased microglial density from childhood into adulthood. It seems possible that some persistent stimulus is the cause of this sustained higher level of microglial density in the subjects with autism. Imaging experiments of quiescent microglia in intact living cortex suggest that they conduct a complete surveillance of the cortex every few hours (Davalos et al. 2005; Nimmerjahn et al. 2005). The greater density, and thus closer spacing of the microglia, in brains of individuals with autism compared with control brains, suggests that this surveillance is more intense in autism.

Sickness behavior results from systemic infection and/or inflammation, driving an increase in signals to the brain that cause changes in metabolism, social withdrawal, appetite suppression and a general ill feeling (Exton 1997; Hart 1998; Perry 2010). Sickness behavior is another example of how a systemic infection or its related

inflammation can alter both behavior and the inflammatory response in the brain. There is evidence that maternal viral infection in the first trimester and bacterial infection in the second trimester are correlated with an increase in offspring reported to have autism (Atladóttir et al. 2010). In a recent microarray analysis of gene expression in brains of individuals with autism compared to controls, Voineagu et al. (2011) found a module of enriched immune and microglial genes, although these genes have not been found in genome wide association studies that have sought to identify genes that predispose to autism. Voineagu et al. (2011) conclude that the enriched gene expression of immune and microglial genes observed in their study has a non-genetic etiology and may reflect internal or external environmental influences, which suggests the possibility that the sustained higher levels of microglia density in people with autism may also be environmentally mediated.

Chez and Guido-Estrada (2010) report that a subset of people with autism have a consistent pro-inflammatory condition of the brain and cerebral spinal fluid and proposed that a systemic infection of the mother may lead to inflammation in the brain and autism. A recent report from Wei et al. (2011) found an increase of IL6 in cerebellar cortex in subjects with autism, which could alter cell migration and disrupt imperative circuits for normal development (Wei et al. 2011). In a mouse model of maternal infection for offspring brain development, it has been reported that offspring from a mother having a single injection of IL6 during pregnancy alters fetal brain development (Smith et al. 2007) which indicates that a maternal infection can impact brain development and may play a critical role in autism. Girard et al. (2010), using a lipopolysaccharide (LPS) mouse model of maternal inflammation, found that a single treatment of an IL-1 receptor antagonist, concurrent with the LPS injection, had the result that the IL-1 receptor antagonist protected against maternal placental inflammation and the offspring had normal brain development. Furthermore, it is well documented that peripheral infection can dysregulate inflammation in the brain and increase monocyte infiltration into the cerebral cortex (D'Mello et al. 2009); it is also reported that people with autism have elevated levels of cytokines (Chez and Guido-Estrada 2010) which may disrupt the homeostatic balance in the cortex resulting in a greater density of microglia.

Are Microglia Predators or Protectors?

The increased microglial densities we observed in the cortices of our subjects with autism appear to be a robust discriminator between the brains of people with autism versus neurotypical brains, and these findings raise a major question. Are markedly increased numbers of microglia among the originators of the pathologic processes in autism, or are they a response (perhaps even a protective one) to some other aspects of this condition? Microglia have neuroprotective functions including the phagocytosis of invading microorganisms and metabolic waste. The increase of microglial densities in individuals with autism could be a function of neuroprotection in response to harmful microorganisms.

In contrast, microglia can also phagocytize synapses and whole neurons, thus disrupting neural circuits. For example, when the axons of motor neurons are cut, the microglia strip them of their synapses (Blinzinger and Kreutzberg 1968; Cullheim and Thams 2007; Graeber et al. 1993). Another example of the disruption of circuitry arises from the direct phagocytosis of neurons. Neurons communicate with microglia by emitting fractalkine, which appears to inhibit their phagocytosis by microglia. Deleting the gene for the microglial fractalkine receptor (Cx3cr1) in a mouse model of Alzheimer's disease has the effect of preventing the microglial destruction and phagocytosis of layer 3 neurons that was observed in these mice in vivo with 2-photon microscopy (Furhmann et al. 2010). In particular, Cx3cr1 knockout mice have greater numbers of dendritic spines in CA1 neurons, have decreased frequency sEPSCs and had seizure patterns which indicate that deficient fractalkine signaling reduces microglia-mediated synaptic pruning, leading to abnormal brain development, immature connectivity, and a delay in brain circuitry in the hippocampus (Paolicelli et al. 2011). In summary, the increased density of microglia in people with autism could be protective against other aspects of this condition, and that a possible side-effect of this protective response might involve alterations in neuronal circuitry.

Microglial Defects as Causes of Disease

By contrast, there are diseases that arise from intrinsic defects in the microglia themselves which can cause stereotypic behavioral dysfunctions. A naturally occurring genetic defect in human microglia is the cause of a remarkable neuropsychiatric disease that was first observed in Japan and Finland, but has subsequently been found throughout the world. Nasu-Hakola disease is caused by a defect in the gene TREM2 or DAP12 which together form a receptor complex which is strongly expressed in microglia but not in astroglia or oligodendroglia (Paloneva et al. 2002; Sessa et al. 2004). In the Allen Brain Atlas, DAP12 is preferentially expressed in olfactory, anterior cingulate, and insular cortices in the mouse (http://mouse.brain-map.org/experiment/show/70523695). These defects in TREM2 or DAP12 impair the capacity of the microglia to phagocytose damaged tissue and increase the secretion of inflammatory cytokines in the olfactory, insular and

cingulate cortices resulting in microglia-mediated dementia specific to these structures (Bianchin et al. 2004; Neumann and Takahashi 2007). Bianchin et al. (2004) report that at around age 35 in affected patients there are: "[i]ncipient personality changes that can only be noticed by relatives and close friends. The behavioral alterations then become progressively more evident during the next months. The patients start to present silly and facetious behavior, lack of insight, social inhibition, and other unrestrained behavior. Sometimes they seem to have a euphoric attitude and are easily distractible, seemingly lacking adequate associated emotional components. As the disease progresses, the patients evolve to a state of profound dementia." The remarkable behavioral specificity of the microglial defect in Nasu-Hakola disease shows that the microglia can influence social behavior in a highly specific manner.

Another stereotypic behavioral defect arising from abnormal microglia is obsessive grooming in mice with a mutation of the gene Hoxb8 (Chen et al. 2010). Hoxb8 is expressed only in the microglia in the adult mouse brain, and these cells originate in spinal bone marrow (Chen et al. 2010). When adult mice with the Hoxb8 mutation were irradiated so as to kill the bone marrow and then received bone marrow transplants with the intact gene, the mice recovered from their excessive grooming pathology, their skin lesions healed, and their fur grew back to normal. When normal mice were irradiated and received bone marrow from donors with the mutated Hoxb8 gene, they developed the excessive grooming pathology. With these experiments and a variety of other elegantly executed controls, Chen et al. (2010) demonstrated that the Hoxb8 mutation with expression restricted to the microglia caused the pathological grooming behavior. This behavior resembles obsessive–compulsive disorder in humans, which involves abnormalities in orbito-frontal and anterior cingulate cortices (Graybiel and Rauch 2000). These structures are also implicated in autism (Allman et al. 2005; Di Martino et al. 2009; Santos et al. 2011; Simms et al. 2009). The excessive grooming in the Hoxb8 mice is also reminiscent of the stereotypical behaviors that are commonly found in a subset of individuals diagnosed with autism (Goldman et al. 2009).

Together with the striking changes in social behavior present in Nasu-Hakola disease, these data suggest that the circuitry of anterior cingulate and orbito-frontal cortices may be particularly sensitive to the disruptive effects of abnormal microglia. A strong association between reduced activity in the anterior cingulate and anterior insular cortices (adjacent to orbito-frontal cortex) in social tasks in subjects with autism versus controls was revealed in a meta-analysis of 24 functional imaging studies (Di Martino et al. 2009), and the reduced activity in these structures in autism may also be related to the apparent vulnerability of these structures to microglial disruption. This vulnerability might also be related to the preferential expression DAP12 in the anterior cingulate and insular cortices. Area FI investigated in our study corresponds to the ventral part of anterior insular cortex. Thus, while changes in microglial density appear to be widespread in brains of autistic individuals, some areas may be more vulnerable than others to its effects.

Visual Abnormalities in Autism

When we began this investigation we anticipated microglial abnormalities in the frontal cortices because many lines of evidence suggest that these structures are involved in autism (Allman et al. 2005; Courchesne and Pierce 2005; Di Martino et al. 2009). We included visual cortex based first on its lack of involvement in prominent social and homeostatic functions and its physical distance from FI. Yet, abnormalities in visual behavior are among the first signs of autism in infancy. Beginning at the end of the first year, the earliest signs of autism include atypical eye contact and visual tracking, and prolonged fixation, a tendency to perseverate visual attention on an original stimulus when presented with a competing stimulus (Zwaigenbaum et al. 2005).

The increased microglial densities in visual cortex may be representative of a pan-cortical microglial phenotype related to the autistic phenotype associated with perceptual integration. In Kanner's original description of autism he emphasized his patients' intense fixation on detail and "inability to experience wholes without full attention to the constituent parts" as a characteristic feature of the disorder (Kanner 1968). Frith (2004) drew attention to the tendency for typically developing children and adults to process information for meaning and gestalt (global) form, often at the expense of attention to or memory for details and surface structure. Happe and Frith (2006) proposed that autistic subjects show "weak central coherence," a processing bias favoring local information, and a relative failure to extract the gist or "see the big picture" in everyday life. The tendency of individuals with autism to focus on detail at the expense of global perceptions has been experimentally verified in many studies and may account in part for impairments in the recognition of faces (Behrmann et al. 2006; Happe and Frith 2006).

This difficulty perceiving the gist or global features of a stimulus configuration by subjects with autism may be analogous to the difficulties experienced by subjects with autism in making rapid intuitive decisions (Allman et al. 2005). A variant of the "weak coherence" theory applied to frontal lobe function and specifically linked to activated microglia and their possible role in altering the development of this structure was proposed by Courchesne and Pierce (2005). Happe and Frith (2006) propose that "weak

coherence" in autism is due to reduced connectivity throughout the brain due to lack of synchronization of neural activity (Brock et al. 2002) or lack of connecting fibers (Just et al. 2004) or lack of top-down connections (Frith 2004). A similar disruption of global connectivity might disrupt the integrative functions of FI in social behaviors as suggested by the reduced activity in this area in subjects with autism versus controls (Allman et al. 2010; Di Martino et al. 2009). An increased density of microglia throughout the cortex in people with autism might contribute to these phenomena through alterations in the neuronal circuitry.

Summary

We found significantly increased microglial densities in individuals with autism in two widely separated and functionally different cortical areas, FI and VC. In light of our findings, as well as increased densities in the cerebellum (Vargas et al., 2005) and dorsal lateral prefrontal cortex (Morgan et al. 2010), we propose that microglial densities are elevated throughout the cerebral and cerebellar cortices in individuals with autism. Future studies are needed to explain the mechanisms responsible for the increased densities and the relationship between this phenomenon and the behavioral manifestations of autism.

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Chapter 3: Microglial and Purkinje Cell Densities in the Cerebellar Cortex in Autism, Rett, Angelman, and Joubert Syndromes

Abstract

Numerous studies report alterations in the cerebellum in autism spectrum disorder (Palmen et al., 2005; Fatemi et al, 2012). The pioneering study by Vargas et al. (2005) revealed evidence for microglial expansion and increased cytokines in the cerebellum of subjects with autism, which suggested the presence of neuroinflammation in this structure. Microglia, the immune cells in the brain, are the first defense against infection, and phagocytize foreign debris. In addition, microglia are essential for normal development and synaptic remodeling. We investigated the neuropathology of the lateral cerebellum and vermis in individuals with autism, as well as Rett syndrome, Angelman syndrome, and Joubert syndrome, since each of these neurodevelopmental disorders have similar behavioral phenotypes as autism spectrum disorder. We found a significant increase in the microglia density in the molecular layer, the location of Purkinje synapses, in individuals with autism and Rett syndrome. In addition, we found a significant reduction in the vermal Purkinje cells in individuals with autism. Future studies dedicated to the neuroinflammation in autism are essential. In particular, investigating the function and modification of microglia on synaptic connectivity in the cerebellum is crucial for developing therapeutics in autism spectrum disorder.

Introduction

Neuroinflammation has been widely reported in autism spectrum disorder (Vargas et al. 2005, Pardo et al. 2005, Zimmerman et al. 2005, Wei et al. 2011, Morgan et al. 2011 and Tetreault et al. 2012). Neuroinflammation is classically defined as an activation of microglia and astrocytes and the increased production of cytokines such as Interleukin-6 (IL6), and Tumor Necrosis Factor-alpha (TNF-alpha) (Monnet-Tschudi et al. 2011). Thomas and colleagues report that a gene network, including IL6 and several other genes, is characteristic of activated versus quiescent microglia (Thomas et al. 2006). Findings show that individuals with autism have significantly increased cytokines in frontal cortex and elevated levels of cytokines in the CSF compared to control cases (Li et al. 2009; Zimmerman et al. 2005). In the development of children with autism there is there is evidence for immune system dysfunction (Ashwood et al. 2006; Chez and Guido-Estrada 2010). The pioneering studies by Vargas et al. (2005) report inflammation is widespread throughout the brains of individuals with autism, finding an increase in inflammatory cytokines in the cerebellum and cerebral cortex in cases with autism (Vargas et al. 2005). In particular, there is evidence that microglia cells are elevated in the cerebellar cortex and cerebral cortex of patients with autism (Vargas et al. 2005, Morgan et al. 2011 and Tetreault et al. 2012). The classical role of microglia is to provide defense against organisms invading the CNS and clear the waste through the process of phagocytosis (Garber and Streit 1990, 2010). Microglia are the brain's immune responders and defenders. In a previous study, investigators reported an increase in HLA-DR labeled microglia in the gray and white matter of the cerebellar cortex in human postmortem

tissue of individuals with autism compared to controls (Vargas et al. 2005). More recently, reports of increased microglia in individuals with autism compared to controls in the visual cortex and fronto-insular cortex, two disparate areas, suggest a global increase in microglia throughout the cerebral cortex (Tetreault et al. 2012). Wei and colleagues (2011) described an increase of IL6 in the cerebellum of individuals with autism and hypothesize that the elevated IL6 activates microglia for the function of synaptic pruning of the Purkinje cells.

Microglia are known to function not only as inflammatory cells and macrophages for the central nervous system but also in synaptic pruning and plasticity (Wake et al. 2009, Schafer et al. 2012). Studies using two-photon imaging of fluorescently labeled microglia in mouse brains show the microglia are actively probing microzones of brain tissue and responding to neuronal stimuli by retracting and expanding their processes (Nimmerjahn et al. 2005, Davalos et al. 2005 and Wake et al. 2009). Synaptic plasticity, the hallmark of neuronal function and network organization, is regulated by the alteration in the number of synapses. In development, synaptic pruning occurs at crucial timing for proper brain function and development of cortical and cerebellar networks; the pruning of synapses is an active process of microglial cells in particular in early stages in development and throughout life (Blinzinger and Kreutzberg 1968; Graeber et al. 1993; Kreutzberg 1996; Paolicelli et al. 2011; Schafer et al, 2012). Microglia make contact with synapses at a frequency dependent on the activity of the neurons (Wake et al. 2009). In a model of neuronal degeneration, microglia contact synapses more frequently and actively remove synapses of degenerating cells (Wake et al. 2009). It appears that individuals with autism have chronic neuroinflammation, which could disrupt the central nervous system

connections and alter networks and contribute to synaptic alteration and cell death (Wood 2003, Streit et al. 2004).

In addition to the neuroinflammation in the cerebellum reported in individuals with autism, there have been several studies that report cerebellar histopathology in individuals with autism. In particular, the Purkinje cells, the only output cells from the cerebellar cortex, have been reported to show reduced size and number in individuals with autism compared to controls (Ritvo et al. 1986, Bauman and Kemper 2005, Bailey et al. 1998, Fatemi et al. 2002, Wegiel et al. 2013). The increased neuroinflammation and reduced numbers of Purkinje cells in the cerebellum of individuals with autism, compared to controls, inspired this study.

We set out to investigate the role of inflammation in the cerebellum in autism spectrum disorder using human postmortem tissue from the lateral cerebellum and the vermis obtained from the NICHD Maryland Brain Bank for Developmental Disorders. We quantified microglial volume densities, Purkinje cell linear densities and granule cell volume densities in the cerebellum of well-phenotyped individuals with autism (n=10) and controls (n=11). In addition, we measured the size and shapes of the microglia somas and Purkinje cell somas in individuals with autism compared to controls. We also investigated several individuals with rare conditions related to autism (Schwichtenberg et al. 2013, Jellinger et al. 1988, Bonati et al. 2007, Clayton-Smith and Laan 2003, Holroyd et al. 1991). We included an individual who was one member of a triplet set, another member of which had autism. This individual had been carefully evaluated for autism but was diagnosed with social anxiety disorder. We also studied three individuals with Rett syndrome, an individual with Angelman syndrome and an individual with Joubert

syndrome. Our goal in this study is to better understand the role of neuropathology of the cerebellum in autism spectrum disorder and other related neurodevelopmental disorders.

Methods

Tissue Samples

NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland-Baltimore provided postmortem formaldehyde-fixed (8% solution) human lateral cerebellum and vermis, as shown in Table 1. The NICHD Brain and Tissue Bank for Developmental Disorders provided detailed clinical records for each individual with autism whose brain we studied, as summarized in the phenotypic descriptions in Table 2. To confirm the diagnosis of autism the medical records of each individual with autism was reviewed by a clinical psychologist (EA) who specializes in autism. For each individual with autism we have at least one thorough clinical description of the subject by either a psychologist or psychiatrist. Most of our subjects with autism had the Autistic Diagnostic Interview-Revised (ADI-R), which is the result of a structured interview with a parent of the individual with autism. In addition, the records include measures of behavioral development, a history of medications and other health issues reported by physicians and clinical psychologists, described in Table 2. For the sibling of an individual with autism, and for individuals with Rett, Angelman, and Joubert syndromes, the medical records are also described in Table 2. In addition, genetic information was provided for two of the individuals with Rett syndrome and the individual with Angelman syndrome. Each of these developmental disorders was specifically chosen for the behavioral similarities to autism and previous reports of abnormalities in the

cerebellum (Schwichtenberg et al. 2013, Jellinger et al. 1988, Bonati et al. 2007, Clayton-Smith and Laan 2003, Holroyd et al. 1991). In particular, investigating behaviorally similar disorders to autism allows for the inquiry of whether there are core features in the neuropathology of the cerebellum across the different disorders.

Sectioning and Staining

Tissues were sectioned in the parasagittal plane at 50 μm on a microtome with a vibrating blade (Microm HM 650V) in 0.1 M phosphate buffer solution (PBS) and stored in well dishes with PBS and sodium azide until used for staining. The microglia were immunocytochemically stained with an antibody specific to IBA1 (ionizing calcium adaptor molecule-1) as previously described in Tetreault et al. (2012). Each case was Nissl stained with 1% cresyl violet for visualization of neurons. Nissl stained sections were used for the Purkinje cell counts, Purkinje cell shape analysis, and granule cell counts.

Quantification of Microglial Densities

Microglial density in the cerebellum was measured blind to phenotype and quantified using the program Stereo Investigator (MBF Bioscience, Williston, VT) with a Reichert Polyvar microscope equipped with a motorized stage and a camera for visualization. The molecular cell layer and granule cell layer microglial were quantified as two separate regions of interest per sample to compare the microglial densities in the different cerebellar layers. A subset of sections was quantified in two separate replications with the same regions of interest. Adjacent sections of cerebellum were quantified as assays for reproducibility. Independent raters also quantified random sections as another assay for reproducibility. The represented density measure is an

average of the blind replicated runs for the section. For the microglial quantification, we used the same method as the quantification for cortex previously reported by Tetreault et al. (2012) to directly compare the microglial density in the cerebellum with the cortex, as shown in Figure 1. Estimated cell counts were performed using the optical dissector probe with a 40X objective (oil immersion NA=1.0) with a dissector height of 16 µm (flanked by 2.0 µm guard zones), a counting frame of 260 µm x 160 µm and a grid size of 425 µm x 425 µm. To avoid oversampling, we used the Gunderson counting rule such that cells intersecting only three of the six surfaces of the dissector cube were counted. Microglial density per mm^3 was calculated by dividing the optical fractionator estimate of the number of cells present in the full thickness of the section within the region of interest by the area of the region of interest and the thickness at which the section was cut to account for tissue shrinkage (Tetreault et al. 2012). When the blind replications are from the exact ROI for the cerebellar microglial counts the correlation is r=0. 9370, P< 0.0001 for the intra-rater reliability.

Microglia Shape Analysis

The investigator, who was blind to case phenotype, outlined the microglial somas using Neurolucida software (MBF Bioscience, Williston, VT). For each case, 50 microglia somas were traced using a 100X objective and analyzed for size and shape for the molecular layer and an additional 50 for the granule cell layer. The cells were randomly chosen using the stereological probe designed for the microglial counts. The measurements for the shape analysis were area, perimeter, Feret maximum, Feret minimum, aspect ratio, compactness, roundness, convexity, shape factor, form factor and

solidity. The average for the 50 cells was taken for each of the measurements for molecular and granule cell layers for each sample. The means for the groups were measured and analyzed.

Purkinje Cell Counts

Linear quantification of the Purkinje cells was measured for each of the cases using Neurolucida software (MBF Bioscience, Williston, VT) and the investigators were blind to the case phenotype. A subset of sections was quantified in two separate replications with same regions of interest. Adjacent sections of cerebellum were classified and quantified for reproducibility. Independent raters quantified and classified blind random sections to replicate the method. The entire Purkinje cell layer was traced, and 100 percent of the cells were counted in the Purkinje cell layer for the section, as shown in Figure 3. The number of Purkinje cells is represented per mm and is calculated by the total number of cells counted in the Purkinje cell layer divided by the total length of the Purkinje cell layer for the section. The reproducibility for the two independent raters of the Purkinje cell counts are significantly correlated with coefficient $r= 0.9866$ and a p-value < 0.0001 for the intra-rater reliability.

Purkinje Cell Shape Analysis

For the Purkinje cell shape analysis, the investigator was blind to case phenotype. The Purkinje cells were traced and analyzed using Neurolucida software (MBF Bioscience, Williston, VT). For each case, 100 Purkinje cells were traced and analyzed for size and shape. The measurements included area, perimeter, Feret maximum, Feret

minimum, aspect ratio, compactness, roundness, convexity, shape factor, form factor and solidity. The average for the 100 cells was taken for each of the measurements for each sample. The means for the groups were measured and analyzed.

Granule Cell Counts

Granule cell density in the cerebellum was measured blind to phenotype and quantified using the program Stereo Investigator (MBF Bioscience, Williston, VT) with a Nikon Eclipse 80i microscope equipped with a motorized stage and a camera for visualization. Samples used for granule cell measurements were the same as for Purkinje cell measurements. The granule cell layer was traced at 10X magnification around one leaflet of the cerebellum, with boundaries at the Purkinje cell layer and the deep cerebellar nuclei. Estimated cell counts were performed using the optical dissector probe at 100X magnification (oil immersion NA=1.0) with a dissector height of 6 μm (flanked by 1.0 μm guard zones), a counting frame of 20 μm x 20 μm and a grid size of 200 μm x 200 μm. The top of the section was refocused at each counting site. To avoid oversampling, the Gunderson counting rule was applied so that cells intersecting only three of the six surfaces of the dissector cube were counted. Granule cell density per mm^3 was calculated by dividing the optical fractionator estimate of the number of cells present in the full thickness of the section within the region of interest by the area of the region of interest and the thickness at which the tissue was cut to account for tissue shrinkage.

Statistical Analysis

All statistical analysis was done using InStat Statistical Software (GraphPad Software, Inc., La Jolla, CA). A Mann-Whitney test with a two-tailed P value was used to compare the microglial densities, Purkinje cell linear measurements, and granule cell densities between the groups, which included individuals with autism and Rett syndrome and the control subjects. In addition, the Mann-Whitney test with a two-tailed p value was used to compare the differences between groups for the microglia cell shape analysis and Purkinje cell shape analysis. Pearson's correlation was used to measure correlations for the microglial cell counts, Purkinje cell counts and the granule cell counts in the individuals, correlations for the cell counts and the ADI-R scores, and correlations for individuals that had both microglial density measurements in the cortex and microglial density measurements in the cerebellum. Possible confounds in the subjects with autism that could alter their microglial density, Purkinje cell linear measurements and granule cell densities were examined. Binary confounds, including whether death was by drowning and whether the subject had seizures, or had taken medication, the subject's brain weight, and the subject's post-mortem interval were tested using the Mann-Whitney test; a possible confounding correlation with post-mortem interval was tested using Pearson's r- squared, as shown in Table 6.

Results

Figure 5 depicts the average microglial densities in frontoinsular cortex (A), visual cortex (B), the cerebellar granule cell layer (C) and the cerebellar molecular layer (D). Previously reported by Tetreault et al. (2012), individuals with autism have significantly greater microglial density in the frontoinsular cortex (18%) and visual cortex (21%) compared to neurotypical cases. In both vermis and lateral cerebellum,

there was no difference in microglial densities in the granule cell layer in individuals with autism compared to controls. There were, however, significantly greater (P=0.0009, two tailed t- test) microglia densities in the molecular cell layer in both vermis and lateral cerebellum in individuals with autism (n=9) compared to controls (n=9). Microglia density was 69% greater in the molecular layer in individuals with autism compared to controls, a more than threefold increase than in FI and VC in individuals with autism compared with controls. Since the NICHD Brain and Tissue Bank for Developmental Disorders provided all the tissue for autistic and control cases, we were able to directly compare microglial counts in the cortex and cerebellum for a number of cases (autistics n=6 and controls n=5) using the same method, as shown in Table 3. The cerebellum for both controls and autistics has significantly greater microglia density (P< 0.0000001, two tailed t-test) compared to the density of microglia in the cortex. When comparing the correlations of microglial densities in the same individuals where both cortical and cerebellar microglia measurements were made, no significant correlations were found. [Figure 5 Top]

Figure 6 exhibits the microglial cell densities for the molecular cell layer and the granule cell layer where each data point represents the average for an individual. The microglial cell densities are an average of the vermis and lateral cerebellum for an individual since there were no significant differences in the microglial densities for the two regions. The individuals with autism (n=9) have significantly greater microglia densities for the cerebellar molecular layer with an average of 24,611 microglial per mm^3 compared to the controls (n=9) with an average of 14,090 microglia per mm^3 (p value is 0.0009, two tailed t-test). The individuals with Rett syndrome (n=3) have a significantly

greater microglia density in the molecular layer, with an average of 29,764 microglia per mm^3, compared to controls (n=9), with an average of 14,090 microglia per mm^3 ($p < 0.0001$, two tailed t- test). In addition, there was no significant difference in the microglial densities when comparing the individuals with autism (n=9) and individuals with Rett syndrome (n=3). We considered the possibility that the high density of microglia in the individuals with Rett syndrome might be due small brain size in these cases. The microglial density in the Rett syndrome cases is 2.1 times that of the controls. The average brain size in the Rett syndrome cases is 927 grams compared with 1404 grams in the controls and thus the Rett syndrome brains are 2/3rds of the weight of the controls, which is not sufficient to explain the microglial density differences.

The individual with Angelman syndrome (n=1), with a microglia density of 37,263 mm^3, and sibling of an individual with autism (n=1), with a 29,764 mm^3 microglia density in the molecular layer of the cerebellum, each had densities similar to the individuals with autism and Rett syndrome. The individual with Joubert syndrome (n=1) had the lowest microglial densities in the molecular layer (6,168 mm^3) of all the subjects. Thus, the microglial densities in the molecular layer for the individuals with Rett and Angelman syndromes are in the high end of the range for the individuals with autism, whereas the densities for the individual with Joubert syndrome are lower than for any control case.

[Figure 6 Top]

There was a significant increase in the microglia soma perimeter (p=0.037, two tailed t-test) and area (p=0.042, two tailed t-test) in the molecular layer in the individuals with autism (n=8) compared to controls (n=8), illustrating that individuals with autism

have larger microglia somas in the molecular layer than controls. The same is true for the individuals with Rett syndrome (n=3) where the microglia soma area (p=0.017, two tailed t-test) and perimeter (p=0.004, two tailed t-test) are significantly larger than controls (n=8) in the molecular layer. There is no significant difference between the individuals with Rett syndrome and autism for the area and perimeter when measuring the microglia soma in the molecular layer, as shown in Figure 7. In addition, the Feret max is significantly (p=0.012, two tailed t-test) larger in the autistics, which indicates that the soma is longer in its principal axis than that of the controls. For the molecular layer, the microglia somas are larger and more irregular in autistic individuals compared to the controls. In the granule cell layer there is no difference in the microglial soma comparing the autistics and controls. The individuals with Rett syndrome have a significantly larger microglia area (p=0.0076, two tailed t-test) compared to the controls in the granule cell layer.

[Figure 7 Top]

There are more microglia in the molecular layer in individuals with autism and the area and perimeter of the microglia somas are larger and more irregularly shaped compared to the controls. However, in the granule layer there is no significant difference, comparing individuals with autism and controls, in microglia density and morphometry. The increase in microglia densities in the cerebellum is specific to the molecular layer, which is the location of vast numbers of synapses on Purkinje dendrites.

There was a significant (p=0.024, two tailed t-test) decrease in the Purkinje cell linear density in the vermis of individuals with autism (n=7) with an average of 8.11 Purkinje cells per mm compared to controls (n=10) with an average of 10.21 Purkinje

cells per mm, as shown in Figure 8. This result is specific to the cerebellar vermis, as there is no significant difference for the Purkinje cell linear measurement in the lateral cerebellum when comparing controls (11.16 Purkinje cells per mm) and individuals with autism (10.22 Purkinje cells per mm.) Within the cerebellar cortex in individuals with autism, the linear density of Purkinje cells was significantly lower ($p=0.017$, two tailed t-test) in the vermis (8.11 Purkinje cells per mm) than in the lateral cerebellum (10.22 Purkinje cells per mm). Figure 9 exhibits the average per case represented as a single data point for the Purkinje cell linear measurements in the vermis. There were no significant differences between the individuals with Rett syndrome compared to the individuals with autism and to the neurotypical subjects. In the individual with Joubert syndrome, the Purkinje cell density was comparable to the high end of the controls. The Purkinje cell linear density in the sibling of an individual with autism was similar to the density in individuals with autism for the vermis.

[Figure 8 Top]

[Figure 9 Top]

For the Purkinje cell morphometry, there was no difference for the perimeter and area between the autistic and control cases but there was a significant difference for a number of measures, which all describe the Purkinje cells as rounder in people with autism, as shown in Table 4.

There were no significant differences in the granule cell densities in individuals with autism, Rett syndrome or controls. There was no correlation for the microglial cell counts and Purkinje cell counts in the cases with autism, Rett syndrome and controls. For the individuals with autism, none of the ADI-R scores correlated with the cell counts or

cell morphometry measurements. Finally, the confounds in the cases with autism did not account for the differences in the microglial density measures, the Purkinje cell counts, granule cell counts and the shape analysis for the microglia and Purkinje cells.

The important findings of this study include an increase in the microglia density in individuals with autism and Rett syndrome specific to the molecular layer in the cerebellum, which is the region of the Purkinje and parallel fiber synapses. Moreover, we report that not only is there an increase in the microglia density in the molecular layer, but that the microglial cells are larger in perimeter and area in individuals with autism spectrum disorder compared to controls, which indicates the microglia are in a different state. In addition, we found that the individual with Angelman syndrome and the individual whose sibling had autism exhibited similar microglia densities in the molecular layer as individuals with Rett syndrome and autism spectrum disorder. We also found a specific and significant decrease in the Purkinje cells in the vermis of individuals with autism compared to controls. Together, these data provide evidence for neuropathological and neuroinflammation alterations in individuals with autism spectrum disorder.

Discussion

Microglia and Neuroinflammation

Our group is the first to report a significant increase in microglia densities and larger microglia somas that are specific to the molecular layer of the cerebellar cortex in individuals with autism and Rett syndrome compared to controls. The specific increase of microglia density in the molecular layer, which is largely comprised of parallel fiber axons synapsing on Purkinje cell dendrites, has several implications for the role of

inflammation in the neuropathology in the cerebellum of individuals with autism, which will be discussed below. The lack of differences in granule cell density and in microglia density in the granule cell layer of the subjects with autism compared to controls are additional lines of evidence suggesting that the abnormalities are particularly localized to the molecular layer of the cerebellum in autism.

Vargas et al. (2005) were the first to report an increase in microglia in the cerebellar cortex in individuals with autism compared to controls, but their methods and results differ from ours in several respects. In Vargas' analysis, the group used the antibody HLA-DR to stain the microglia and measured the microglia with an area fraction quantification method based on the Delesse sampling procedure (Gundersen et al. 1988). In the area fraction quantification method, the fraction of a two-dimensional area covered by a specific cell type is measured, but the method does not provide a three-dimensional volume density. In contrast, we quantified IBA1 immunostained microglia in separate determinations for the molecular and granule cell layers using the optical fractionator method, which provided volume densities for these layers. Despite these differences, both studies point to an increase of microglia in the cerebellar cortex in individuals with autism. These findings elucidate the neuropathology of the cerebellum in autism and suggest that a better understanding of the role of the inflammation in the cerebellum could aid in the understanding the behaviors in autism.

We also found that the microglia somas were larger in the individuals with autism and Rett syndrome suggesting that the microglia were in the activated state. Kozlowski and Weimer (2012) used the method of automated image analysis to study the morphology of fluorescently labeled microglia in mice, which had been challenged with a

standard method for inducing microglial activation. The method involved intravenous injections of lipopolysaccharide (LPS), an inflammatory agent from the outer cell wall of bacteria. Kozlowski and Weimer observed a 23% increase in the size of microglia following injections of 1 milligram of LPS per kilogram of body weight after 24 hours. This result is close to the increased size we observed in autistic (20.6%) and Rett (19.6%) microglia compared to controls. Thus, the increased microglia sizes in autistic and Rett syndrome microglia are comparable to those obtained in experimental microglial activation with the standard LPS protocol for inflammatory challenge suggesting that the microglia may be continuously activated in these conditions. It should be noted, however, that in the mice challenged with LPS and in the individuals with autism and Rett syndrome, there is considerable variance in the sizes and therefore presumably the activation states of individual cells in their microglial populations.

We are able to directly compare microglia density measures in the cerebral cortex and in the cerebellar cortex since we used the same stereological method and many of the same individuals in the prior study (Table 3). In our previous study we found on average an increase of 20% in microglia density in the cortex in individuals with autism compared to controls whereas in the molecular layer of the cerebellar cortex there is a 69% increase in the microglia in individuals with autism compared to controls. Thus there are increased densities in both the cerebral and cerebellar cortices, but the magnitude of the effect is about 3.5 times greater in the cerebellum. Suzuki et al. (2013) measured the microglia activation in living individuals with autism using positron emission tomography and radiotracer for microglia—[11C](R)-(1-[2-chrorophynyl]- N-methyl- N-[1-methylpropyl]-3 isoquinoline carboxamide) ([11C](R)-PK11195)M and found increase of

binding potentials in several brain regions in individuals with autism compared to neurotypical subjects. The cerebellum, together with the brainstem and orbitofrontal cortex, showed the greatest increases in microglial binding in their study. Thus the increases in microglia size and/or activity in the cerebellum have been demonstrated for subjects with autism by two very different methods.

These observations lead to the following questions. Why is there an increase in microglia density in the brains of individuals with autism? Is the increase in microglia neuroprotective or deleterious in the individuals with autism? What is the relationship between the microglia and neurocircuitry?

Microglia are known to become activated due to infection, traumatic brain injury, stroke and neurodegenerative diseases; microglia in the quiescent stage are actively monitoring their environment and then upon activation engulf and eliminate debris in the phagocytic phase (Lin and Bergles, 2004, Vargas et al. 2005, Wake et al. 2009). The pioneering studies of Davalos (2005) and Nimmerjahn (2005) showed, using the method of *in vivo* imaging of microglia, that quiescent microglia on a minute-to-minute basis constantly probe their environment in the healthy mouse cerebral cortex, which laid the foundation for studies to understand the function of microglia in the healthy brain. Microglia are known to respond to a number of neurotransmitters, including GABA, glutamate, and dopamine, where excitatory neurotransmission has been shown to increase microglial process motility and inhibitory neurotransmission-reduced microglia movement (Barger and Basile, 2001, Nimmerjahn et al. 2005, Grinberg et al. 2011). Using live two-photon imaging of fluorescently labeled microglia and neurons, microglia processes make brief (5 min.) and direct contact with synapses in the resting state, and

after transient brain ischemia the microglia make prolonged (1 hr.) contact with the synapses, often with a receding bouton (Wake et al. 2009). Using the unique feature of postnatal development of the visual system and high resolution imaging of microglia, investigators report that during the peak critical period (P28), microglia in layer II and VI contacted spines, synaptic terminals and synaptic clefts within the visual cortex upon visual stimulation (Tremblay et al. 2010).

Microglia are essential for the normal development of the brain. Microglia sculpt the population of Purkinje cells in the normally developing cerebellum (Marin-Teva et al. 2004). Paolicelli (2011) reported that microglia actively engulf synaptic components and are essential for synaptic pruning in the development of the normal mouse brain (Paolicelli et al. 2011). Microglia are also essential for the synapse elimination and pruning that occur in the normal development of the retino-geniculate pathway in mice (Schafer et al, 2012). Microglia are necessary for the masculinization of the male mouse brain and behavior, where the microglia are activated in the hypothalamic preoptic area (POA) of the male brain and promote the formation of more numerous spines than are present in the POA in females (Lenz et al. 2013). These findings demonstrate evidence that microglia are active in the healthy brain and function as a key player in normal development of neural circuits through synaptic growth and pruning, axon guidance and apoptosis.

Maternal infection increases the risk of neurodevelopmental disorders, specifically autism (Atladóttir et al, 2010, Brown and Patterson, 2011). A mouse model of maternal immune activation using a poly (I:C) injection on embryonic day 9.5 and/or 12.5 reported a deficit in the linear density of Purkinje cells specific to lobule VII of the

cerebellum and indicates the alteration of the Purkinje cells is caused by maternal activation of the immune system occurring during embryonic development (Shi et al. 2009). A recent study describes that valproic acid, a common pharmaceutical drug used to treat epilepsy, has been significantly associated with an increase in autism spectrum disorder, even after adjusting for maternal epilepsy (Christensen et al. 2013). An *in vitro* study of primary adult human microglia found that valproic acid reduced the phagocytic activity of microglia and the expression of microglial markers (Gibbons et al. 2011). Nasu-Hakola disease is a dementia caused by a defect in the TREM2 or DAP12 gene which forms a receptor complex and is strongly expressed in microglia (Paloneva et al. 2002; Sessa et al. 2004). This genetic defect reduces the phagocytic activity of the microglia, impairs the clearance of debris in the cortex and induces deficits in social behavior as an early manifestation of Nasu-Hakola dementia (Bianchin et al. 2004; Neumann and Takahashi 2007). Mutations of TREM2 are also found in a subset of patients with late onset Alzheimer's disease (Jonsson et al, 2013; Guerreiro et al, 2013). These data support the hypothesis that suppressing microglial phagocytosis can alter brain development and lead to neurodegeneration later in life. Future studies focused on understanding the microglial phagocytic activity could have numerous benefits for understanding neurodevelopmental and neurodegenerative disorders.

Purkinje Cells

We also report a reduction in the Purkinje cell linear density in the vermis of individuals with autism compared to vermis in controls and to the lateral cerebellum in individuals with autism. In Table 5, we show the previous work for the Purkinje cell qualitative and quantitative reports for individuals with autism and Rett, Angelman and

Joubert syndromes, which we investigated because subjects with these disorders often exhibit the behavioral features of autism. It is also been reported that individuals with Rett syndrome exhibit reductions in Purkinje cells; however, we did not observe a reduction in the three individuals in our study.

Exciting new research using a genetic mouse model of Rett syndrome, the MECP2 knockout, produces behavioral and synaptic phenotypes similar to autism (Chen et al. 2001, Guy et al. 2001). Investigators reported that replenishing a MECP2-knockout with wild-type microglia reduced a number of behavioral and physiological deficits and restored normal lifespan (Derecki et al. 2012). The authors report that following bone marrow transplants carrying normal MECP2 gene into MECP2 knockout mice, 43% of the cerebellar microglia are derived from the wildtype, which is a much higher fraction than engrafts to other brain structures, especially to the neocortex, where it was only 5% (Derecki et al. 2012). This preferential engrafting to the cerebellum implies that that the engrafted wildtype microglial in the cerebellum may be largely responsible for the restorative effects of the transplants (Derecki et al. 2012). In addition, the benefits mediated by wild type microglia were diminished when their phagocytic activity was inhibited pharmacologically by using annexin V to block phosphatidylserine residues on apoptotic targets preventing recognition and engulfment by tissue resident phagocytes (Derecki et al. 2012). These results imply that the phagocytic activity is beneficial in Rett syndrome. The MECP2-null mouse provides a remarkable insight into the neuropathology of autism spectrum disorder and serves as a guide for future investigations to understand the relationship between the microglia and neurons in the cerebellum.

The individual with Angelman syndrome we investigated had a deletion on chromosome 15 Q. 2Q 13, which most often is the ubiquitin E3 ligase (UBE3A) gene. The microglia density in the molecular layer of the individual with Angelman syndrome was higher than any of the individuals with autism; the Purkinje cell density was in the autistic range. The histology of the brain in individuals with Angelman syndrome has been reported for only one case in the literature and this was qualitatively normal with respect to the cerebellar cortex (Kyriakides et al. 1992). In mice, the maternal copy of UBE3A is strongly expressed in Purkinje cells and the paternal copy of UBE3A is hardly expressed at all in the cerebellum (Dindot et al. 2008). In normal mice, the maternal copy of UBE3A is weakly expressed in the granule cells or in the molecular layer, which included the microglia; therefore the direct action of the genetic defect in the cerebellum of individuals with Angelman syndrome is in the Purkinje cells and not in the granule cells or microglia. The UBE3A knockout in mice has no effect on the density the Purkinje cell somas or the branching pattern of the Purkinje dendrites; however there was a reduction in the spine density on the Purkinje cells (Dindot et al. 2008). The primary defect in the UBE3A maternal knockout in the cerebellum was a reduction in the spine density on the dendrites of the Purkinje cells (Dindot et al. 2008). These results suggest that the increased microglia density we observed in the molecular layer of individuals with Angelman syndrome might be secondary to defects in the Purkinje cells' spines. This mechanism in Angelman syndrome is important to understand for a more comprehensive approach to the mechanism of autism and the UBE3A provides an excellent mouse model to study autism spectrum disorder in future investigations. There are also significant enrichments in copy number variations for UBE3A in genome-wide

association studies of individuals with autism compared to controls (Glessner et al, 2009; Bucan et al, 2009), which further implicates this gene in autism.

In addition, we studied the cerebellum of an individual with Joubert syndrome. Joubert syndrome is a rare genetic disorder, which can be caused by mutations of more than 20 genes which effect cerebellar development. In particular, it is characterized by an underdevelopment of the cerebellar vermis and malformed brainstem (Joubert et al. 1969). This case comparison was valuable since there are reports of hypoplasia of the vermis in individuals with autism, which was specifically true in this individual case (Courchesne et al. 1988, Scott et al. 2009). The previous histological reports for Joubert syndrome include only four individuals and describe mixed results in the neuropathology. In the original report of Joubert syndrome, the authors described no reduction in Purkinje cells and an increase in the astrocytes in the molecular layer (Joubert et al. 1969). Three more recent individual case reports found reduced Purkinje cells in the individuals with Joubert syndrome and increases in astroglia but none of the studies analyzed the microglia (Ishikawa, et al. 2008; Maria, 1999; Yachnis and Rorke 1999). The microglia densities in the individual with Joubert syndrome in our study were exceptionally low in the molecular layer, lower in fact than in any of the controls, which differs markedly from the individuals with autism, Rett syndrome and Angelman syndrome. By contrast, the Purkinje cell densities in the individual with Joubert syndrome are in the high normal range. Although we only studied a single individual with Joubert syndrome, these differences in the neuropathology suggest that this neurodevelopmental disorder is distinct from the other conditions. In addition, this shows that having a specific defect in the development of the vermis does not lead to increased microglial densities, and that

different pathobiological mechanisms are involved in autism, and in Rett and Angelman syndromes. This finding also suggests that neuroinflammation in the molecular layer of the cerebellum in autism, Rett syndrome, and Angelman syndrome may be specific to the pruning and plasticity of the Purkinje cell dendrites in multiple cerebellar regions including the vermis and lateral cerebellum.

Purkinje cells are the only efferent neurons from the cerebellar cortex; in the mouse cerebellum there are about 175,000 parallel fiber synapses on an individual Purkinje cell dendritic tree (Napper and Harvey, 1998). Long term depression (LTD) in activity at the parallel fiber-Purkinje cell synapses is the main mechanism for the Purkinje cell plasticity and is the mechanism necessary for the eye blink conditioning, which is a form of learning that is dependent on the cerebellum (Ito 1986). LTD lasts only a few hours and a major question has been how the transient change is translated into a long-term change in synaptic connectivity (Ito 2012). In view of the capacity of microglia to monitor and alter synapses, it is possible that these cells have a role in converting the synaptic modifications in LTD into long-term structural changes in connectivity. There is a report of an increase in the dendritic spines of individuals with autism in the cerebral cortex using the Golgi method (Hutsler and Zhang 2009). Extensive investigation of the dendritic spines in the cerebellar cortex of individuals with autism would provide a greater understanding for the relationship between the Purkinje cells and the microglia. Perhaps the selective increases in microglial density in autism, Rett and Angelman syndromes in the synapse-rich molecular layer might reflect a dysfunction in this learning-related process in these conditions.

The Cerebellum in Autism

A number of reports indicate that cerebellar hypoplasia is a feature of autism (Courchesne et al. 1997, Scott, et al. 2009, Bolduc, et al. 2011). Using magnetic resonance imaging (MRI), Courchesne reported that the cerebellar vermal lobules 6 and 7 were significantly smaller in patients with autism and described it to be a result of developmental hypoplasia rather than shrinkage or deterioration after full development of the individuals (Courchesne et al. 1988). By contrast, in an MRI study of the cerebellum that included 35 individuals with autism and age matched controls, no abnormalities in cerebellar lobules 6 and 7 in autistic individuals were detected, but the volume of the total cerebellum was significantly increased in individuals with autism (Piven, et al. 1997). The authors proposed that their finding differed from Courchesne and colleagues because Courchesne did not match for IQs between the individuals with autism and controls (Piven, et al. 1997). A recent MRI study of individuals with various autism spectrum disorders found that the mid-sagittal area of the vermis was decreased specifically to the high-functioning autism group compared to controls and that neither IQ nor age predicted the size of the vermis within the various autism groups. (Scott, et al. 2009). In addition, these authors found that the total volume of the vermis was decreased across the combined group of all individuals with ASD (Scott, et al. 2009). A more recent study identified decreases in vermis volume correlated with global impairments in development, cognition, expressive language, fine and gross motor skills, and behavior problems, as well as a higher rate of positive testing for ASD (Bolduc, et al. 2011). The largest MRI study to date, based 89 individuals with autism spectrum disorder and controls, observed that the neuroanatomic networks correlated with ASD included the cerebellum (Ecker et al. 2012). The most recent MRI analysis of the cerebellum for

individuals with autism reports that there is a specific reduction in the grey matter of the vermis and CRUS-II in low functioning individuals with autism and it is associated with social and interaction deficits in ASD (Riva et al. 2013). In an early MRI study, investigators reported a progressive cerebellar atrophy increasing in age in individuals with Rett syndrome in contrast to individuals with autism (Murakami et al. 1992). Numerous reports provide evidence that individuals with autism exhibit motor deficits in addition to the classical symptoms of autism (Vilensky et al. 1981, Green et al. 2009). Subjects with autism display in gait disturbances indicating a specific dysfunction in the motor system (Vilensky et al. 1981). It is reported that autistic children often have a variety of postural instabilities, greater clumsiness, and altered motor coordination (Bauman and Kemper 2005, Molloy et al. 2003). In a recent study designed to understand the relationship of motor coordination deficits in autism, the investigators identified 83 studies that focused on motor coordination, arm movement, gait, and postural instability deficits in autism, and reported that individuals with autism had substantial motor coordination deficits across a wide range of behaviors, and described motor abnormalities as a cardinal feature of autism (Fournier et al. 2010). More importantly, these are additional lines of evidence that the cerebellum is altered in autism and that not only do the motor dysfunctions account for the cerebellar alterations but the cognitive and social deficits in autism can be addressed by the cerebellar abnormalities. Cerebellar lesions are involved in several neuropsychological behaviors including impairments in language, executive functions, mood and affect (Schmahmann & Sherman, 1997). In a study reviewing the developmental malformations of the cerebellum, vermis malformations induced affective and social disorders and were more associated with autism spectrum

symptoms (Tavano et al. 2007). In particular, the cerebellum is described as a common site for altered connectivity in individuals with autism (Belmonte et al. 2004). There have been a number of functional neuroimaging studies that have shown activation for several non-motor tasks (Gao et al. 1996). Buckner's massive study on the functional connectivity of the cerebellum based on one thousand subjects reported that the cerebellum contains at least two large, homotopic maps of the entire neocortex (Buckner et al. 2011). In particular, the prefrontal cortex and Broca's area are over-represented in the cerebellar cortex whereas the somato-motor cortex is underrepresented in the cerebellar cortex (Buckner et al. 2011). A recent study explains that the cerebellum operates as a general co-processor, whose outcome is dependent on the connectivity of brain regions, impacting cognitive functions, sensory and motor processing (D'Angelo et al. 2012). Kawato et al. (1987) described the concept of a "forward model" which is a feedback-optimized program for the manipulation of a controlled object where a robust model provides smooth motor performance based on experience (Ito 2012). This concept of a forward model could be applied to the cerebellum for learning different social cues and behaviors and if there is a disruption in the forward model the appropriate behaviors would not be efficiently learned and the responses would then become difficult and effortful. Describing a patient with a unilateral cerebellar lesion, Holmes (1939) wrote: "One intelligent and observant patient remarked, 'The movements of my left arm are done subconsciously, but I have to think out each movement of the right (affected) arm. I come to a dead stop in turning and have to think before I start again.' It is significant that practice may improve the performance of the test." This highlights the process of a disrupted cerebellar forward model where the patient described a contrast between the

unaffected arm performing the task as an automatic response and the affected arm required effortful concentration on the task due to the cerebellar lesion.

Temple Grandin, as a person with autism, commented (Grandin, 2005): “Each day, I collect more data to place in my library of experience. When I encounter a new social situation, I have to search my memory for a similar experience that I can use as a model for my next action. As I fill my database with more and more information, I become better and better at handling different social situations. I have to rehearse how to deal with a person before I interact with him or her. I have a very difficult time when I am confronted with unexpected social surprises. For common social interactions with clients, I use programmed, prerehearsed responses. Everything is done with logic.” Her very insightful description of her difficulties with social interactions and her reliance on conscious effortful processing may serve as a powerful illustration of the limitations of not having an effective forward model for social interactions. Together these data describe the importance of the cerebellar connectivity for cognitive, behavioral, sensory, and motor processing in individuals with autism. In particular, a primary alteration in the Purkinje cell layer and neuroinflammation in the molecular layer containing the Purkinje cell synapses could disrupt the critical connectivity for processing cognitive, sensory and motor behaviors in individuals with autism.

Conclusion

We report an increase in the microglia density in the molecular layer of the cerebellum, the location of the Purkinje synapse dendrites, in individuals with autism, Rett and Angelman syndromes, which may be the key to understanding these neurodevelopmental disorders. In particular the increase in microglial density in the

molecular layer is another line of evidence for neuroinflammation in autism. Perhaps the capacity of microglia to monitor and alter synapses in the cerebellum could be a mechanism for converting the synaptic modifications in LTD into long-term structural changes in connectivity. A detailed examination of the dendritic spines of the Purkinje cells in the cerebellar cortex of individuals with autism would provide a greater understanding of the relationship between the Purkinje dendritic spines, connectivity and microglia function and modification. Moreover, these findings support the utility of the MECP2 and UBE3A knockout mice since the neuropathology in the human Rett and Angelman subjects was similar to that of individuals with autism. Both the MECP2 and the UBE3A mice are excellent models for exploring the relationship between neuroinflammation and neuronal circuits in the brain. Importantly, answering the question of whether the inflammation is harmful or beneficial would be a great advance in understanding the pathology in autism and could provide possible therapeutics. In addition, these findings support the hypothesis that the cerebellar neuropathology is a core feature in autism and accounts for a number of the social impairments, behavioral symptoms and motor deficits in individuals with autism. Future studies dedicated to cerebellar connectivity in autism could provide advancement in understanding autism. The utility of the networks involved in the cerebellar forward model for cognition, social and motor behaviors could aid in a greater understanding of autism spectrum disorders.

CHAPTER 4: SUMMARY AND CONCLUSIONS

In this thesis we describe an increase in microglial densities in the cerebral and cerebellar cortices in autism. In our first study, we found an average increase of 20% in microglial densities in individuals with autism in fronto-insular cortex and visual cortex, two disparate cortical regions, which indicates a global increase in microglia densities in subjects with autism compared to neurotypicals (Tetreault et al., 2012). In our second study, we examined subjects with a variety of neurodevelopmental disorders, including autism, Rett, Angelman, and Joubert syndromes, and a sibling of an autistic individual, to determine whether the microglia densities, Purkinje cell linear densities and the neuropathological states are specific to any of the disorders. We found a much larger elevation in microglia densities (compared to the cerebral cortex) in individuals with autism and Rett syndrome, specific to the molecular layer in the cerebellum, which is the region of the Purkinje and parallel fiber synapses. In addition, the Angelman syndrome case and the sibling of an individual with autism both had microglia densities in the range of the individuals with autism and Rett syndrome. By contrast, the Joubert syndrome case has a lower density than any of the controls. We also found a significant deficit in the Purkinje cells in the vermis of individuals with autism compared to controls. The Purkinje cell density in the vermis was also significantly lower than in the lateral cerebellum in the autistic subjects indicating that the abnormality is specific to the vermis. Together, these data provide evidence for neuropathological and neuroinflammation alterations in individuals with autism spectrum disorder. Moreover, we report that not only was there an elevation in the microglia density in the molecular layer, but that the microglial cells were larger in perimeter and area in individuals with

autism spectrum disorder compared to controls, which suggests that the microglia are chronically activated in the molecular layer.

The microglia density in the Joubert's syndrome case in our study was exceptionally low in the molecular layer, lower in fact than any of the controls, which differs markedly from the autism, Rett syndrome and Angelman syndrome cases. In addition, the average microglia soma was larger in the Joubert case compared to the autism and Rett syndrome cases in both the molecular and granule cell layers. These findings suggest that the microglia are not different in the granule cell and molecular layers of the Joubert case. In addition, this single Joubert case had the largest microglia soma size, which suggests that the microglia may have been filled with inclusions and may be in the end state of gliosis, which would be consistent with vermal hypoplasia. By contrast, the Purkinje cell density in the Joubert syndrome case was in the high normal range. Although we only studied this single case, these differences in the neuropathology suggest that this neurodevelopmental disorder is distinct from the other conditions. In addition, the Joubert findings show that having a specific defect in the vermis does not necessarily lead to increased microglial densities, and that different pathobiological mechanisms are likely to be involved in this condition compared to autism, Rett and Angelman syndromes.

Since microglial density is significantly elevated across all the disorders (except Joubert syndrome) compared to neurotypicals, it appears that the increase in microglia density is not specific to autism spectrum disorder but rather is a feature common to a number of developmental disorders. However, microglia in individuals with autism and Rett syndrome have an increased soma size, suggesting a chronically activated state,

which could indicate possible environmental factors, perhaps in combination with genetic predisposition, leading to a systemic infection or an alteration in the phagocytic function of the microglia. There are several other lines of evidence for an elevated state of inflammation in individuals with autism. There are several reports of an elevated cytokines in the cerebral spinal fluid in individuals with autism as compared to neurotypicals (Vargas et al., 2005; Zimmerman et al., 2005; Chez et al., 2007). Individuals with autism also show a significantly elevated expression of C-reactive protein (CRP), indicative of an inflammatory response, and there is a positive correlation between CRP and the severity of autism (Khakzad et al., 2012). A recent study in Finland, including a national cohort of over a million samples of prenatal sera measuring the level of CRP, indicates there is a significant association between elevated levels in the mother's serum and the likelihood that her child will become autistic (Brown et al., 2013). For the maternal CRP levels in the highest quintile compared to the lowest quintile, there is a significant, 43% elevated risk for autism (Brown et al., 2013).

Maternal infection and immune activation are also associated with autism (Brown et al., 2004, Patterson et al., 2012). In a model of maternal immune activation (MAI) in which the viral mimic polyinosine:cytosine (poly(I:C)) is injected in pregnant mice, the offspring exhibit autism-like behaviors, with deficits in social interaction, verbal and olfactory communication and prepulse inhibition, elevated stereotyped/repetitive behaviors, as well as a deficit in cerebellar Purkinje cells (Shi et al., 2009; Malkova et al., 2012). In addition, Hsiao et al., (2012) report that MIA increases interleukin-6 (IL-6) in the placenta and fetal brain, which reduces placental growth hormone, possibly causing harmful effects on fetal brain development. A recent examination of metabolic

conditions, such as diabetes, hypertension and obesity during pregnancy and their association with autism, reported that mothers with obesity were 1.6 times more likely to have a child with autism spectrum disorder (Krakowiak et al., 2012). In obesity there are increases in proinflammatory cytokines: specifically, there are elevated levels of IL-6 and TNF-alpha which can alter fetal development (Krakowiak et al., 2012).

Thus, there are a number of maternal risk factors that are associated with increased incidence of autism in the offspring. Using a model of peripheral organ inflammation, cerebral microglia recruit monocytes into the brain through the release of TNF-alpha (D'Mello et al., 2009). It is possible that systemic infection leads to elevated levels of microglia in individuals with autism. These data highlight that there is an elevation of systemic inflammation and it is plausible that maternal inflammation is a risk factor, which can lead to chronic neuroinflammation and increased microglia density in autism.

Another potential mechanism for the increase of microglia density in autism and Rett syndrome is an alteration in the phagocytic function of the microglial cells. Introducing into MECP2-knockout mice wild-type microglia via bone marrow transplantation reduces a number of behavioral and physiological deficits and restores normal lifespan in this mouse model with autistic features (Derecki et al., 2012). The transplanted wild-type microglia were far more likely to take up residence in the cerebellum than in the cerebral cortex in these mice, which indicates that these cerebellar microglia play a large role in the restoration of function in these animals (Derecki et al., 2012). It is notable that the benefits of wild-type microglia diminished when their phagocytic activity was inhibited using annexin V to block phosphatidylserine residues

on apoptotic targets, which prevents recognition and engulfment by phagocytes (Derecki et al., 2012). These results imply that the phagocytic activity of the transplanted microglia is central to the restorative effect and that impaired microglial phagocytosis may be crucial to Rett syndrome. Therefore, the elevated microglial density in the autism and Rett syndrome cases could be due to a compensation for dysfunction in the microglial phagocytic activity. Maternal use of valproic acid, a drug used to treat epilepsy, is significantly associated with an increase in autism spectrum disorder in the offspring (Christensen et al., 2013). Consistent with the hypothesis that autism may arise from impaired microglial phagocytosis, valproic acid reduces the phagocytic activity of primary, adult human microglia and the expression of microglial markers *in vitro* (Gibbons et al., 2011).

There is additional evidence that impaired microglia phagocytosis is the cause of neurological diseases. Nasu-Hakola dementia is caused by a defect in the TREM2 or DAP12 genes, whose proteins form a receptor complex that is strongly expressed in microglia (Paloneva et al., 2002; Sessa et al., 2004). This genetic defect reduces the phagocytic activity of microglia, impairs the clearance of debris in the cortex and induces deficits in social behavior as an early manifestation of Nasu-Hakola dementia (Bianchin et al., 2004; Neumann and Takahashi 2007). Mutations of TREM2 are also found in a subset of patients with late onset Alzheimer's disease (Jonsson et al., 2013; Guerreiro et al., 2013). In a more recent study of Alzheimer's disease, the CD33 susceptibility loci that are risk factors for Alzheimer's disease are linked to defective microglia phagocytosis of amyloid and result in increased numbers of microglia in the brains of individuals with the susceptibility loci (Bradshaw et al., 2013).

The passage of macrophages from the blood into the brain may also be impeded in autism. Individuals with autism exhibit reduced levels of soluble platelet endothelial molecule-1 and p-selectin, both of which facilitate leukocyte migration and blood brain barrier permeability, which could influence macrophages influx into the brain (Onore et al., 2012). This, in turn, could be another defect where healthy microglia cannot aid in the dysfunction and leads to an alteration in phagocytic activity, and elevated dysfunctional microglia in individuals with autism.

These data support the hypothesis that suppressing microglial phagocytosis can alter brain development and lead to neurodegeneration later in life. Future studies focused on understanding the microglial phagocytic activity could have numerous benefits for understanding neurodevelopmental and neurodegenerative disorders.

It is interesting that the Angelman case had an elevated microglial density similar to the autism and Rett syndrome cases, but the microglial soma size in Angelman syndrome was in the range of the neurotypicals. These findings suggest that there are more microglia but they may not be chronically activated in Angelman syndrome. As described earlier, knocking out the maternal copy of UBE3A in mice has no effect on the density of the Purkinje cell somas or the branching pattern of the Purkinje dendrites; however there was a reduction in the spine density on the Purkinje cells (Dindot et al., 2008). Since UBE3A is strongly expressed in the Purkinje cells but hardly expressed in other cell types in the cerebellum (Dinot et al., 2008; Allen Brain Atlas), it is likely that the defect arises from an abnormality within the Purkinje cells themselves and may be related to the mechanisms of long term depression (LTD) which will be discussed below. The mouse model of Angelman syndrome had impaired learning, fear conditioning, and

motor deficits (Huang et al., 2013). Increasing the number of UBE3A gene copies is also linked to autism, and increasing the UBE3A gene in the mouse threefold results in mice that exhibit the core autism behaviors including defective social interaction, impaired communication and increased stereotypic behaviors as well as altered gluatamatergic synaptic transmission, resulting in reduced excitatory transmission (Smith et al., 2011). Experience-driven neural activity generates UBE3A transcription, in which UBE3A alters excitatory synapse development by reducing the number of the AMPA receptors at excitatory synapses (Greer et al., 2010). Thus, the reduction in the glutamate receptors in the Angelman mice could explain the reduction of the dendritic spines on the Purkinje cells. In the Angelman syndrome individual, the increased density of microglia could have been a response to the need for increased synaptic pruning due to defective parallel fiber-Purkinje cell synapses. The overexpression of UBE3A in some individuals with autism and the mouse model of this condition might also impair these synapses.

Microglia make contact with synapses at a frequency dependent on the activity of the neurons (Wake et al., 2009). In a model of neuronal degeneration, microglia contact synapses more frequently and actively remove synapses of degenerating cells (Wake et al., 2009). In particular, the increase in microglia density could be related to the reduced number of dendritic spines on the Purkinje cells through the mechanism of long-term depression (LTD). LTD of the Purkinje synapses lasts only a few hours and a major question has been how the transient change is translated into a long-term change in synaptic connectivity (Ito, 2012). In view of the capacity of microglia to monitor and alter synapses, these cells might have a role in converting the synaptic modifications in LTD into long-term structural changes in connectivity. Interestingly, the UBE3A materal

knockout in the mouse model of Angelman syndrome reduces LTD in the visual cortex of mice (Yashiro et al., 2009). UBE3A expression is linked to the presence of the AMPA glutamate receptors at the synaptic interface (Greer et al., 2010), and LTD at the parallel fiber-Purkinje cell synapses appears to depend on the reduction in the number of AMPA receptors (Ogasawara et al., 2008). Thus the maternal UBE3A knockout in Angelman syndrome may impair LTD. Future studies of two-photon imaging of microglia and Purkinje cells could advance the understanding of the relationship of neuron-microglia interaction in the normal state, and in addition illuminate the mechanisms for synapse elimination and possibly the elevated microglia densities in Angelman syndrome.

The sibling of an individual with autism had an elevated microglia density and increased microglia soma size, which were similar to autism cases. This individual was a twenty-two-year-old male and a member of a set of triplets in which another member had been diagnosed with autism. The sibling case tested for autism, but did not meet the diagnostic criteria. He lacked normal eye contact, had social awkwardness, exhibited stereotyped nervous behaviors and felt socially isolated. He met the diagnostic criteria for obsessive-compulsive disorder (OCD) and social anxiety disorder but not for major depressive disorder. However, his death was a suicide. Elevated rates of both major depression and social phobia are found among parents and siblings in the families with an autistic child (Smalley et al., 1995). Since this individual had elevated microglia density and soma size similar to the autism cases, this suggests that the microglia changes could be relevant for social anxiety and OCD.

Is elevated of microglia density in autism a biomarker for the disorder?

Although this study examined ten autistic cases, it will be necessary to evaluate many more autism brains, because of the heterogeneity in neuropathological observations and specifically in microglia pathology. Second, even though microglia density is elevated in the cerebral and cerebellar cortex in autism (Vargas et al., 2005; Morgan et al., 2011, Tetreault et al., 2012), we have evidence for an increase in microglial density in both Rett and Angelman syndromes and in the sibling of an autistic individual in the cerebellar cortex. This indicates that the elevation of microglia is not specific to autism, but rather, it is a common feature of several neurodevelopmental disorders. In addition, there are several lines of evidence for increased neuroinflammation and microglia activation in mental illnesses including schizophrenia (Bayer et al., 1999; Wierzba-Bobrowicz et al., 2004), sickness behavior (Perry et. al., 2010) and major depressive disorder (Trzonkowski et al., 2004). In general, it appears that elevated microglia and neuroinflamamtion is a feature of several neuropsychiatric diseases, but the anatomical patterns of increased densities may vary in different disorders.

Would an anti-inflammatory treatment aid in reducing the symptoms in autism?

The next logical step would be to treat developmental and psychiatric patients with autism using an anti-inflammatory medication. A recent clinical trial of minocycline in patients with autism showed no clinical improvements after six months of testing (Pardo et al., 2013). This study was a very small case study (N=10) and notably, the baseline microglia activation was not measured using imaging techniques. The peripheral immune status was not assessed. Ideally, it would be important to know whether the individuals are suffering from neuroinflammation prior to the minocycline treatment, which can be achieved using positron emission tomography and radiotracer for

microglia—[11C](R)-(1-[2-chrorophynyl]- N-methyl- N-[1- methylpropyl]-3 isoquinoline carboxamide) ([11C](R)-PK11195). Measuring the microglia *in vivo* would provide a baseline and a post treatment measure of the microglia would provide insight into whether the minocyline is actually targeting the microglia, which is the main source of the neuroinflammation in the brain. Although there was no difference in the clinical trials for autism after six months, there are, however, benefits for two minocycline trials double blind placebo-controlled in schizophrenia (Levkovitiz et al., 2010; Chaudry et al., 2012). Both of these studies were performed with large number of participants, 54 and 114, and reported beneficial effects on cognitive function and negative features of schizophrenia with the use of the drug in the early stages of schizophrenia (Levkovitiz et al., 2010; Chaudry et al., 2012). In a recent trial testing the an antibody against TNF, a proinflammatory cytokine, to treat psoriasis revealed a decrease in depression in major depressive disorder, as a side effect and this reduction was unrelated to the decreased psoriasis symptoms (Raison & Miller, 2013). It is possible to have another minocycline trial in individuals with autism under different conditions, such as including participants with early autism diagnosis, measuring the level of activated microglia pre- and post-treatment, including greater number of subjects, and testing with double blind placebo-controlled groups, which may yield better results and may suggest a beneficial therapeutic for individuals with autism.

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Tables

Table 1 Individuals examined for microglial densities, Purkinje cell linear measurements and granule cell densities in the cerebellum. The tissue source is NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, MD. PMI=post-mortem interval. X=density measurements were made for this structure.

ID	GUID	Age	Sex	Cause of Death	Brain Weight	PMI	section	Vermis	Lateral Cerebellum
Autistic									
M4021	NDAR_INVUX2	3	M	drowning	1330g	15 hrs.	4	X	X
M4029	NDAR_INVRX2	3	M	drowning	1130g	13 hrs.	4	X	X
UMB5308	NDAR_INVGG735JVA	4	M	skull fractures	1310g	21 hrs.	10		X
UMB4671	NDAR_INVVY9	4	F	fall from 9th story	1320g	13 hrs.		X	
UMB797	NDAR_YX624FEY	9	M	drowning	1320g	13 hrs.		generic CB	
UMB4305	NDAR_WL137ER1	12	M	Serotonin Syndrome	1360g	13 hrs.	2, 10	X	X
UMB4899	NDAR_INVGW5	14	M	drowning	1450g	9 hrs.	4	X	X
UMB5278	NDAR_YH540PL	15	F	drowning with seizure	1417g	13 hrs.	4	X	X
UMB5294	NDAR_INVYN399YKW	19	M	suicide by hanging	1560g	16 hrs.			X
UMB4999	NDAR_INVVM9	20	M	cardiac arrhythmia	1427g	14 hrs.	4	X	X
Sibling of Autistic									
UMB5378	NDAR_INVRP817TM7	22	M	suicide by hanging	1500g	8 hrs.	2, 10	X	X
Control									
UMB1791	NDAR_INVXL388UKV	2	F	drowning	1200g	12 hrs.	2	X	
UMB1284	NDAR_INVPP10	3	F	drowning	1250g	11 hrs.	4	X	X
UMB4670	NDAR_INVEA3	4	M	commoito cordis	1470g	17 hrs.	10		X
UMB1708	NDAR_INVRJ569TT1	8	F	compressional asphyxia	1320g	20 hrs.	10		X
UMB5387	not provided	12	M	drowning	1750g	13 hrs.	4	X	X
UMB5077	NDAR_INVFE727ZB7	16	F	multiple injuries	1330g	13 hrs.	3	X	
UMB4591	NDAR_INVVH3	16	F	multiple injuries	1330g	14 hrs.	4	X	X

UMB4727	NDAR_INVAD6	20	M	multiple injuries	1330g	5 hrs.	4	X	X
UMB1542	NDAR_INVXZ7	22	M	multiple injuries	1510g	4 hrs.	4	X	X
Rett									
UMB4882	NDAR_INVFK280UCW	17	F	complications of disorder	871g	18 hrs.	4	X	
UMB1815	NDAR_INVVH323KHT	18	F	complications of disorder	930g	5 hrs.	4	X	
UMB4516	NDAR_INVJE188DKF	20	F	natural	980g	9 hrs.	4	X	
Angelman									
UMB1754	NDAR_INUJ814JV0	4	M	drowning	1360g	24 hrs.	8		X
Joubert									
UMB5486		5	F	complication of disorder	1035g	6 hrs.	2, 10	X	X

Table 2 This table is adapted from Tetreault et al. (2012) and new cases are shown in the table which include individuals with autism, the sibling of an autistic, the Rett cases, an Angelman case and a Joubert case. Below is a phenotypic description of the autistic subjects including age, gender, seizure status, medications, medical history, cause of death, PMI, and [1]ADI-R (Autism Diagnostic Interview-Revised) test description and cutoffs: Qualitative Abnormalities in Social Interaction (A=10), Qualitative Abnormalities in Communication (B=7), Stereotyped Patterns (C= 3), and Abnormal Development (D=1). All subjects have a psychological evaluation and a Bayley or a developmental test. [2]Minio-Paluello et al, 2009. [3]Matson et al, 2008. [4]Goldberg et al, 2003. [5]Walters et al, 1990. [6]MacDonald et al, 2006.

Patient ID	Age & Gender	PMI	Cause of Death	Seizure Disorder	Medication	Additional Medical Hist.	ADI-R[1]	Neuropath Report or Autopsy	Patient Summary	Respirator or Traumatic Death State
Autistics										
M4021	3 years Male	15 hours	drowning	no	none reported	none reported	none reported	none provided	Rigid routine, many repetitive[6] and aggressive behaviors. No gestures for communication, auditory sensitivity.	none reported
M4029	3 years Male	13 hours	drowning	no	none reported	none reported	none reported	none provided	Autistic regression[4] at 24 months, aggressive behaviors, negative response to several sensory stimuli and ran from sounds.	Found in a canal and resuscitated with CPR. Five hours on respirator.

UMB5308	4 years Male	21 hours	skull fractures motor vehicle accident	no	none reported	none reported	A: 17 B: 14 C: 4 D: 3	neuropath; moderte edema, cerebral and cerebellar hemispheres reported intact	Walked at 11 months, no language, only babbled and no use of words, parallel play with others, often ignored others, no imitation, sometimes had eye contact, inappropriate laughing, smiled often, avoided large groups, lacked social cues, followed routines, hand flapping, head banging	Immediate death when struck by a motor vehicle.
UMB4671	4 years Female	13 hours	fall 9 stories	no	none reported	diaper rash	A: 26 B:13 C: 3 D: 5	autopsy; noted normal brain	Lacked body self awareness and awareness of others[2] (observed by mother); could not identify body parts when tested by a psychologist. Normal hearing. Cognition delayed; no socialization.	none reported

UMB797	9 years Male	22 hours	drowning	no	Desipramine	ADHD; seizure associated with medication, migraines	A: 24 B: 20 C: 6 D: NA	neuropath; noted normal brain	No hypersensitivity to sensory stimuli, 8 cortical regions stained with H&E, no microgliosis in cortex, VC had unusually large Meynert cells and an irregular shaped claustrum.	Overdose of desipramine a week prior to death; no revival.
UMB4305	12 years Male	13 hours	serotonin syndrome	yes	Clonazepam Depakote Olazapine Quetiapine	pervasive development disorder NOS psychosis NOS ADHD	A: 25 B: 15 C: 8 D: 4	neuropath; necrosis effects; large macrophage & astrocytes in RH.	Very aggressive, destructive and abusive behavior without provocation; special education, lived in group home, unmanageable behavior. Lacked bladder control.	Large contusion in right frontal lobe; cystic necrosis.
UMB4899	14 years Male	9 hours	drowning	yes	Clonidine Trileptal Zoloft	none reported	A: 22 B: 14 C: 8 D: 4	neuropath; normal brain and cortex	Loss of verbal skills at 1 year; high levels of sensory interest, compulsions and stereotypy[6]. Autistic regression[4].	Found in bottom of pool, CPR revived, on life support for 24 hours.

UMB5278	15 years Female	13 hours	drowning	yes	Depakote Keppra Prozac	none reported	A: 22 B: 11 C: 5 D: 5	none provided; noted normal brain	Noted to have mild autism, high functioning & at grade level, seizures from infancy, second cousin has autism, had spontaneous speech and aggressive behavior.	none reported
UMB5294	19 years Male	16 hours	suicide by hanging	no	Zoloft Seroquel Welbutrin Luvox Ritalin Refused to take medications prior to death	Asperger's diagnosis, ADD, high cholesterol, asthma, depressed mood and first cousin had autism	A: 17 B: 19 C: 5 D: 2	neuropath; neurons mildly reduced & contracted in cerebellum, mild WM edema	High functioning. Was attending college at time of death. Hyperactivity from a young age, no imitation, special ed in early years and mainstreamed in high school, spoke using a script to order food in a restaurant, repeated commercials, only spoke about his topic of interest, not aware of emotions of others, hypersensitive to taste, food, smells and light.	

UMB4999	20 years Male	14 hours	cardiac arrhythmia	no	Amoxicillin Depakote Naltrexone Risperdal	severe mental retardation, aggression, compulsion	none reported	neuropath; normal brain, increase in leptomengial cells of meninges and gliosis	Self injurious behavior including severe head banging[3], treated with Naltrexone[5], stereotypy[6], sensitive GI tract[7] and no communication.	none reported
Sibling of Autistic										

UMB5378	22 years Male	8 hours	suicide by hanging		Strattera (discontinu-ed before death)	social anxiety disorder		neuropath; normal brain without neuropath findings	Was a member of a set of triplets, one of whom was diagnosed with autism and the second had a history of depression and Asperger-like symptoms. Subject was evaluated for autism and ADOS performed. Social difficulties, lack of eye contact, grimacing, head and leg shaking, anxiety, obsessive thoughts, difficulties completing writing assignments due to severe self-criticism. However, ASD diagnosis rejected in favor of social anxiety disorder due to patient's history. High intelligence; very superior verbal ability.	
Rett										

UMB4882	17 years Female	18 hours	complications of disorder	yes	valium	Rett syndrome C763T nonsense mut. hetro. cerebral palsy		neuropath; hypoplasia of the brain & patchy moderate PC loss, more prominent in the folia	Quadriplegic cerebral palsy, severe scoiliosis, hypertonia and contracture of extremities	
UMB1815	18 years Female	5 hours	complications of disorder	yes	Tegretol Baclofen	Rett syndrome Hetro. IVS3-2A>G cerebral palsy scoliosis		neuropath; hypoplasia of the brain, multifocal mal-development contusion to the right temporal tip	Nonverbal and nonambulatory; she had crying and laughing spells that were unprovoked.	
UMB4516	20 years Female	9 hours	natural	yes	Depakene Tegretol Dilantin	Rett syndrome no genetic information. Mother had 10 month pregnancy complicated by influenza		neuropath; slight cerebral, cerebellar and nigral degeneration	At 16 months she stopped talking, engaged in stereotyped behaviors, ataxia, unexpected laughing spells.	
Angelman										

UMB1754	4 years Male	24 hours	drowning	yes		Angelman syndrome 46, XY, del (15) (q11.2q13)		Some loss of cerebral cortical neurons especially in layer 3, very mild and focal loss of Purkinje and granule cells	Clinical presentation started at 9 months and included slowing of motor and language abilities. He had spasticity. Ataxia upper limb and gait. Bilateral arachniod cysts.	
Joubert										
UMB5486	5 years Female	6 hours	complication of disorder	no	Ergocal-ciferol, iron, periactin	Joubert syndrome		Neuropath; showed hypoplastic vermis, dilation of 4th ventricle, "molar tooth sign", grey matter heterotopia, focal white matter rarefaction.	Dysphagia, fed by gastric tube, sleep apnea, retinal dystrophy, hearing impairment, didn't use arms for grasping, nephronophthisis, kidney failure	

Table 3 Individuals with autism and neurotypical cases that are in common for the microglial density measurements in the cerebellum, frontoinsular cortex and visual cortex. X=microglia density measurements were made for this structure.

ID	GUID	Age	Sex	FI	VC	Vermis	Lateral Cerebellum
Autistic							
M4021	NDAR_INVUX206VRV	3a	M	X	X	X	X
M4029	NDAR_INVRX268EH4	3b	M	X	X	X	X
UMB4671	NDAR_INVVY9	4	F	X	X	X	
UMB4899	NDAR_INVGW538MM3	14b	M	X	X	X	X
UMB5278	NDAR_YH540PL	15	F	X	X	X	X
UMB4999	NDAR_INVGW5	20	M		X	X	X
Control							
UMB4670	NDAR_INVEA3	4b	M	X	X		X
UMB5387	not provided	12	M	X	X	X	X
UMB4591	NDAR_INVVH3	16	F	X	X	X	X
UMB4727	NDAR_INVAD6	20b	M	X	X	X	X
UMB1542	NDAR_INVXZ7	22a	M	X	X	X	X

Table 4 Purkinje morphometry in individuals with autism and controls. The Purkinje cells in individuals with autism are more round then the controls as shown in the measurements below.

Measurement	Definition	P value	Results
Feret Max (μm)	Largest dimension of the contour.	0.04311	Control's Feret max is greater than the autistics which indicates the contour is less round for the controls.
Aspect Ratio	The degree of flatness of the contour. Aspect ratio approaching 1 indicates a rounder contour.	0.02791	Autistic individual's value is closer to 1 which indicates the cells are rounder.
Compactness	The relationship between the area and maximum diameter. Compactness for a circle is 1.	0.0415	Autistic individual's value is closer to 1 which indicates the cells are more compact.
Shape Factor	The complexity of the contour. A small shape factor is a small perimeter as compared to area. A circle has a small shape factor of 3.54.	0.0347	The autistic individual's have a smaller shape factor indicating the shape is closer to a circle.
Form Factor	As the contour approaches 1 the shape is approaches a perfect circle.	0.0223	The individuals with autism are closer to the value of 1 indicating the shape is closer to a perfect circle.
Solidity	A circle, square or an ellipse has a value of 1.	0.0206	Individuals with autism have a greater solidity value and are more round.

Table 5 Previous Purkinje cell studies in autism. List of abbreviations: Autistic=AU Control=CN Mentally Retarded=MR Purkinje cell=PC

Group	Cerebellar Region	Method	Finding
Williams et al. (1980)	entire cerebellum	N=1 AU Single case confirmed autism. Tissue was formalin fixed paraffin and celloidin embedded blocks, cellular and fiber stains. Qualitative observations.	Qualitative analysis a single case with autism exhibited a decrease in number of Purkinje cells.
Bauman and Kemper (1985)	vermis, lateral and inferior cerebellar hemispheres	N=1 AU N=1 CN Single autistic case report, celliodin blocks and Yakovlev staining protocol. Qualitative observations.	Qualitative assessment of Purkinje cells. Described shrinkage and fewer Purkinje cells in autistic individual.
Ritvo et al. (1986)	vermis and cerebellar hemispheres	N=4 AU N= 4 CN Brains were obtained from UCLA School of Medicine and all cases met the DSM-III criteria for autism, tissue was formalin fixed and sectioned at 10 microns, Nissl stained, coded for phenotype. Linear measurement of Purkinje cells was made in 2mm intervals.	Total Purkinje cell counts were significantly reduced in vermis and cerebellar hemisphere of each autistic case compared to controls in both regions.
Bauman and Kemper (1990)	cerebellum	N=1 AU N=2 CN Nissl stained cerebellum. Qualitative observations.	Decrease in Purkinje cells in a single case using a microscopic qualitative analysis.
Bauman et al. (1991)	cerebellum	N=5 AU N=4 MR Microscopic and neuroanotomic observations of Nissl stained cerebellum.	Qualitative analysis describing a decrease in Purkinje cell numbers in autistics N=5. N=2 autistics marked PC loss N=3 autistics with mild PC loss.
Fehlow et al. (1993)	cerebellum	N=1 AU N=2 CN Single case report.	Purkinje cell loss in lobules VI and VII.
Bauman et al. (1993)	cerebellum	N=6 AU N=5 MR DSM III Confirmed autism diagnosis, whole brain serial histological sections, cerebellar Purkinje cell analysis. Qualitative observations.	Qualitative analysis decrease in Purkinje cell numbers in autistics N=6. N=2 autistics with larger PC size and N=2 autistics with smaller PC size.

Bailey et al. (1998)	vermis and cerebellar hemispheres	N=6 AU N=7 CN ADI Criteria for autism diagnosis, 14 microns cut sections, Nissl stained, left and right hemispheres stained and hemispheres randomly chosen for quantification, Purkinje cells counted as number per unit area of the Purkinje cell layer.	Significant decrease in Purkinje cell density in all autistic adults compared to the control cases (N=5).
Fatemi et al. (2002)	cerebellar hemispheres	N=5 AU N=5 CN Brains were obtained from Autism Research Foundation, investigator blind to phenotype, fresh frozen tissue coronal sectioned at 14 microns, Nissl stained, linear measurement of of Purkinje cell cross sectional area of 75 randomly chosen Purkinje cells using Stereoinvestiagor software. Total Purkinje cell counts per slide were divided by total granule and molecular layer areas to obtain granular and molecular layer based Purkinje cell densities.	24% decrease in autistic Purkinje cell size. No difference in density Purkinje cell in autistic cases when compared to the controls.
Whitney et al. (2008)	cerebellar hemispheres mixed, lobule crus II located inferior to the horizontal fissure	N=6 AU N=4 CN Tissue was obtained from Harvard Brain Tissue Center, Brain Bank at Duke University and University of Maryland Brain Bank. Formalin fixed cerebella, fresh frozen and sectioned at 30 microns and shrinkage to 7.5 microns, immunostained for Calbindin and counterstained with thoinin, Purkinje cells quantified in Neurolucida, stereological quantification of 10 sections per case counted on average 694 PC per brain.	No statistical difference between the number of the Purkinje cells in autistic individuals compared to controls. N=3 autistic cases which had decrease PC compared to the mean number of PC in the controls. Whitney et al., (2008) reports that a subset of individuals with autism have a reduction in PCs compared to controls.
Wegiel et al. (2013)	cerebellum	N= 12 AU N=10 CN Tissue was obtained from NICHD brain and tissue bank, Harvard brain bank and brain and tissue bank at New York State Institute. All individuals ADIR reported and met criteria for autism. Tissue was embedded in celloidin, sectioned at 200 μm and Nissl stained. Stereology was used to quantify the Purkinje cells. The nuclear radii were used to measure the Purkinje sell size. The mean number of Purkinje cells examined in the entire cerebellar cortex was 253 cells per case.	The average volume of Purkinje cells were significantly (P=0.01) reduced by 26% in individuals with autism (N=12) compared to controls (N=7). There was a significant (P=0.001) decrease of 25% of Purkinje cells individuals with autism (N=12) compared to controls (N=7).

X et al. (2013)	lateral cerebellum and vermis	N=10 AU N=9 CN Tissue obtained from NICHD Maryland Brain Bank. All ADIR confirmed autistic cases, sectioned at 50 μm on a viabratome, Nissl stained sections, investigator blind to phenotype, linear qauntification of PC per mm of entire section (100% quantification of PC in PC layer) in Neurolucida software, average of 857 PC counted per brain, 100 PC per case randomly chosen for shape and size analysis using Neurolucida software.	Significant decrease (P=0.024) in Purkinje cells in cerebellar vermis of individuals with autism (N=7). Purkinje cells are more round in several size measures in individuals with autism (N=10) compared to controls (N=9) in vermis and lateral cerebellum.

Table 6 Confounds were tested for individuals with autism. Drowning, seizures, medications, PMI, brain weight, and age do not account for increase in microglial densities. In addition, there are no correlations with the ADIR and the the microglial counts in the individuals with autism. For the Rett's individuals, there brain size is reduced compared autistics but it does not account for the microglial densities when measuring ML total density vs. brain weight (r=0.411 P=0.365 (ns).

Confound	Molecular Layer Microglial Density
Drowning	Autistics that drowned vs. not drowned P=0.658 (not significant) two tailed t-test
Seizures	Autistics that had seizures vs. no seizures P=0.507 (not significant) two tailed t-test
Medications	Autistics that were on medications vs. not P=0.161 (not significant) two tailed t-test
Postmortem Interval (PMI)	**Is PMI correlated with microglial density?** ML autistics, total density vs. PMI, N=9, Pearson's r=0.344, P=0.182 (ns) ML controls, total density vs. PMI, N=9, Pearson's r=-0.197, P=0.305(ns)
Brain Weight	**Is brain weight correlated with microglial density?** ML autistics, total density vs. BW, N=9, Pearson's r=-0.075, P=0.423(ns) ML controls, total density vs. BW N=9, Pearson's r=--0.584, P=0.305(ns)
Age	**Is age correlated with the microglial density?** ML autistics, total density vs. Age, N=9, Pearson's r=-0.186, P=0.315 (ns) ML controls, total density vs. Age, N=9, Pearson's r=-0.090 P=0.408(ns)

Figures

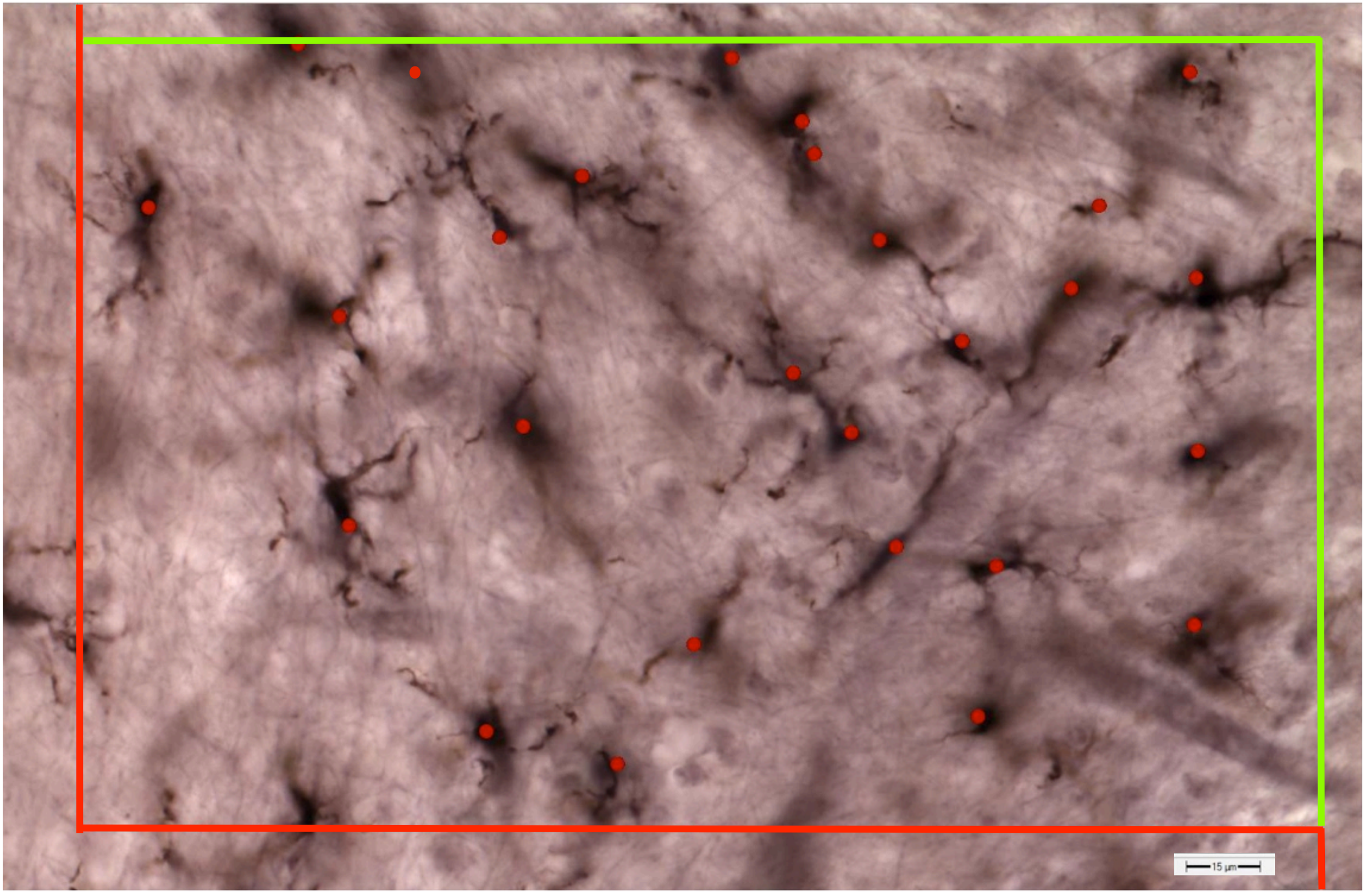


Figure 1 Stereological procedure for quantifying and identifying microglia in control and the brains of individuals with autism; ame method as described in Tetreault et al, 2012. Above is a photomicrograph from the 14 year old autistic male.

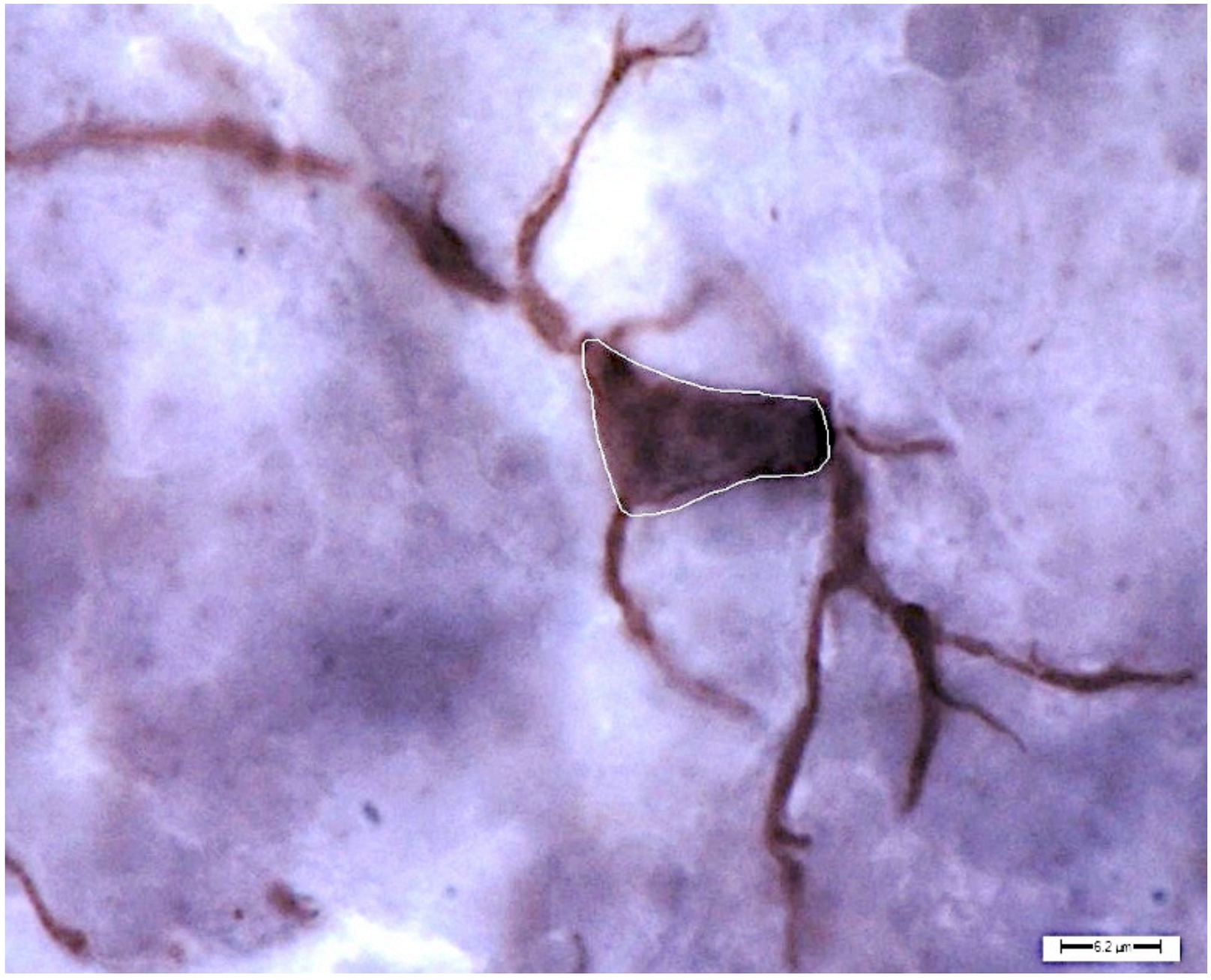


Figure 2 Method for microglia shape analysis. For each case, 50 microglia somas were traced using a 100X objective and analyzed for size and shape for the molecular layer, and an additional 50, for the granule cell layer. The cells were randomly chosen using the stereological probe designed for the microglial counts. The measurements for the shape analysis were area, perimeter, Feret maximum, Feret minimum, aspect ratio, compactness, roundness, convexity, shape factor, form factor and solidity. Above is a photomicrograph from the 22 year old autistic male.

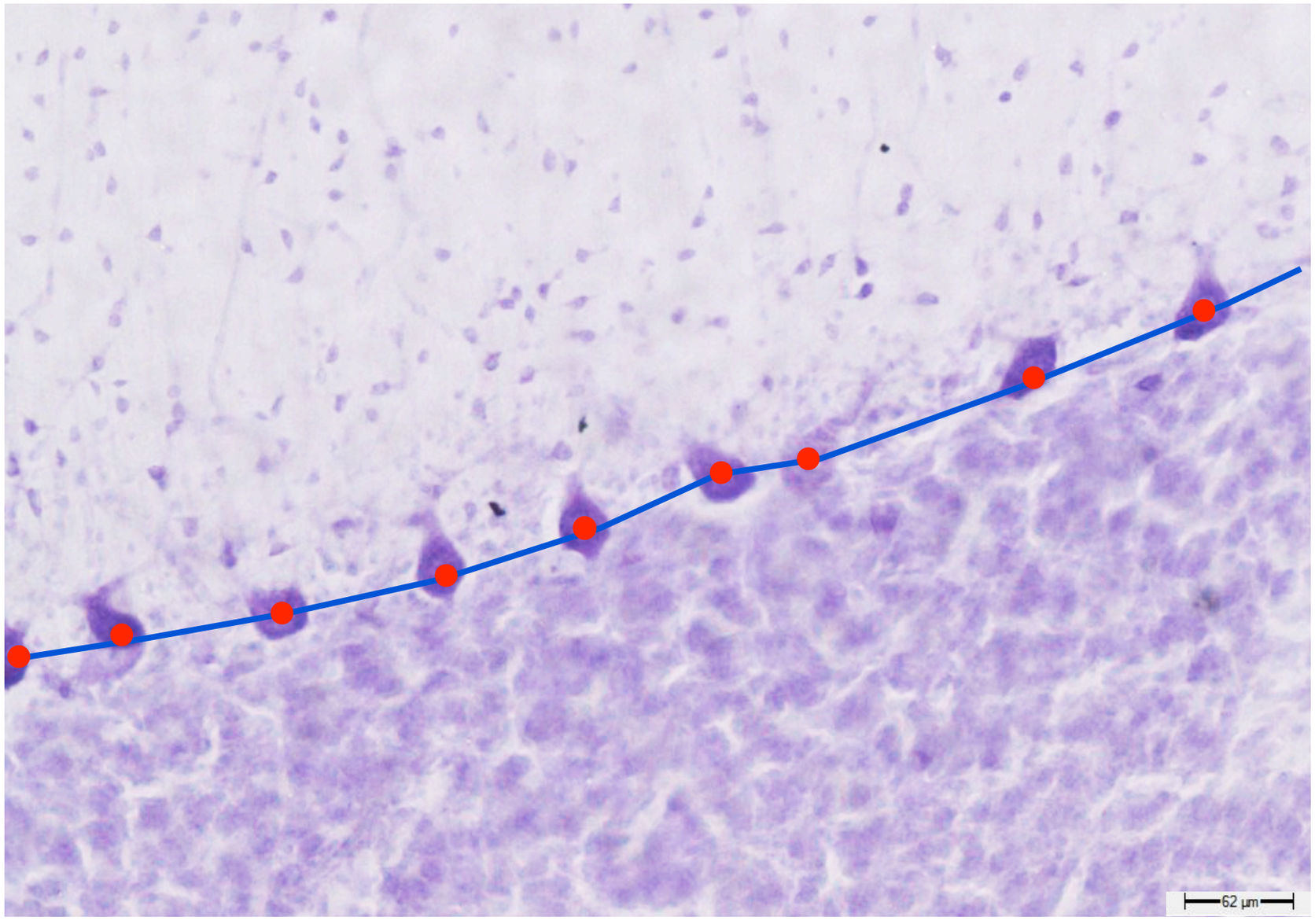


Figure 3 Linear quantification of the Purkinje cells was measured for each of the cases using Neurolucida software (MBF Bioscience, Williston, VT) and the investigators were blind to the case phenotype. The entire Purkinje cell layer was traced, and 100 percent of the cells were counted in the Purkinje cell layer for the section. The number of Purkinje cells is represented per mm and is calculated by the total number of cells counted in the Purkinje cell layer divided by the total length of the Purkinje cell layer for the section. Above is a photomicrograph from a three year old control male cerebellum taken with Nikon Elements Software.

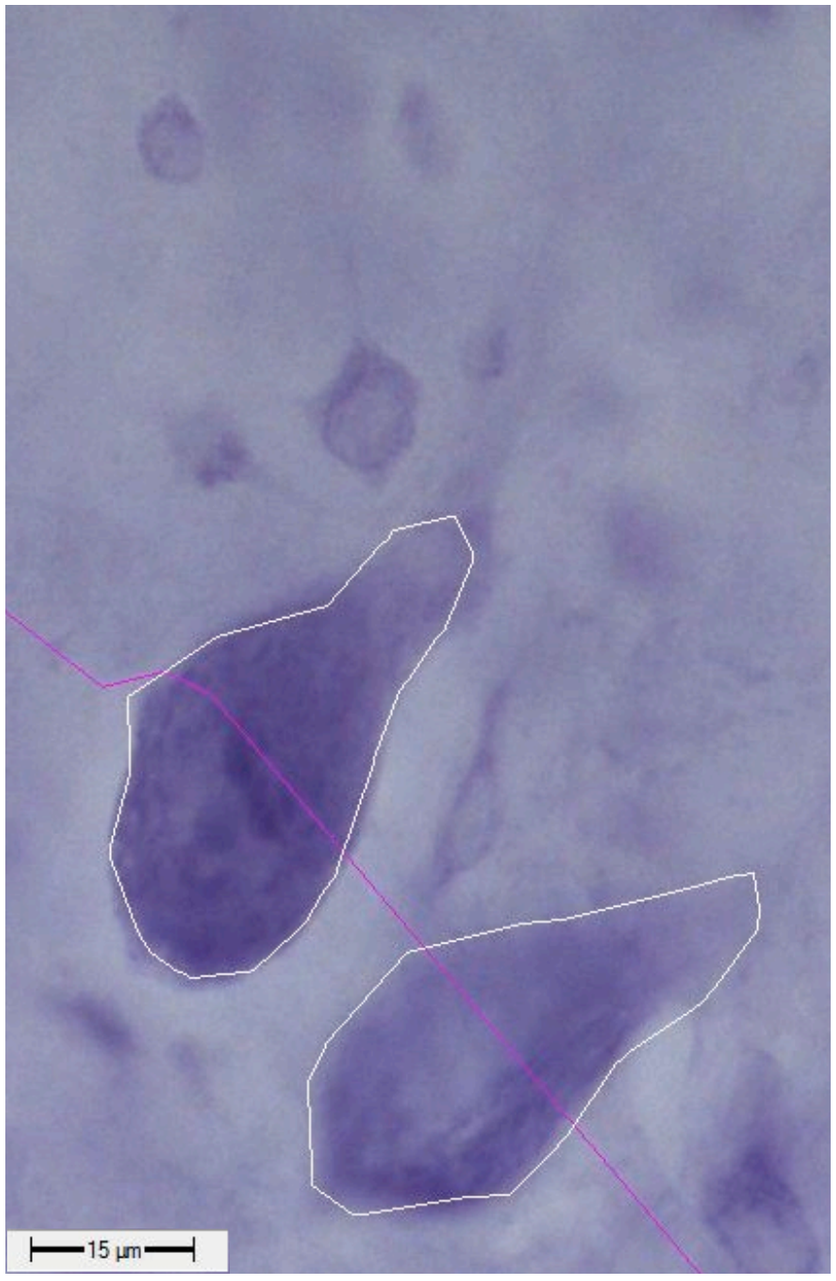


Figure 4 Purkinje shape analysis. The Purkinje cells were traced and analyzed using Neurolucida software (MBF Bioscience, Williston, VT). For each case, 100 Purkinje cells were traced and analyzed for size and shape. The measurements included area, perimeter, Feret maximum, Feret minimum, aspect ratio, compactness, roundness, convexity, shape factor, form factor and solidity. The average for the 100 cells was taken for each of the measurements for each sample. The means for the groups were measured and analyzed. Above is a photomicrograph from the 22 year old control male.

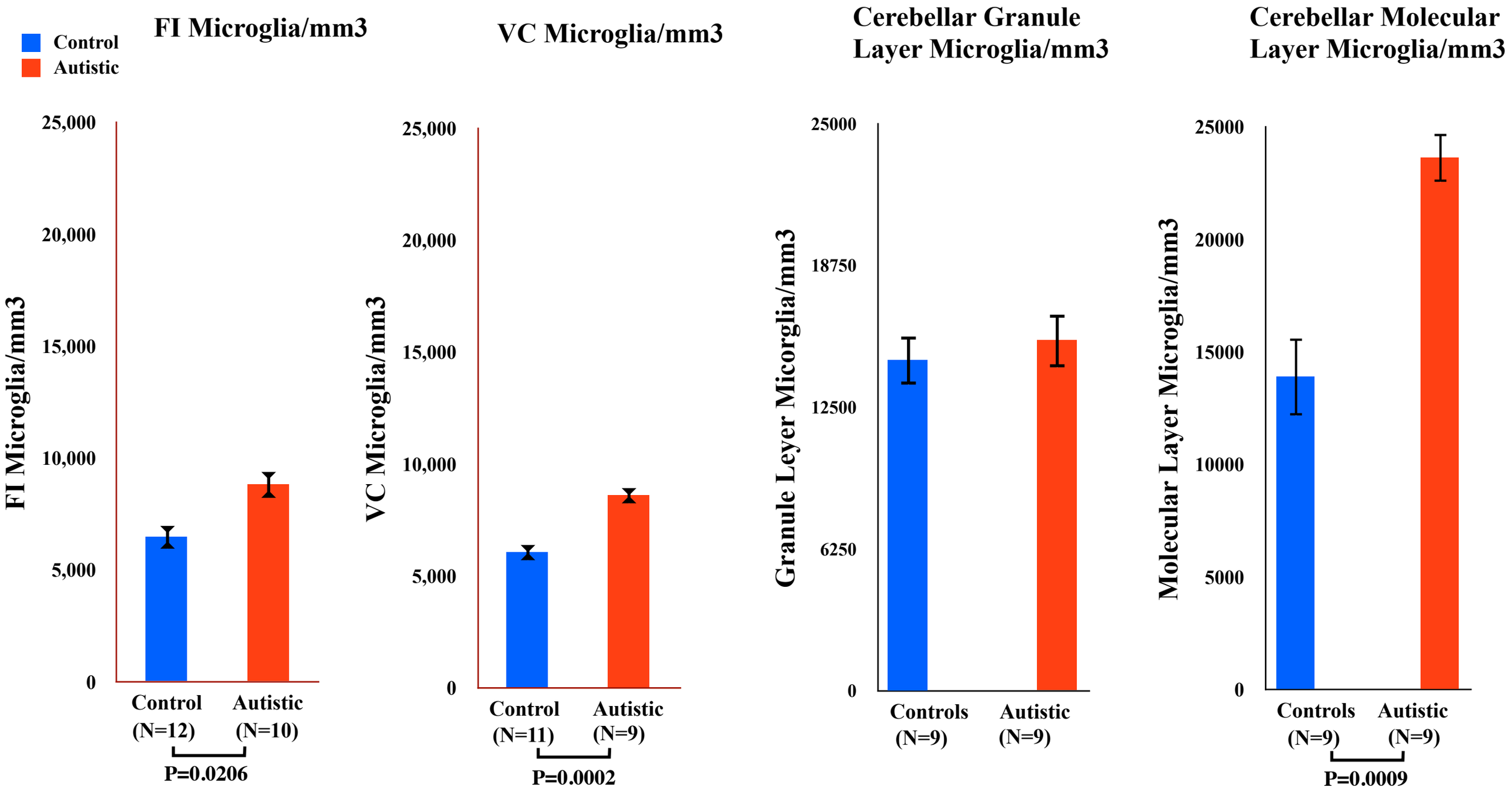


Figure 5 Average microglial cell counts in FI, VC and cerebellum (GL& ML). Autistic FI density is 18% greater than controls and in VC it is 21% greater than controls (Tetreault et al., 2012). The density in the cerebellar granule layer shows no significant difference between autistic and controls, whereas in the cerebellar molecular layer, individuals with autism have significantly (P value is 0.0009, two tailed t-test) greater density of microglia, by 69%. Error bars represent the standard error of the mean.

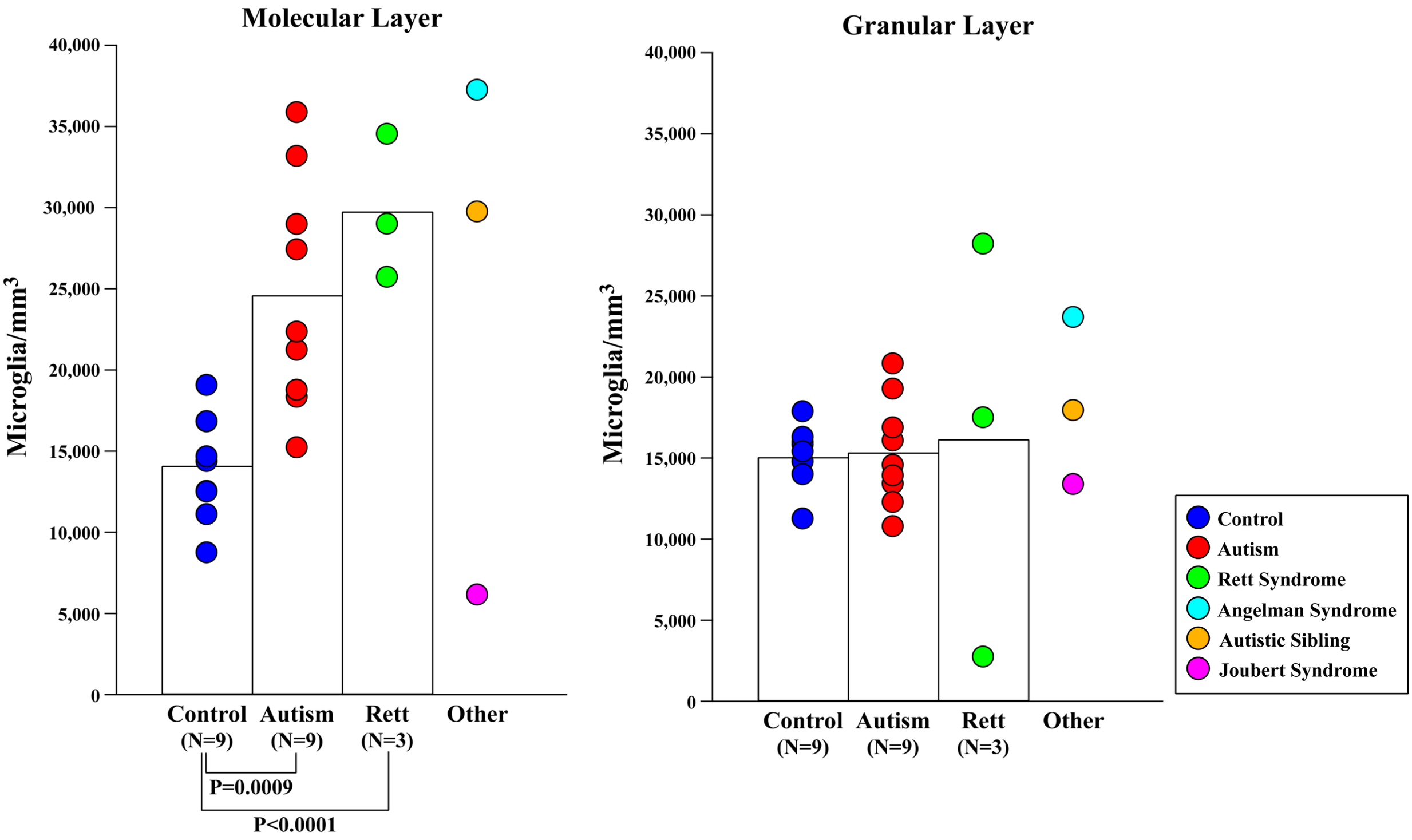


Figure 6 The individuals with autism (N=9) have significantly (p value is 0.0009, two tailed t-test) greater microglial densities for the cerebellar molecular layer compared to the controls (N=9). The individuals with Rett syndrome (N=3) have significantly greater microglial density in the molecular layer compared to controls (N=9) $P < 0.0001$. The cases are averaged for the microglial cell counts for vermis and lateral cerebellum since there was no difference in the microglial densities for the two regions. The individual with Angelman syndrome (N=1) and sibling of an individual with autism (N=1) display microglial densities similar to the individuals with autism for the molecular layer. The individual with Joubert syndrome (N=1) exhibits abnormally low microglial densities for the molecular layer compared to the controls. Note, the increase in microglial densities is specific to the molecular layer where the Purkinje synapses are located.

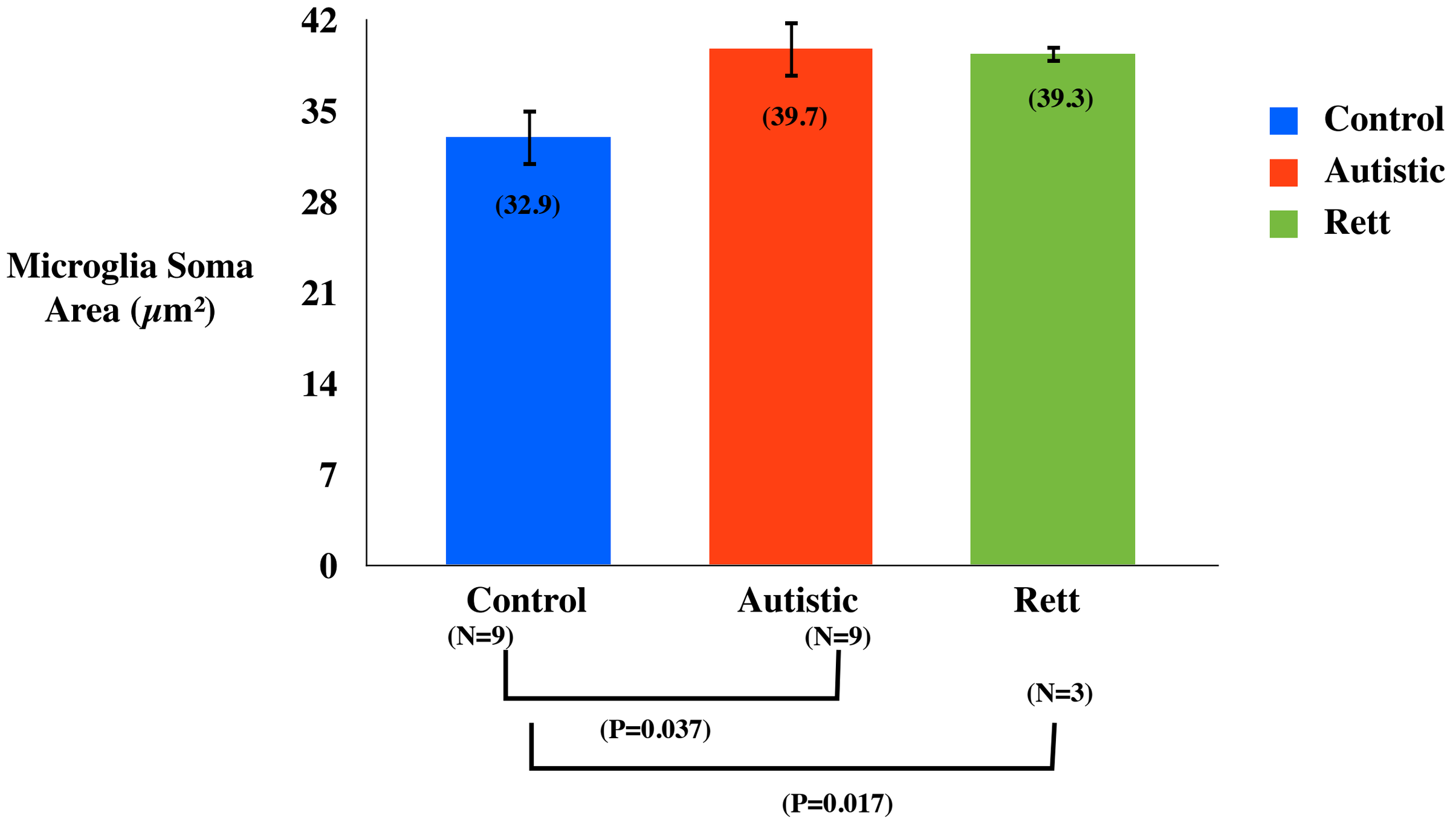


Figure 7 In addition, we found a significant (P=0.042) increase in the soma perimeter of the microglia in individuals with autism (25.8μm) and Rett (26.2μm) (P=0.0043) compared to controls (22.2 μm) indicating the microglial cells are activated in the individuals with autism and Rett syndrome.

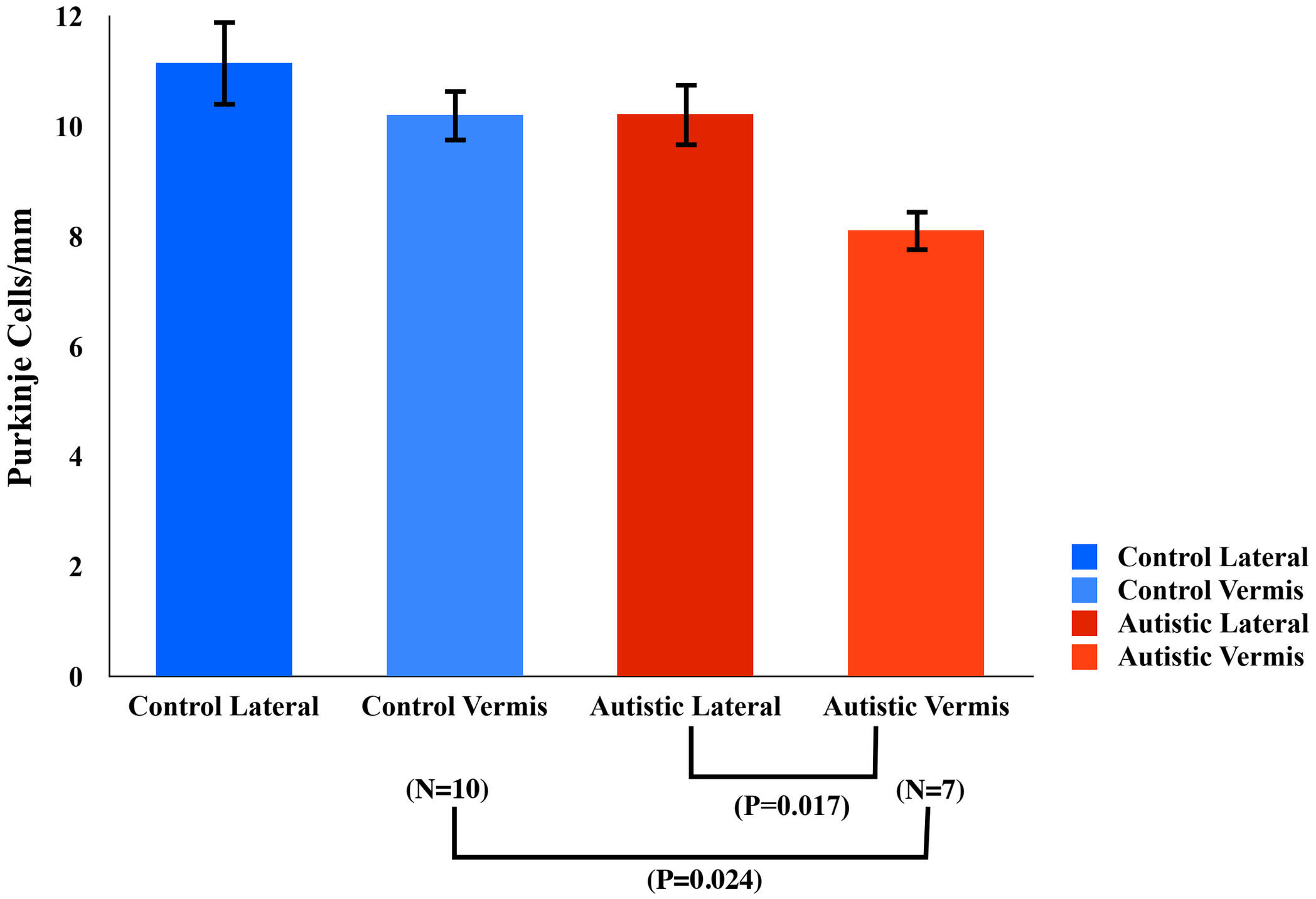


Figure 8 Average Purkinje cells/mm for individuals with autism and controls in lateral cerebellum and cerebellar vermis. When comparing the vermis of individuals with autism and controls there are significantly fewer Purkinje cells in individuals with autism (N=7) compared to controls (N=10) using a two tailed t-test (P value is 0.024) whereas there is no difference in lateral cerebellum for the individuals with autism compared to the controls. In addition, there was a significant (P=0.017) reduction in Purkinje cells in the cerebellar vermis compared to lateral cerebellum in the individuals with autism. Error bars represent the standard error of the mean. There is no difference for the microglial density for the lateral cerebellum when comparing individuals with autism to control. There is no correlation between the number of Purkinje cells/mm and the microglial densities.

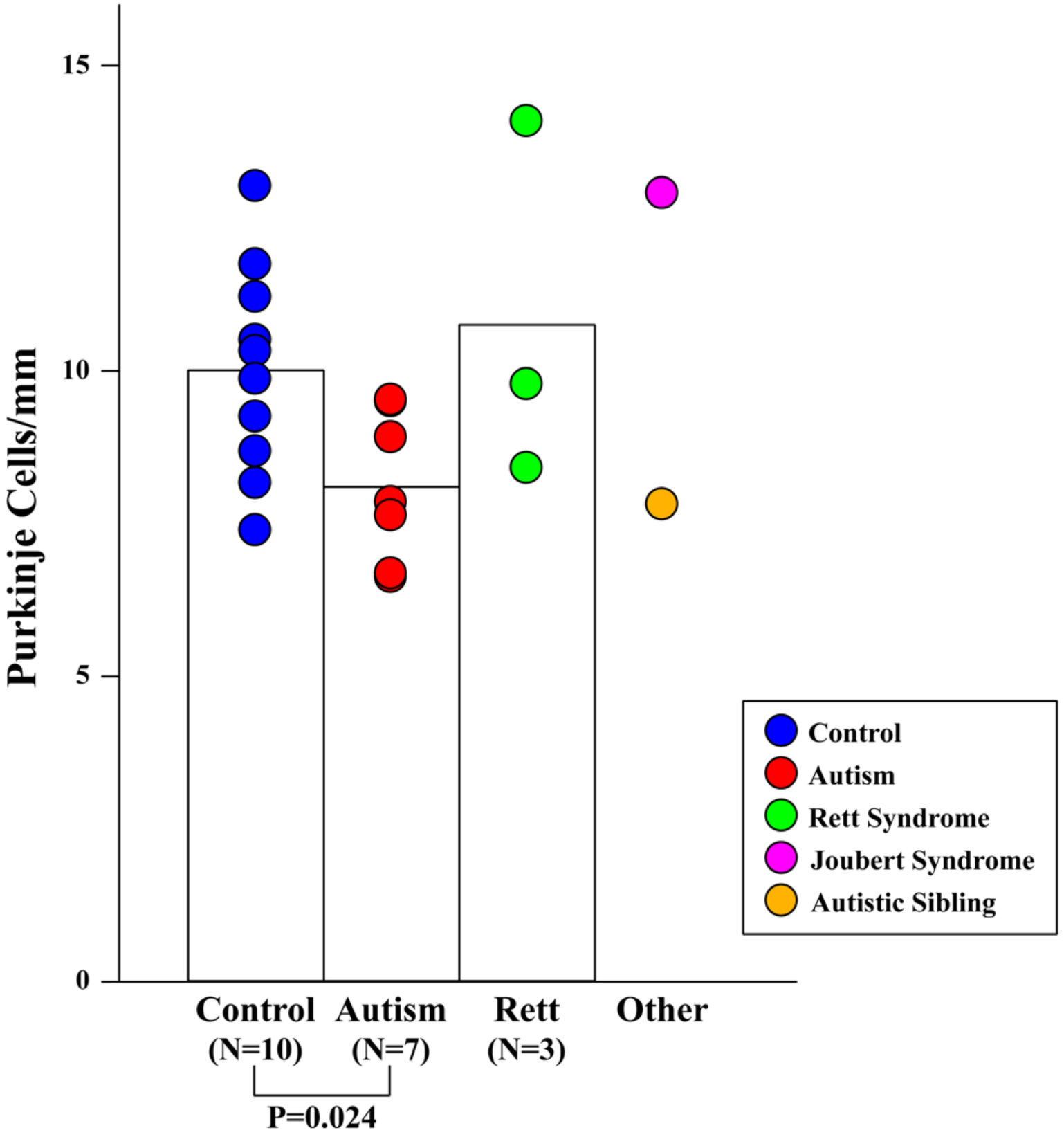
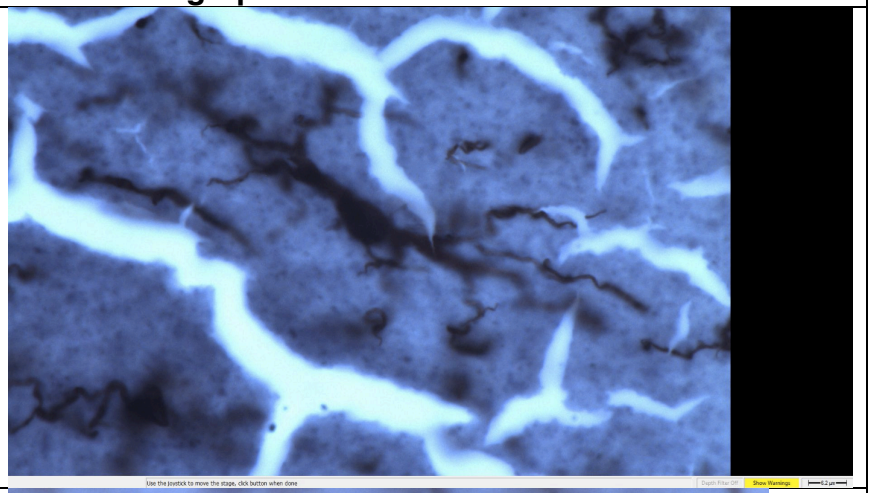
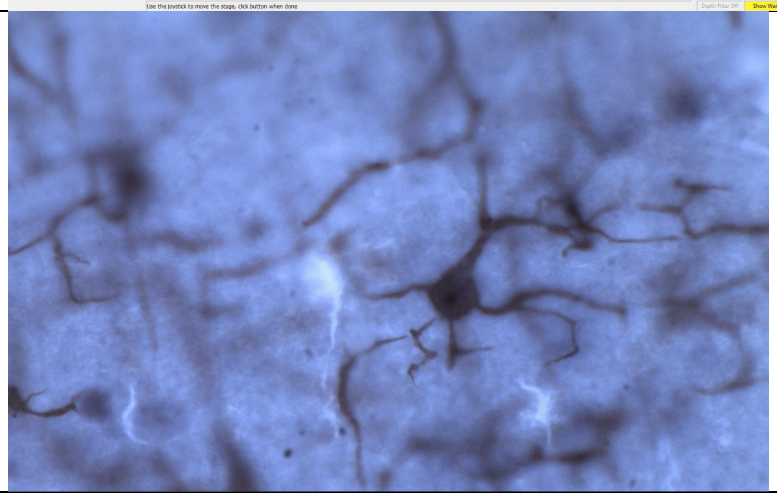
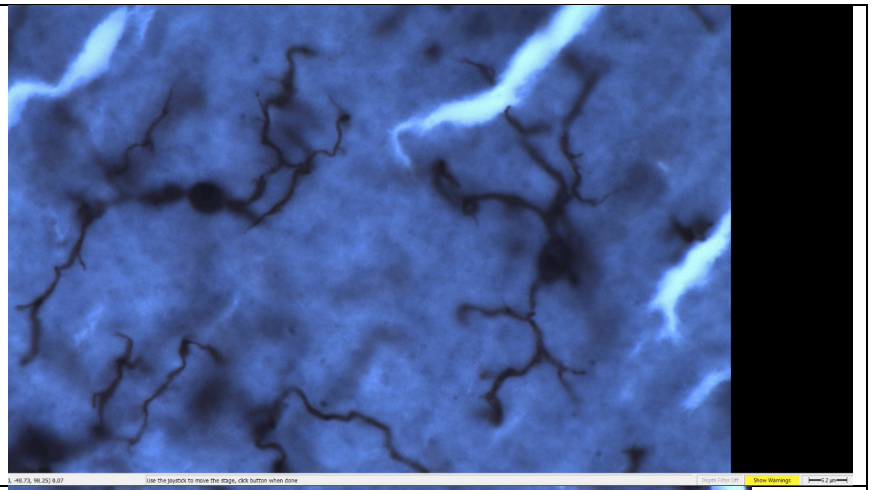
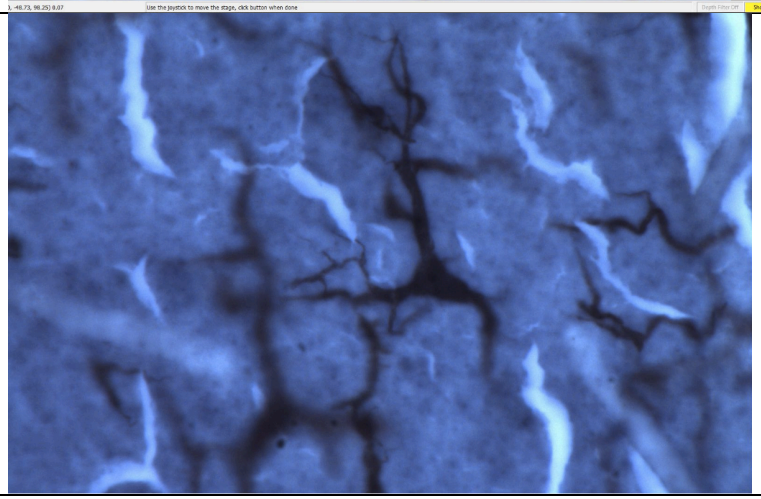
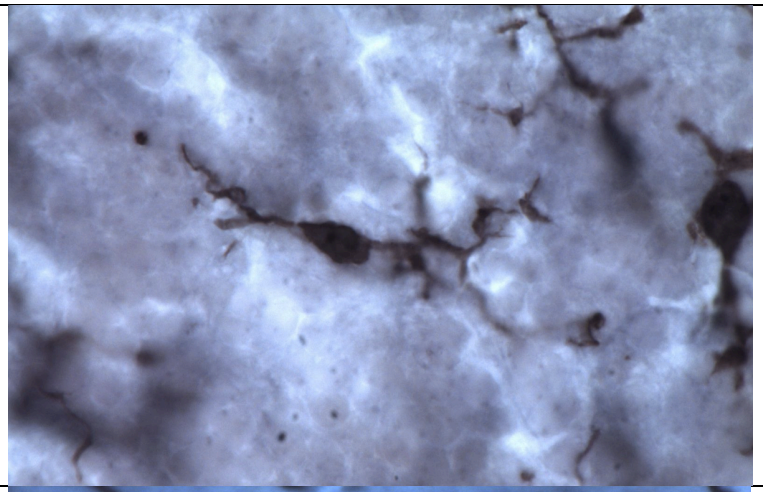
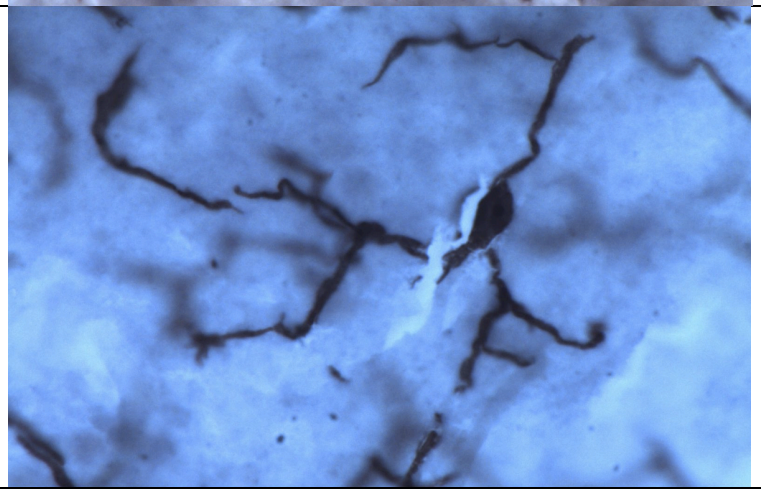


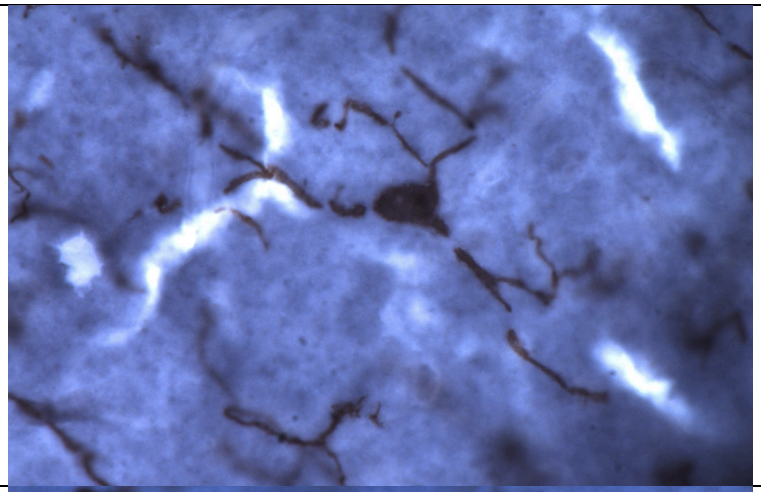
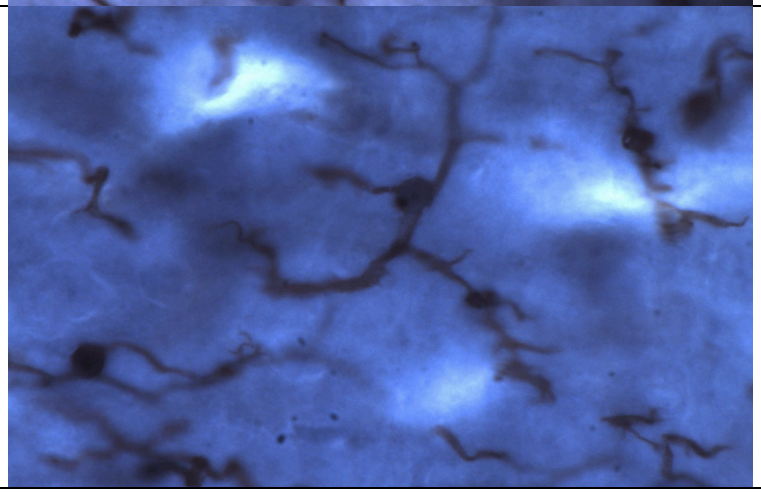
Figure 9 Purkinje cells/mm for in cerebellar vermis where each data point represents a single case. There are significantly fewer Purkinje cells in individuals with autism (N=7) compared to controls (N=10) using a paired, two tailed t-test (P value is 0.024). The autistic sibling is within the range of the autistic cases for the number of Purkinje cells/mm. Note, the Joubert case is within the control range for Purkinje cells.

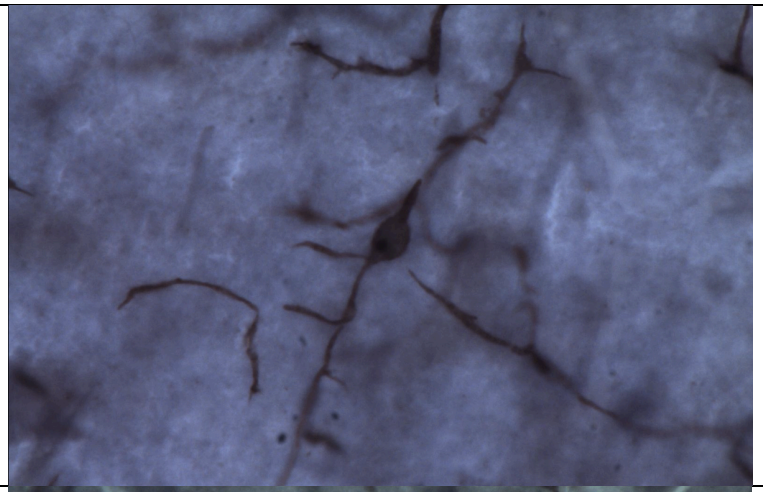
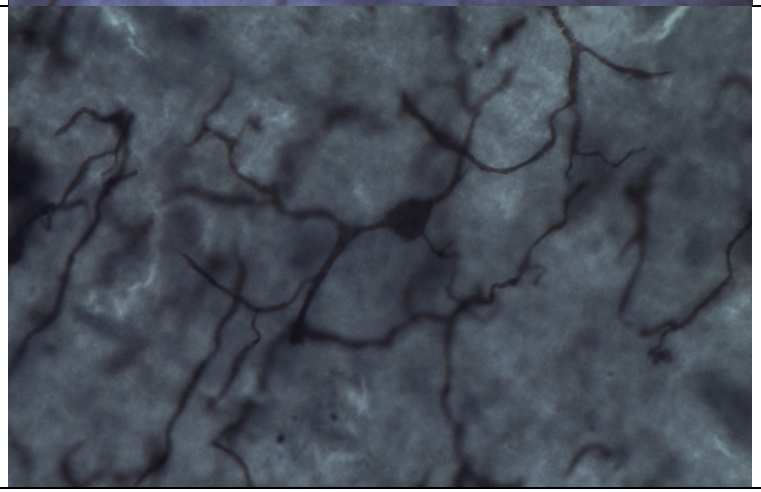
Appendix

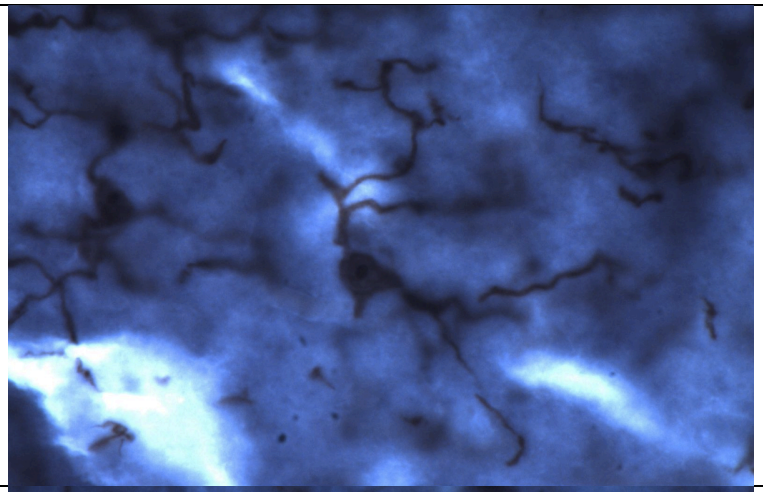
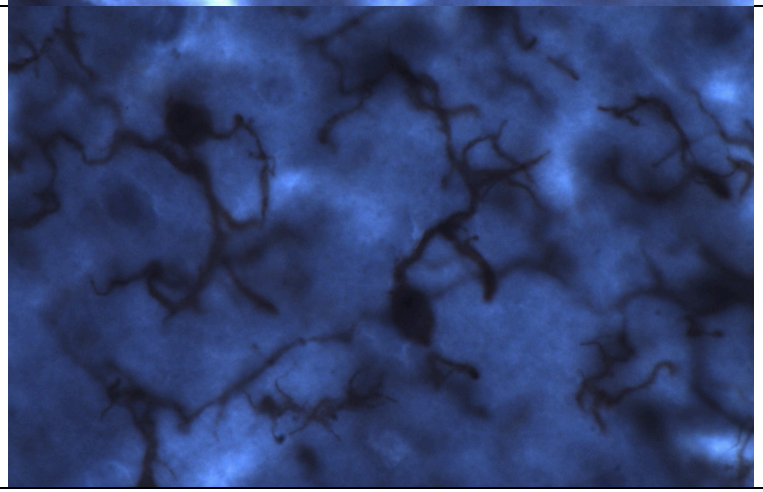
Type	Identification	Photomicrograph
Control	CB_CN_2_M_MB1791	
	LC_CN_3_F_MB1284	

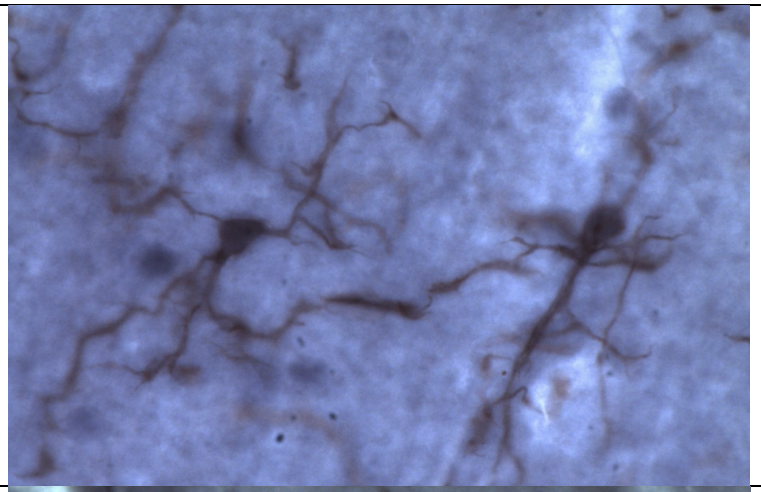
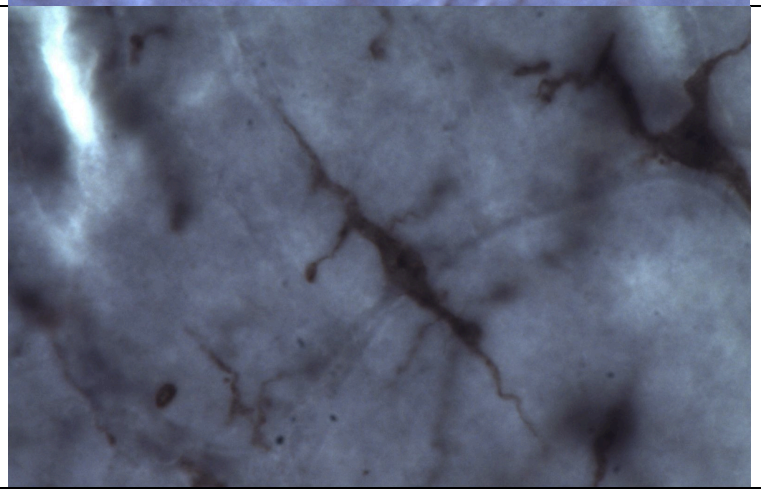
	CB_CN_4_M_MB4670	
	CB_CN_8_F_MB1708	

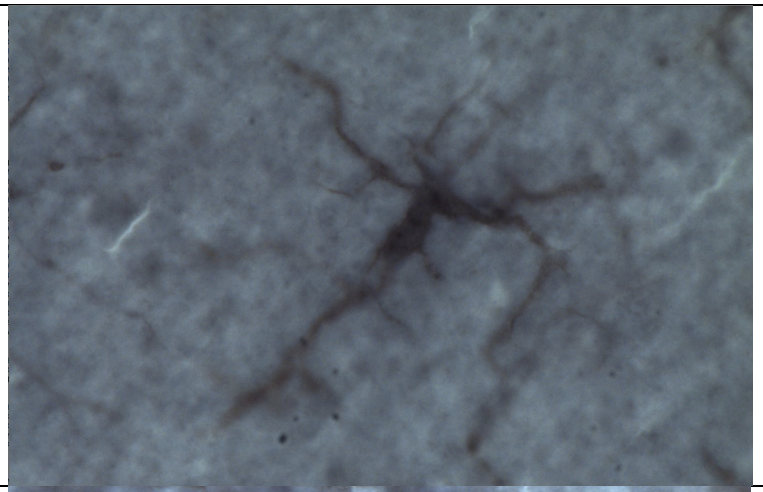
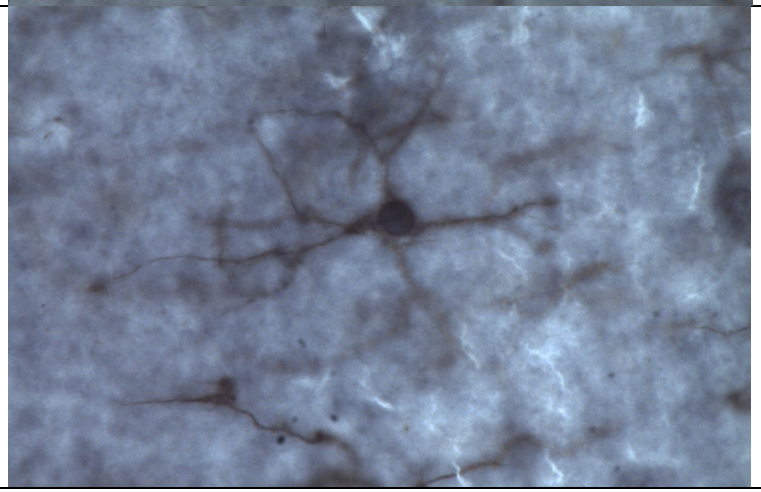
	LC_CN_12_M_MB5387	
	CB_CN_16_F_MB4591	

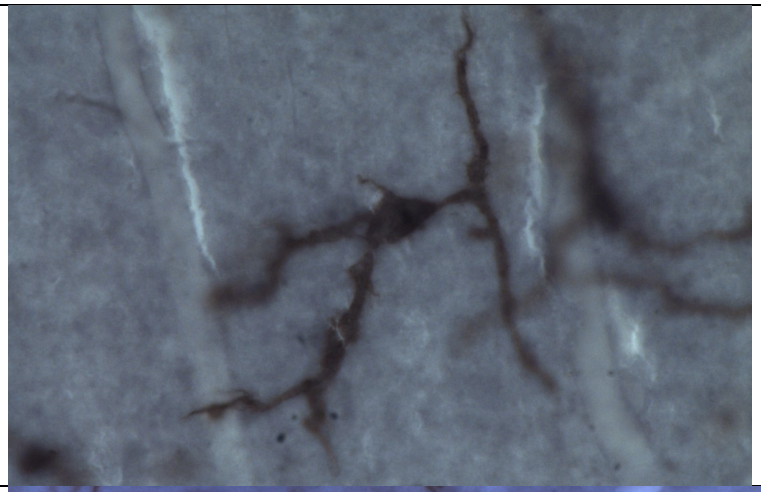
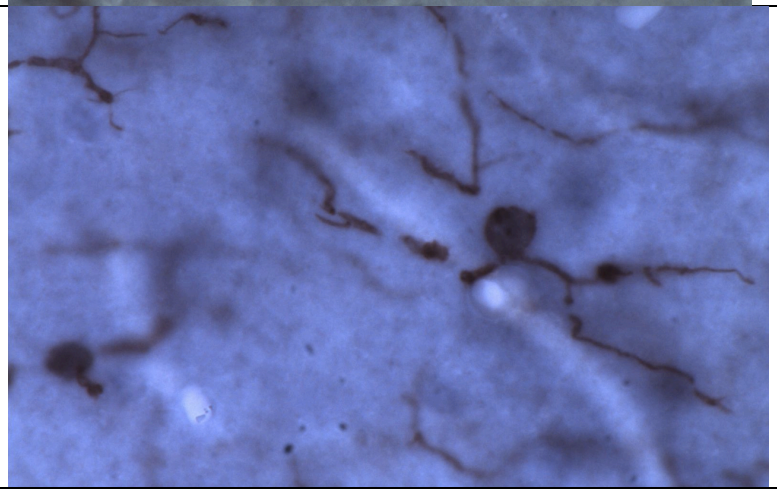
	CB_CN_16_M_MB5077	
	CB_CN_20_M_MB4727	

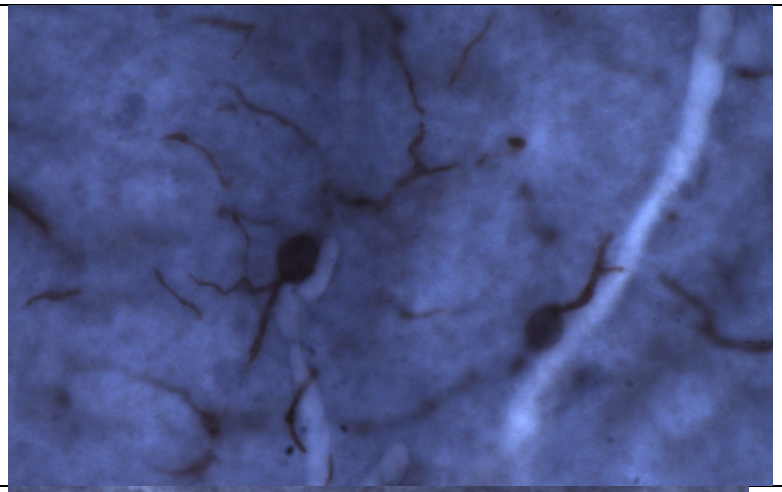
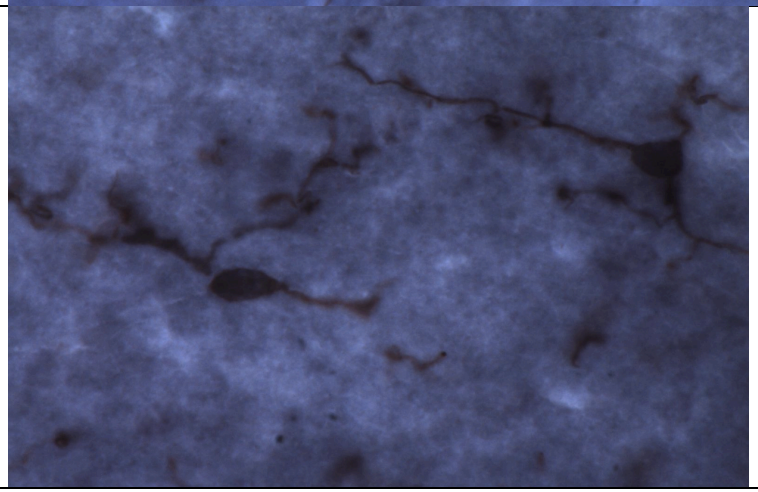
	LC_CN_20_M_MB4727	
	LC_CN_22_M_MB1542	

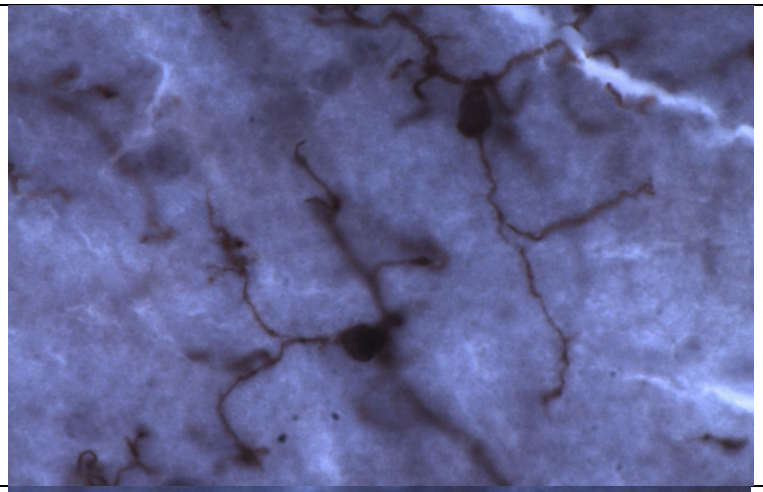
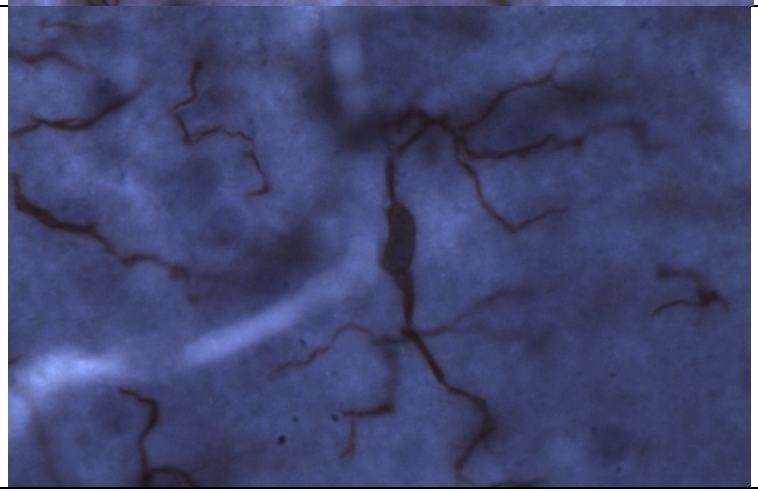
	CB_CN_22_M_MB1542	
Autistic	CB_AU_3_M_MB4021	

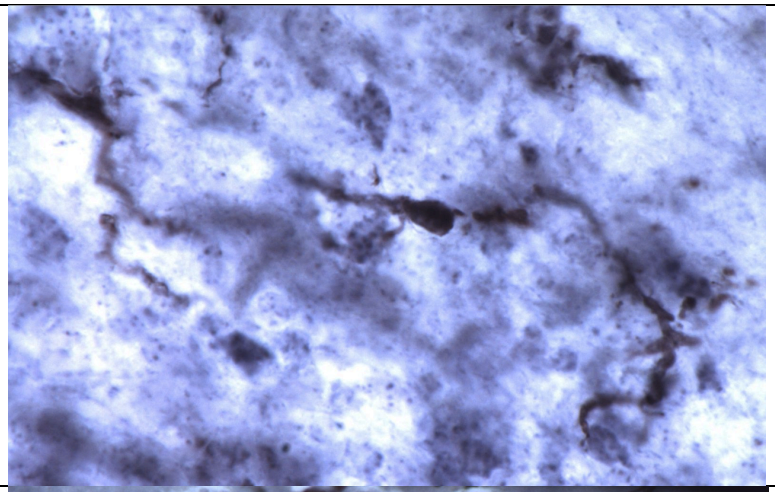
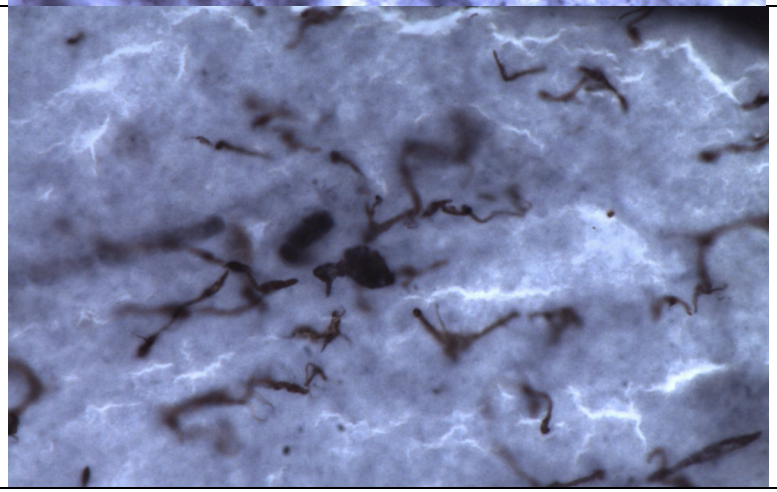
	LC_AU_3_M_MB4021	
	CB_AU_3_M_MB4029	

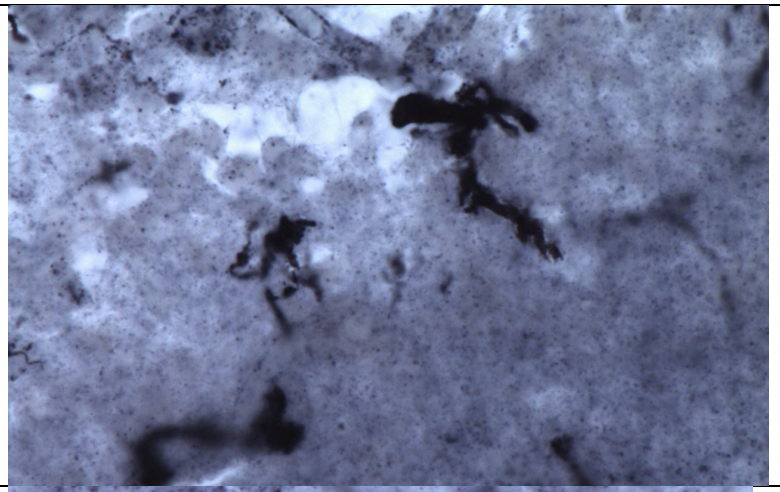
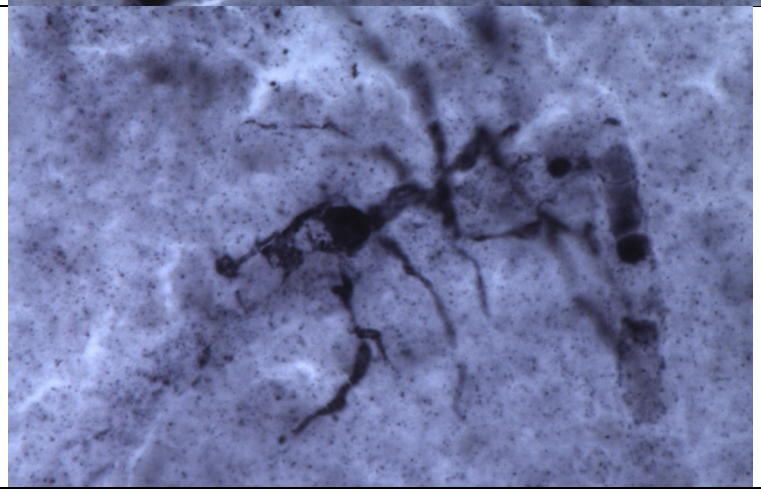
	LC_AU_3_M_MB4029	
	LC_AU_12_M_MB4305	

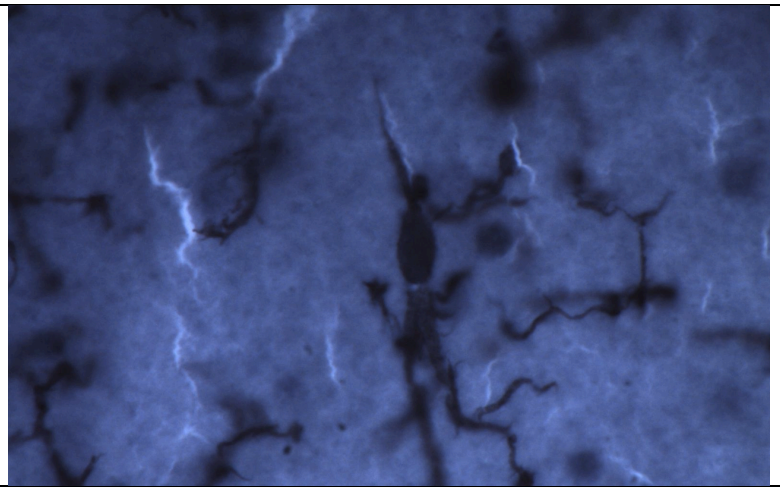
	LC_AU_14_M_MB4899	
	LC_AU_20_M_MB4899	

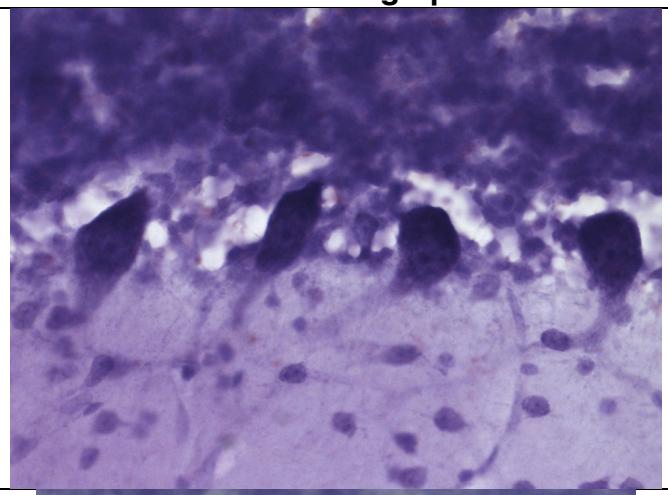
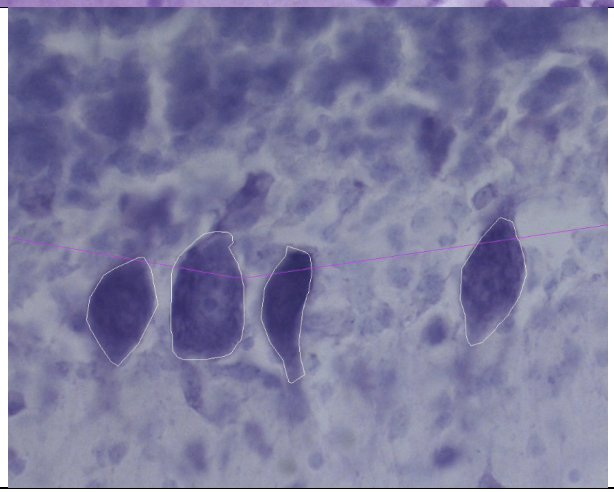
	LC_AU_20_M_MB4999	
Autistic Sibling	BBB: PC_AU_22_M_MB5378	

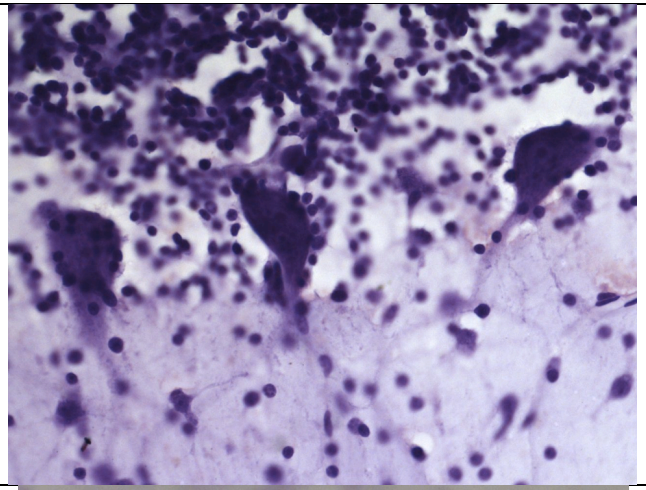
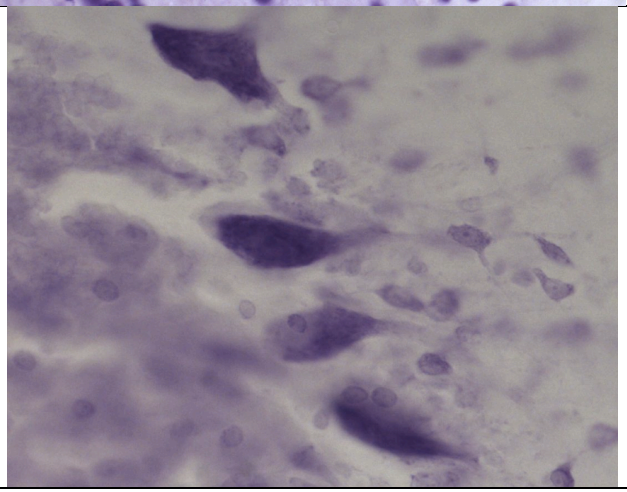
	CCC: PC_AU_22_M_MB5378	
	LC_AU_22_M_MB5378	

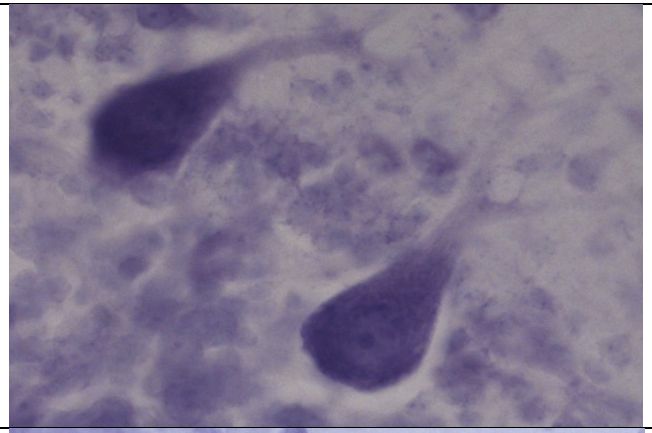
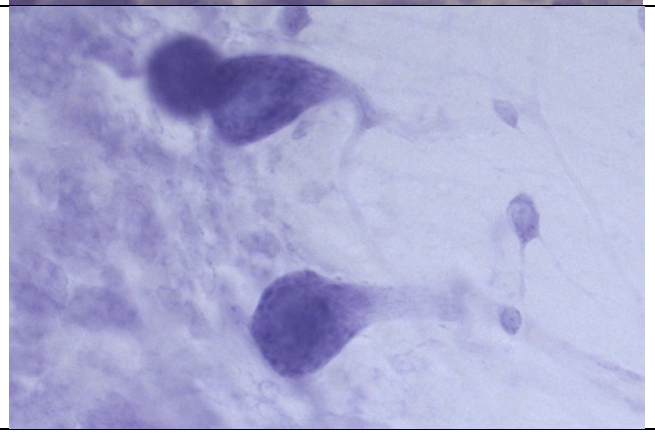
Rett's	CB_R_20_M_MB4516	
	CB_R_18_F_MB1815	

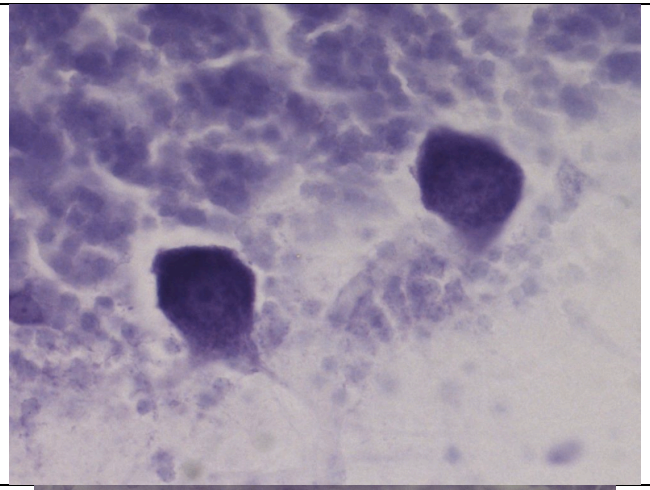
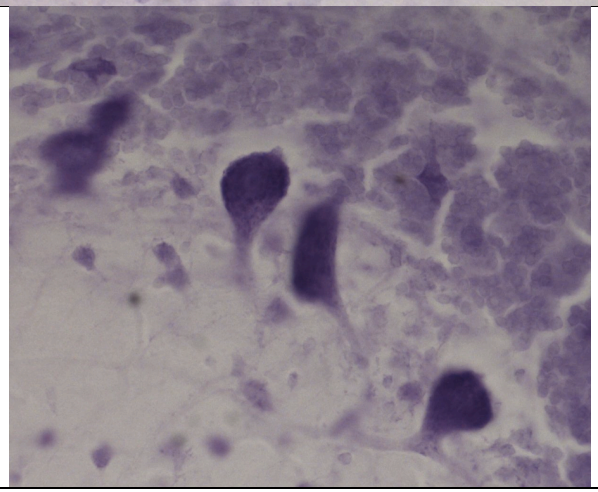
	CB_R_17_F_MB4882	
Joubert's	CB_R_17_F_MB4882	

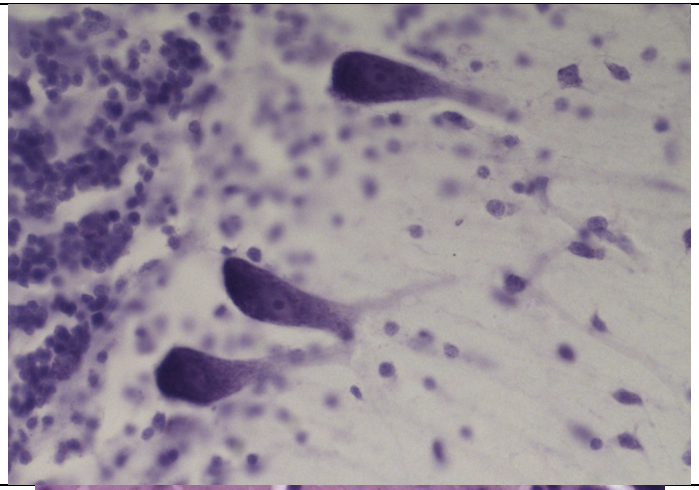
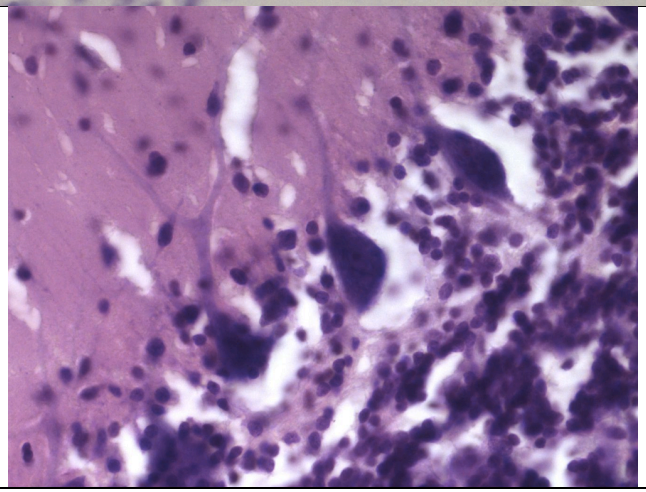
Angelman's	LC_Angel_MB1754	

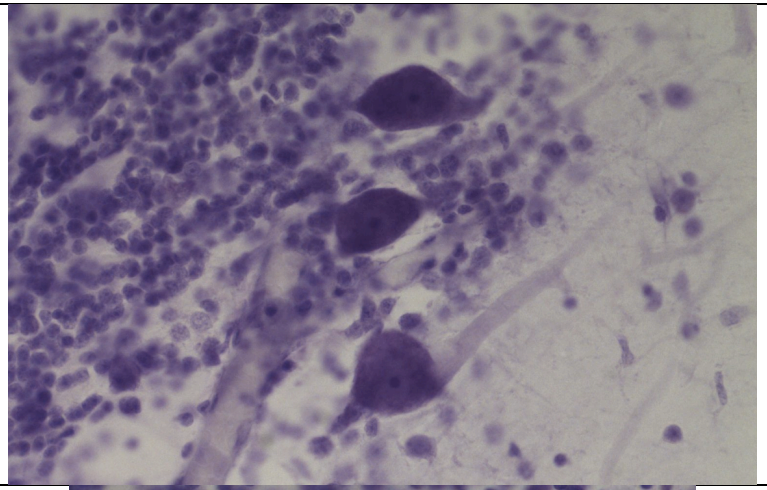
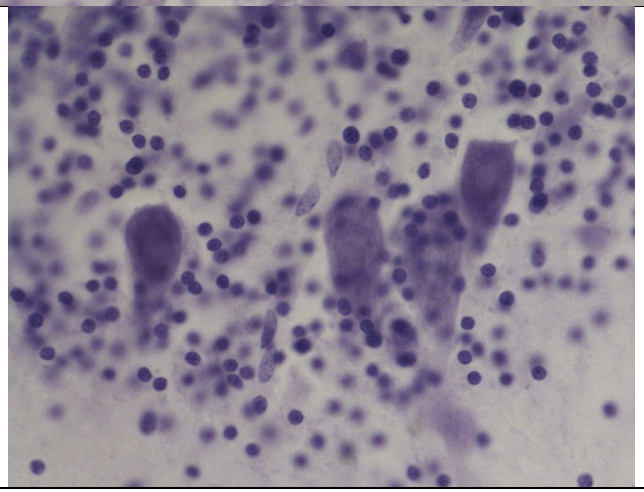
Type	Identification	Photomicrograph
Control	CB_CN_2_M MB1791 vermis	
	CB_CN_3_F MB 1284 vermis	

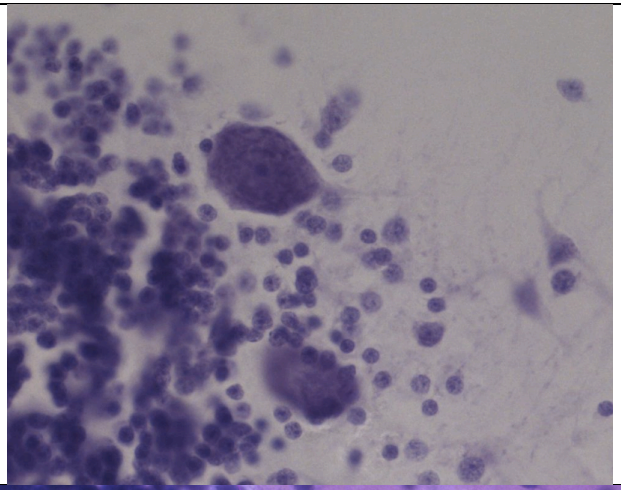
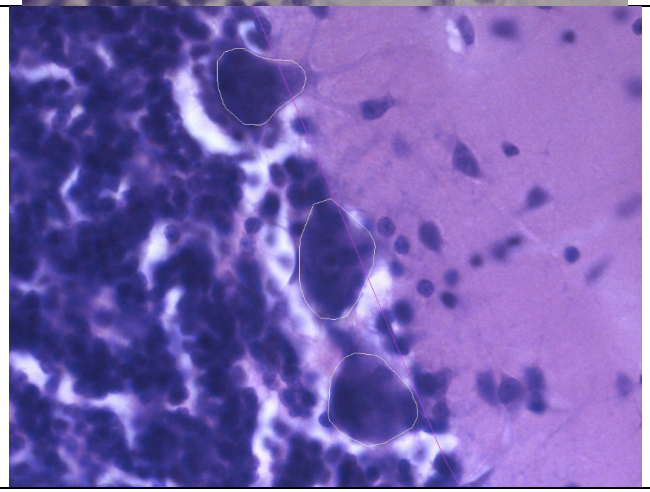
	CB_CN_16_M MB5077 vermis		
	CB_CN_16_F MB4591 vermis		

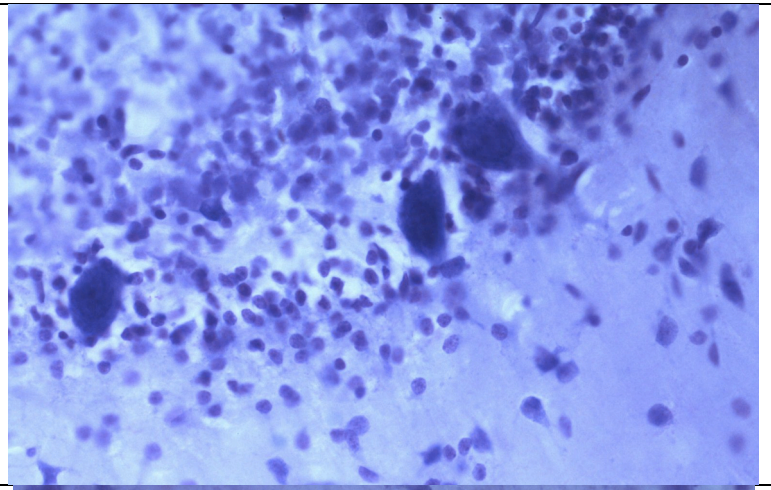
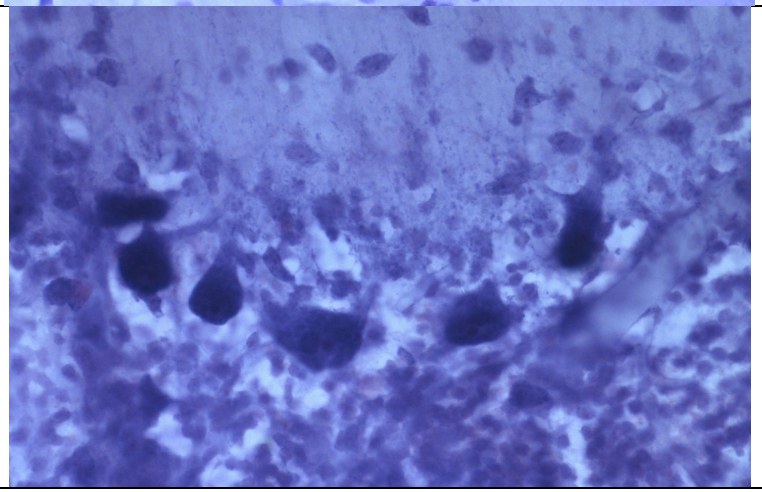
	CB_CN_20_M MB4727 vermis		
	CB_CN_22_M MB1542 vermis		

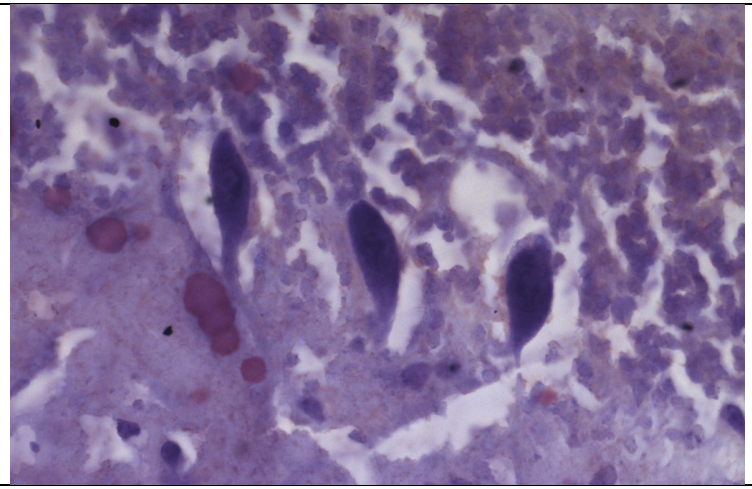
Autistic	CB_AU_3_M MB4021 vermis	
	CB_AU_3_M MB4029 vermis	

	CB_AU_4_F MB4671 vermis	
	PC_AU_12_M_4305 vermis	

	CB_AU_14_M MB4899 vermis	
	CB_AU_15_F MB5278 vermis	

	CB_AU_20_M MB 4999 vermis		
	PC_AU_22_M_5378 vermis		

Rett's	CB_R_17_F MB4882	
	CB_R_18_F MB1815 post vermis	

	CB_R_20_M MB4516		

Autistic	Age	Brain Weight (g)	PMI (h)	Purkinje Vermal Cell Counts	Purkinje Lateral Cell Counts	Microglia Cell Counts ML	Microglial Cell Counts GL
M4021	3	1330	15	7.865413129	11.73569585	21243.29772	14592.99535
M4029	3	1130	13	7.646776501	12.04058681	18358.76686	13435.80712
UMB5308	3	1310	21	No tissue	7.857826801	27429.69403	20848.47471
UMB4671	4	1320	13	6.69408674	No tissue	18788.50962	10807.59115
UMB797	9	1175	13	No tissue	10.81282991	35886.04286	12289.92183
UMB4305	12	1360	13	9.538720398	9.289495568	22371.15226	16895.44401
UMB4899	14	1450	9	9.516067156	8.609003571	15241.17122	13938.36685
UMB5278	15	1417	13	8.925452442	12.14054967	28988.6059	16090.85909
UMB5294	19	1560	16	No tissue	15.34439645	No tissue	No tissue
UMB4999	20	1427	14	6.636811134	8.053696223	33192.44021	19294.24675
Sibling of an Autistic							
UMB5378	22	1500	8	7.828454321	9.689803795	29764.18009	17967.08225
Control							
UMB1791	2	1200	12	10.33585472	No tissue	16856.26353	14013.29603
UMB1284	3	1250	11	10.51957003	15.25332409	16830.20377	11280.74478
UMB4670	4	1470	17	No tissue	8.921328937	8769.916599	15841.43178
UMB1708	8	1320	20	No tissue	8.992850935	12523.84025	14792.30201
UMB5387	12	1750	13	No tissue	9.597261729	11121.02806	15422.21561
UMB5077	16	1330	13	7.401600185	No tissue	14680.23174	16318.56466
UMB4591	16	1330	14	11.22031343	13.22794285	19084.71562	15989.05109
UMB4727	20	1330	5	9.262843321	13.14935218	12552.84258	14025.7618
UMB1542	22	1510	4	8.695774397	11.31411579	14393.23396	17887.83002
Rett							
UMB4882	17	871	18	9.795804125	No tissue	29008.05413	2755.922427
UMB1815	18	930	5	14.09865894	No tissue	34546.08577	28230.54796
UMB4516	20	980	9	8.42495273	No tissue	25738.40037	17515.97064
Angelman	4	1360	24	No tissue	9.5712970474828	37263.52756	23700.88462
Joubert	5	1035	6	12.919871233917	13.810325931048	6168.029371	13403.24101

Microglia Molecular Layer	Area (µm²)	Perimeter (µm)	Feret Min (µm)	Feret Max (µm)	Aspect Ratio	Compactness	Shape Factor	Form Factor	Roundness	Convexity	Solidity	Area error coeff.	Perimeter error coef
Control													
AA: CB_CN_16_M MB5077	26.6200708	19.5684615	4.96076923	7.13461538	0.81932846	0.70800692	3.81230769	0.86984615	0.67631154	0.98117231	1.00702692	1.43873769	1.49565538
DD: CB_CN_16_M MB5077	33.8380736	22.2509091	5.56272727	8.14545455	0.81073636	0.69799818	3.84554545	0.85509091	0.66275455	0.98113545	1.00961	1.63585727	1.61507182
FF: CB_CN_8_F MB1708	25.300212	19.672	4.486	7.582	0.765462	0.627044	3.9356	0.823	0.597238	0.99034	1.004128	1.44635	0.504404
JJ: CB_CN_2_M MB1791	21.8121672	17.5137931	4.48275862	6.42931034	0.81921207	0.70894828	3.78637931	0.87948276	0.67588793	0.99136724	1.00411552	1.28811379	0.43568966
MM: CB_CN_16_F MB4591	36.9441513	24.6205128	5.46410256	9.5957265	0.7325359	0.60016923	4.05649573	0.77880342	0.54666154	0.98261282	1.03002051	1.81045299	1.87400598
GGG: LC_CN_20_M MB4727	38.2302891	25.2921875	5.446875	9.953125	0.71805313	0.57813281	4.108125	0.76109375	0.52728281	0.98815938	1.02587188	1.85970625	0.65320313
III: LC_CN_22_M MB1542	39.7041468	25.8467742	5.46451613	10.2322581	0.71682097	0.57012258	4.11048387	0.76354839	0.52704516	0.98945806	1.03024677	1.90020806	0.63540806
KKK: LC_CN_16_F MB4591	34.4538082	22.2986301	5.73835616	8.19178082	0.81328082	0.71200822	3.81356164	0.86780822	0.66677671	0.98913699	1.00579863	1.63973425	0.53538767
NNN: LC_CN_12_M_MB5387	24.1280855	19.5727273	4.36727273	7.51454545	0.74765273	0.60601818	4.02127273	0.79327273	0.56960545	0.98548	1.01644364	1.43884	0.48305818
RRR: LC_CN_20_M MB4727	44.0326687	27.3925373	5.73731343	10.7985075	0.71290448	0.56213582	4.12820896	0.75850746	0.5192597	0.98331493	1.03714776	2.01377463	0.66566567
TTT: LC_CN_3_F MB1284	37.1185414	24.6547486	5.42999768	9.48784186	0.73867664	0.6028804	4.07739599	0.7746927	0.55748381	0.98197558	1.03169359	1.81280721	0.86709121
Autistic													
CC: CB_AU_9_M MB797	33.0348717	21.9641509	5.39245283	8.20377358	0.79675283	0.67377264	3.84188679	0.85792453	0.64166132	0.98946792	1.01036792	1.61533679	1.61824151
KK: CB_AU_3_M MB5308	43.9260441	28.8135593	5.71186441	10.7559322	0.71366271	0.56254407	4.37288136	0.69491525	0.52116102	0.93490169	1.0346339	2.11820169	0.65921525
AAA: LC_AU_20_M MB4999	32.5282152	22.0478261	5.38695652	8.06086957	0.80962609	0.68978043	3.88347826	0.84369565	0.66426304	0.97963261	1.01222609	1.62192174	1.28240435
EEE: LC_AU_14_M MB4899	44.1964854	28.1926829	5.7195122	11.2829268	0.68147805	0.53876829	4.25829268	0.71243902	0.47581707	0.98270976	1.05630244	2.07314878	0.69488049
FFF: LC_AU_12_M MB4305	39.4280885	23.7826923	5.99230769	8.71923077	0.81941731	0.70318462	3.80576923	0.87442308	0.67877692	0.98800192	1.00492885	1.74894808	0.55990192
LLL: LC_AU_3_M MB4021M	36.2032036	23.8290909	5.39272727	9.17818182	0.74748727	0.60297636	3.97290909	0.80563636	0.56590364	0.9866	1.01278727	1.75207091	0.58980364
MMM: LC_AU_15_F MB5278	31.7209267	21.36	5.42333333	7.715	0.82854167	0.71746167	3.813	0.872	0.69207167	0.98086	1.00162333	1.57075833	0.51282667
OOO: LC_AU_14_M MB4899	49.6636176	29.3176471	6.09019608	11.5058824	0.70208627	0.55179412	4.18960784	0.73215686	0.50345294	0.98229412	1.05571569	2.15598431	0.73353725
PPP: LC_AU_3_M MB4029M	53.9819772	34.0368421	6.50526316	12.7894737	0.66191228	0.5341386	4.64947368	0.62	0.4502614	0.93694737	1.09860526	2.50301579	0.82725439
QQQ: LC_AU_3_M MB4021M	37.2980373	28.3898305	5.56610169	9.27966102	0.75437627	0.62392373	4.67271186	0.6840678	0.57712034	0.89601017	1.02687288	2.08784576	0.59859153
SSS: LC_AU_3_M MB4029M	43.1013475	27.7576271	5.85762712	10.9254237	0.69714407	0.56783898	4.22898305	0.72135593	0.49788136	0.98328814	1.05965763	2.04067966	0.71579661
WWW: LC_AU_20_M MB4999	31.7579121	21.2051724	5.57586207	7.60344828	0.84066207	0.74488966	3.7787931	0.88603448	0.71249828	0.98742241	1.00330345	1.55896207	0.53677241
Sibling of an Autistic													
BBB: PC_AU_22_M MB5378	39.4210128	25.6382979	5.33404255	10.1744681	0.70896809	0.54518511	4.09553191	0.76489362	0.51197872	0.98842766	1.03320213	1.88531915	1.57825745
CCC: PC_AU_22_M MB5378	35.8794929	24.2803571	5.39285714	9.14285714	0.7543125	0.61428214	4.06946429	0.77642857	0.58120893	0.97107679	1.01758214	1.78518571	0.55328036
JJJ: LC_AU_22_M MB5378	37.534002	23.696	5.706	8.91	0.786654	0.661478	3.8792	0.8418	0.626216	0.988818	1.011806	1.74224	0.600106
Rett													
BB: CB_R_20_M MB4516	39.3444886	26.3427711	5.4373494	10.2355422	0.69907952	0.55752048	4.26084337	0.71042169	0.4998488	0.97497711	1.0558253	1.93701747	1.67225482
OO: CB_R_18_F MB1815	40.651222	25.5381356	5.82372881	9.70338983	0.75461102	0.62405678	4.01822034	0.79279661	0.57932542	0.9770322	1.02622966	1.87757627	1.93842119
QQ: CB_R_17_F MB4882 Sec. 1 vermis	38.0762589	26.7933775	5.29470199	10.4788079	0.67388874	0.53268146	4.3913245	0.67509934	0.46520927	0.96865497	1.08319272	1.96998212	1.59371126
Joubert													
NN: CB_JS_5_F MB5486 Sec. 10 lat. cerebell.	56.6219794	36.6990196	6.35686275	14.4333333	0.60338333	0.47313137	4.95068627	0.55803922	0.3783402	0.94826569	1.19015686	2.69859804	2.19809314
PP: CB_R_17_F MB4882 Sec. 1 vermis	41.2520175	32.11	5.29	12	0.62003	0.47599	5.052	0.52375	0.397255	0.9011725	1.1249025	2.3605025	1.6363825
Angelman													
VVV: LC_Angel_MB1754	31.7579121	21.2051724	5.57586207	7.60344828	0.84066207	0.74488966	3.7787931	0.88603448	0.71249828	0.98742241	1.00330345	1.55896207	0.53677241
Microglia Granule Layer													
Control													
AA: CB_CN_16_M MB5077	31.0921944	21.8014085	5.20985915	8.00985915	0.79339014	0.6705662	3.93549296	0.82070423	0.63759437	0.97307324	1.01784507	1.60320986	1.60004225
DD: CB_CN_16_M MB5077	35.1566724	22.9921053	5.59473684	8.39605263	0.80253026	0.68130789	3.89328947	0.83763158	0.65019737	0.97592632	1.00819605	1.69055395	1.39973553
FF: CB_CN_8_F MB1708	26.5583671	20.2842105	4.57236842	7.82236842	0.75861974	0.61729342	3.95697368	0.81276316	0.58591447	0.98966316	1.01214868	1.49065132	0.54752632
JJ: CB_CN_2_M MB1791	26.7032456	20.2736842	4.61754386	7.77368421	0.75947193	0.61626842	3.95333333	0.81649123	0.58734211	0.98707018	1.01200702	1.4909	0.52258772
MM: CB_CN_16_F MB4591	40.8455079	27.6634921	5.7015873	10.552381	0.69965873	0.5668746	4.32984127	0.69285714	0.49959683	0.95708254	1.065511111	2.03369683	1.70582698
GGG: LC_CN_20_M MB4727	38.0869813	26.1770833	5.37291667	10.3770833	0.68264792	0.54042708	4.266875	0.70791667	0.47618333	0.98221875	1.06402292	1.92495833	0.69781667
III: LC_CN_22_M MB1542	40.2231725	26.9627451	5.40980392	10.727451	0.67940392	0.53352549	4.27882353	0.70039216	0.47097059	0.98601373	1.06015882	1.98214314	0.6824098
KKK: LC_CN_16_F MB4591	38.4742439	24.8298246	5.45789474	9.75263158	0.72674912	0.57768246	4.01912281	0.78666667	0.53581579	0.98887018	1.01780877	1.82555088	0.61428246
NNN: LC_CN_12_M_MB5387	47.0563909	30.6309091	5.72727273	12.32	0.63905818	0.48904	4.49890909	0.644	0.41802182	0.96983273	1.09887091	2.25226	0.75690727
RRR: LC_CN_20_M MB4727	41.9709967	27.9733333	5.67833333	10.9116667	0.68521333	0.54556167	4.32016667	0.69566667	0.47901167	0.97074667	1.06294833	2.05696667	0.69659333
TTT: LC_CN_3_F MB1284	42.920982	28.3945252	5.7683868	10.7930583	0.70114931	0.56731045	4.35499637	0.69983144	0.50465863	0.95974839	1.06482088	2.08638508	1.08307919
Autistic													
CC: CB_AU_9_M MB797	37.2453423	24.228866	5.55773196	9.22680412	0.75380412	0.62415361	3.99989691	0.79731959	0.57676495	0.9842268	1.02587113	1.78136082	1.4809299
KK: CB_AU_3_M MB5308	53.6573574	33.0333333	6.62777778	11.8814815	0.709011111	0.581961111	4.51148148	0.66407407	0.51219074	0.92855185	1.05415741	2.42901481	0.72835
AAA: LC_AU_20_M MB4999	31.0921944	21.8014085	5.20985915	8.00985915	0.79339014	0.6705662	3.93549296	0.82070423	0.63759437	0.97307324	1.01784507	1.60320986	1.60004225
EEE: LC_AU_14_M MB4899	33.3298381	25.1452381	4.90238095	9.98809524	0.66714048	0.5205619	4.39166667	0.6752381	0.4580619	0.97573095	1.08445714	1.84889286	0.64972381
FFF: LC_AU_12_M MB4305	43.3136262	27.6	5.85714286	10.7642857	0.70708095	0.57562143	4.20595238	0.72952381	0.51046905	0.98314286	1.06478571	2.02927143	0.66680238
LLL: LC_AU_3_M MB4021M	37.8083462	24.7788462	5.43846154	9.70384615	0.72616346	0.58287115	4.04826923	0.77788462	0.53630577	0.98656538	1.02253269	1.82223077	0.60158654
MMM: LC_AU_15_F MB5278	37.5569585	24.6075472	5.54150943	9.47924528	0.73534717	0.60398113	4.05339623	0.77849057	0.54943585	0.98535283	1.03138679	1.80941887	0.60777547
OOO: LC_AU_14_M MB4899	51.3329667	32.8912281	6.14561404	13.1087719	0.62350702	0.48657719	4.61912281	0.60070175	0.39504561	0.96195088	1.11779825	2.4181386	0.86735614
PPP: LC_AU_3_M MB4029M	47.50085	29.9160714	6.24642857	11.1589286	0.7017125	0.57769286	4.38571429	0.67410714	0.50025714	0.94682857	1.07083036	2.19975179	0.74875536
QQQ: LC_AU_3_M MB4021M	43.608549	31.054902	5.88823529	10.7666667	0.70365294	0.57352745	4.75058824	0.60568627	0.5062	0.88977843	1.06269608	2.28335686	0.69225294
SSS: LC_AU_3_M MB4029M	32.5577681	22.89375	5.22291667	8.75416667	0.73097917	0.6151125	3.94	0.76958333	0.55370833	0.98785625	1.01098333	1.64254583	0.61892292
WWW: LC_AU_20_M MB4999	32.732631	22.6603448	5.03965517	8.8362069	0.73891207	0.58908621	3.97827586	0.80206897	0.55238276	0.99032414	1.01546379	1.66646379	0.5664931
Rett													
BB: CB_R_20_M MB4516	42.5280317	26.7698413	5.99047619	10.0333333	0.73834603	0.61385556	4.13444444	0.74603175	0.55119048	0.96766032	1.03942698	1.96832222	1.58812381
OO: CB_R_18_F MB1815	46.5067188	28.2630435	6.0942029	10.8971014	0.71625435	0.57922754	4.16594203	0.74014493	0.52152464	0.97784638	1.05837681	2.07820145	1.89535072
QQ: CB_R_17_F MB4882 Sec. 1 vermis	44.9542295	29.9965909	5.68295455	11.9295455	0.64132045	0.50360114	4.55340909	0.63159091	0.42337841	0.972325	1.10951477	2.20545	1.72624886
Sibling of Autistic													
BBB: PC_AU_22_M MB5378	42.3330807	26.0526316	5.81578947	10.0280702	0.74231404	0.59954386	4.02035088	0.79122807	0.56052456	0.98925789	1.03095263	1.91552281	0.74889298
CCC: PC_AU_22_M MB5378	44.6929102	27.6142857	5.91632653	10.7285714	0.71515918	0.57134694	4.14387755	0.74571429	0.52084286	0.97787551	1.02868367	2.03096122	0.69110816
JJJ: LC_AU_22_M MB5378	41.6390173	26.9038462	5.74615385	10.5076923	0.69885	0.56297692	4.20230769	0.72692308	0.49676154	0.98468077	1.05689231	1.978	0.67796154
Joubert													
NN: CB_JS_5_F MB5486 Sec. 10 lat. cerebell.	53.7309788	35.5020202	6.25858586	13.6424242	0.61728687	0.48592323	4.90565657	0.55373737	0.39283131	0.93671919	1.17044747	2.61017071	2.33090606
PP: CB_R_17_F MB4882 Sec. 1 vermis	47.50085	29.9160714	6.24642857	11.1589286	0.7017125	0.57769286	4.38571429	0.67410714	0.50025714	0.94682857	1.07083036	2.19975179	0.74875536
Angelman													
VVV: LC_Angel_MB1754	34.1859373	23.1149254	5.29701493	8.79850746	0.75351642	0.62360597	4.00358209	0.79358209	0.57677463	0.98178955	1.01515522	1.69995672	0.58233881

Contour Name	Area (µm≤)	Perimeter (µm)	Detected Objects	Feret Min (µm)	Feret Max (µm)	Aspect Ratio	Compactness	Object Markers	Shape Factor	Form Factor	Roundness	Convexity	Solidity	Area error coeff.	Perimeter error coef
Control Vermis															
1: CB_CN_22_M MB1542 vermis	584.58066667	99.78172043	0	22.450537634	38.501075269	0.7154129032	0.6005655914	0	4.1638709677	0.7366666667	0.5204408602	0.9825677419	0.9527301075	18.342715054	
2: CB_CN_3_F MB 1284 vermis	645.50380172	100.52931034	0	23.499137931	38.294827586	0.75835	0.6346301724	0	3.9818103448	0.8037068966	0.584725	0.9874931034	0.9765362069	18.479143966	
7: CB_CN_16_F MB4591 vermis	614.77498261	99.753913043	0	21.935652174	38.907826087	0.7277591304	0.5843156522	0	4.0493913043	0.7747826087	0.538126087	0.9869895652	0.9745617391	18.337304348	3.6757434783
10: CB_CN_20_M MB4727 vermis	782.875	113.12333333	0	24.023333333	45.238888889	0.7020555556	0.5442833333	0	4.077	0.7644444444	0.50037111111	0.99105	0.98045111111	20.793742222	3.5850666667
AA: CB_(CN)_16_M MB5077 vermis	672.88085217	102.23534483	0	22.957758621	39.928448276	0.7306215517	0.5908905172	0	3.9486206897	0.7935344828	0.5467318966	0.9945146552	0.9768732759	18.642396552	3.0389672414
CC: CB_CN_2_M MB1791 vermis	581.8710084	101.21932773	0	19.544537815	41.851260504	0.6606840336	0.4922663866	0	4.2493277311	0.7135294118	0.4483294118	0.9918596639	0.9747571429	18.606162185	3.3634848739
EE: CB_CN_16_F MB4591 vermis	800.60685586	109.55675676	0	26.803603604	40.627927928	0.7900198198	0.6738954955	0	3.8997297297	0.832972973	0.631536036	0.9860099099	0.9840891892	20.139478378	3.6281234234
JJ: CB_CN_16_M MB5077 vermis	678.79601626	100.20081301	0	24.443902439	37.657723577	0.7792642276	0.657899187	0	3.8896747967	0.836504065	0.614695122	0.9936121951	0.9842398374	18.418996748	4.3753203252
PPP: CB_CN_16_M MB5077 vermis	573.23704854	91.361165049	0	21.818446602	35.144660194	0.7688330097	0.6328990291	0	3.8633009709	0.8477669903	0.5992067961	0.9951747573	0.991776699	16.79302233	4.6941417476
KK: CB_CN_2_M MB1791 vermis	602.80033594	99.265625	0	20.95625	39.84140625	0.6960445313	0.5420992188	0	4.0975	0.7559375	0.4909789063	0.9925	0.9718820313	18.247907813	4.0386085938
UUU: CB_CN_16_F MB4591 post vermis	555.09581915	89.889361702	0	22.091489362	33.742553191	0.78905	0.6672829787	0	3.8532978723	0.850212766	0.6296606383	0.9920989362	0.9885712766	16.524023404	5.6679234043
RRR: CB_CN_2_M_MB1791 vermis	529.80555556	92.762037037	0	18.930555556	38.143518519	0.6811527778	0.5068546296	0	4.0890740741	0.7615740741	0.4718925926	0.9956796296	0.9869777778	17.051913889	4.9690555556
Control Lateral Cerebellum															
4: CB_CN_4_M MB4670 lateral	691.25585833	104.94166667	0	23.916666667	40.2125	0.7419475	0.6117691667	0	4.04125	0.7819166667	0.5602266667	0.98324	0.9741066667	19.2907875	
BBB: LC_CN_12_M_MB5387	674.902	101.95263158	0	21.913684211	40.750526316	0.7220715789	0.5508705263	0	3.9542105263	0.8102105263	0.5268684211	0.9950989474	0.9896210526	18.741411579	5.3251778947
CCC: LC_CN_16_F_4591 lateral	670.97844444	97.9111111111	0	23.6	37.725252525	0.7765030303	0.6360555556	0	3.8073737374	0.8705050505	0.6085464646	0.9975959596	0.997330303	17.998634343	2.2876525253
EEE: LC_CN_22_M_1542 lateral	622.86252632	97.390526316	0	21.504210526	37.986315789	0.7437926316	0.5757926316	0	3.9213684211	0.82	0.5575884211	0.9909989474	0.9896863158	17.902696842	5.1809210526
GGG: LC_CN_20_M_4727 lateral	775.95897368	107.98947368	0	24.085087719	42.19122807	0.7511078947	0.5868552632	0	3.8953508772	0.8333333333	0.5703394737	0.9953008772	0.9896666667	19.851285088	5.6604649123
II: CB_CN_8_F MB1708 lateral	929.60593878	120.17346939	0	28.214285714	45.669387755	0.758344898	0.6320367347	0	3.9639795918	0.8051020408	0.5818663265	0.9897428571	0.9709989796	22.090744898	5.3764346939
KKK: LC_CN_3_F MB1284 lateral	727.68475758	104.6	0	23.117171717	41.777777778	0.7288272727	0.5601282828	0	3.9029292929	0.8285858586	0.5347131313	0.9942767677	0.9922929293	19.228033333	5.5713676768
AAA2: CB_CN_8_F MB1708 lateral	648.88502586	101.11206897	0	22.18362069	39.907758621	0.7265258621	0.5734293103	0	4.0020689655	0.7929310345	0.5368224138	0.9962741379	0.986762931	18.586515517	2.4486448276
Autistic Vermis															
3: CB_AU_20_M MB 4999 vermis	802.87467778	113.06666667	0	26.018888889	43.393333333	0.7483922222	0.62043	0	4.0073333333	0.79211111111	0.57046	0.9873477778	0.9817055556	20.78461	
5: CB_AU_4_F MB4671 vermis	624.83116935	106.03467742	0	20.081451613	43.857258065	0.6611717742	0.4864532258	0	4.2637096774	0.7125	0.450966129	0.9928524194	0.9766443548	19.491295161	3.6670282258
6: CB_AU_3_M MB4021 vermis	868.99047619	113.22095238	0	27.847619048	42.219047619	0.7890152381	0.6706942857	0	3.876	0.8420952381	0.6295495238	0.9902247619	0.9867733333	20.812666667	3.9614838095
8: CB_AU_3_M MB4029 vermis	614.77498261	99.753913043	0	21.935652174	38.907826087	0.7277591304	0.5843156522	0	4.0493913043	0.7747826087	0.538126087	0.9869895652	0.9745617391	18.337304348	3.6757434783
9: CB_AU_15_F MB5278 vermis	488.16476531	86.880612245	0	20.287755102	33.717346939	0.7467112245	0.6194346939	0	3.9541836735	0.81	0.5647816327	0.9917469388	0.9856632653	15.970661224	3.2507969388
11: CB_AU_14_M MB4899 vermis	992.39010989	119.0010989	0	30.010989011	44.195604396	0.8044043956	0.6872912088	0	3.8036263736	0.871978022	0.6514967033	0.991510989	0.9931967033	21.875143956	3.9035263736
HHH: PC_AU_12_M_4305 vermis	593.44978378	95.847747748	0	21.182882883	37.731531532	0.7354675676	0.5768882883	0	3.9487387387	0.8115315315	0.5474972973	0.9922873874	0.9898009009	17.618954955	4.9224756757
Autistic Lateral Cerebellum															
CCC2: LC_ASD_19_M_MB5294 lateral	670.97844444	97.9111111111	0	23.6	37.725252525	0.7765030303	0.6360555556	0	3.8073737374	0.8705050505	0.6085464646	0.9975959596	0.997330303	17.998634343	2.2876525253
FFF: LC_AU_15_F_5278 lateral	429.26195833	78.988541667	0	18.995833333	30.209375	0.777621875	0.6413416667	0	3.8339583333	0.859375	0.6104010417	0.996546875	0.99105625	14.520882292	4.6846385417
III: LC_AU_14_M 4899 lateral	734.97954545	104.44363636	0	23.661818182	40.651818182	0.7580245455	0.5981472727	0	3.8736363636	0.8421818182	0.5811145455	0.9952454545	0.9923545455	19.199851818	5.5576254545
JJJ: LC_AU_12_M_4305 lateral	643.44780808	97.378787879	0	22.990909091	37.609090909	0.7669757576	0.6277616162	0	3.8597979798	0.8488888889	0.5948787879	0.9956292929	0.9892272727	17.900765657	4.6346717172
LLL: LC_AU_3_M_M4029M lateral	517.00266667	88.272916667	0	20.448958333	33.866666667	0.7613197917	0.6162010417	0	3.9067708333	0.8288541667	0.5865645833	0.9939239583	0.9848635417	16.226722917	4.9658177083
MMM: LC_AU_20_M_4999 lateral	647.97445263	95.595789474	0	23.676842105	35.829473684	0.8031536842	0.6728715789	0	3.7847368421	0.8807368421	0.6507242105	0.9949873684	0.9908357895	17.571730526	5.8256810526
XXX: CB_AU_3_M_MB5308 Nissl lat. cereb.	570.178	92.169902913	0	20.866990291	36.346601942	0.7414436893	0.586568932	0	3.9024271845	0.8288349515	0.5553582524	0.9980834951	0.9948126214	16.94346699	1.9749262136
ZZZ: CB_AU_9_M MB797 generic cereb.	437.62831858	78.340707965	0	19.913274336	29.238938053	0.8096362832	0.6910858407	0	3.762920354	0.8904424779	0.6608141593	0.9983787611	0.9972327434	14.400631858	1.9539061947
DD: CB_AU_3_M MB5308 lateral	646.87280702	101.65789474	0	21.731578947	40.189473684	0.7124859649	0.5522429825	0	4.0622807018	0.7706140351	0.5160017544	0.9913070175	0.9787754386	18.68692807	3.3895947368
GG: CB_AU_9_M MB797 generic cerebellum	672.71721705	100.08062016	0	24.034108527	38.407751938	0.7681224806	0.6391062016	0	3.8733333333	0.8413953488	0.5959953488	0.9915767442	0.989348062	18.396582946	4.2134426357
LL: CB_AU_9_M MB797 generic	772.89516807	109.90588235	0	24.901680672	42.905882353	0.7349529412	0.5923941176	0	3.983697479	0.7984033613	0.547312605	0.9940865546	0.9838352941	20.203836134	3.9874865546
NNN: LC_AU_3_M_M4021M lateral															
Sibling of Autistic															
AAA: PC_AU_22_M_5378 vermis	526.85	88.371	0	21.849	33.04	0.782778	0.667068	0	3.882	0.8388	0.61856	0.99414	0.981647	16.245501	4.68154
DDD: LC_AU_22_M_5378 lateral	473.24011458	82.810416667	0	20.128125	31.263541667	0.7850458333	0.6576885417	0	3.8552083333	0.8522916667	0.6245677083	0.9920072917	0.9891770833	15.221547917	5.787434375
Rett Vermis															
SSS: CB_R_17_F MB4882 post vermis	494.03846154	83.175	0	21.734615385	30.378846154	0.8271278846	0.7238403846	0	3.7596153846	0.8911538462	0.6887432692	0.9939721154	0.9919855769	15.288986538	5.1074913462
TTT: CB_R_20_M MB4516 post vermis	622.24519231	96.366346154	0	22.468269231	37.170192308	0.7583009615	0.6164538462	0	3.9081730769	0.8296153846	0.5836644231	0.9946894231	0.9895682692	17.714075	5.1314740385
WWW: CB_R_18_F MB1815 post vermis	423.17773267	79.721782178	0	18.514851485	30.707920792	0.7582217822	0.6150633663	0	3.9126732673	0.8272277228	0.5832831683	0.9928594059	0.9875227723	14.655058416	4.349890099
YYY: CB_R_17_F MB4882 Nissl post vermis	437.62831858	78.340707965	0	19.913274336	29.238938053	0.8096362832	0.6910858407	0	3.762920354	0.8904424779	0.6608141593	0.9983787611	0.9972327434	14.400631858	1.9539061947
BB: CB_R_17_F MB4882	540.53040909	87.912727273	0	22.712727273	32.088181818	0.82045	0.7171181818	0	3.7960909091	0.8747272727	0.6781618182	0.9909381818	0.9906318182	16.16125	4.0926136364
FF: CB_R_18_F MB1815	553.09177477	92.364864865	0	21.454954955	35.354954955	0.7519261261	0.6179108108	0	3.9665765766	0.804954955	0.5725126126	0.9919603604	0.9754693694	16.978851351	4.1211720721
MM: CB_R_20_M MB4516	778.27823894	109.18230088	0	25.118584071	42.531858407	0.7456566372	0.6048238938	0	3.9337168142	0.8168141593	0.5626743363	0.9916371681	0.9883973451	20.069947788	4.3414123894
Joubert															
QQQ: CB_JS_5_F MB5486 Lat	348.12870588	76.370588235	0	14.823529412	31.610784314	0.6711980392	0.4822264706	0	4.1164705882	0.7491176471	0.4566490196	0.9941647059	0.9871215686	14.038301961	4.5443
VVV: CB_JS_5_F MB5486 vermis	383.40694231	78.261538462	0	16.721153846	31.225961538	0.7197605769	0.5596019231	0	4.0158653846	0.7906730769	0.5280355769	0.9928432692	0.9851201923	14.386275	5.0393009615
Angelman															
BBB2: LC_Angel_MB1754	604.04120755	96.596226415	0	21.578301887	38.069811321	0.7347415094	0.5860179245	0	3.9672641509	0.8068867925	0.5487858491	0.9930169811	0.9856075472	17.757011321	6.5764009434