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NEURONAL AND GLIAL CELL NUMBER IS ALTERED IN A CORTICAL LAYER-SPECIFIC MANNER IN AUTISM

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Abstract

Autism Spectrum Disorder (ASD) is a neurodevelopmental condition characterized by impaired social communication and repetitive behaviors. Changes in the number of specific cell types in the cerebral cortex could produce a dramatic alteration in the regulation of cortical circuits and thus behavior in ASD.

We investigated whether there are layer-specific changes in the number of neurons, astrocytes, and oligodendrocytes in the prefrontal cortex in postmortem human brains from ASD subjects. We quantified the number of specific cell types in the prefrontal cortex (Brodmann areas (BA) 9, 46 and 47) of 10 subjects with ASD and 10 age-matched control subjects. We found that the number of neurons was increased, and the number of astroglial cells was decreased in layer II of all three prefrontal areas. Area BA47 was most widely affected presenting with an increased number of neurons and a decreased number of astrocytes in layer II and deeper layers of the cortex. Among other possibilities, the alterations in neuron and glial cell number we report here are consistent with a failure of radial glial cells to shift daughter cell production from neurons to astrocytes during prenatal cortical development in ASD. The data provided here are key anatomical findings that shed light on ASD pathogenesis.

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Author contributions:

NYM performed the experiments and quantifications. CF performed the experiments and quantifications and contributed to project design and writing of the manuscript. TH provided support with human tissue cutting. BD assisted with the statistical analysis and the tissue gathering. VF designed and supervised the project, wrote the manuscript. All authors contributed intellectual contents and critical review of the paper.

Conflict of interest statement:

The authors declare no conflict of interest.

Ethical approval:

All the experiments on postmortem human tissue were approved under the IRB exception number 859356–1.

LAY ABSTRACT

The cerebral cortex affected with Autism spectrum disorder (ASD) present changes in the number of neurons and glia cells, possibly leading to a dysregulation of brain circuits and affecting the behavior in ASD. However, little is known about cell number alteration in specific layers of the cortex. We found an increase in the number of neurons and a decrease in the number of astrocytes in specific layers of the prefrontal cortex in postmortem human brains from ASD subjects. We hypothesize that this may be due to a failure in neural stem cells to shift differentiation from neurons to glial cells during prenatal brain development in ASD. These data provide key anatomical findings that may contribute to the bases of ASD pathogenesis.

INTRODUCTION

Autism spectrum disorder (ASD) is a neurodevelopmental condition characterized by the impairment of social interaction, communication, and repetitive behaviors. About 1% of children in the US are affected by ASD, with boys being 4 to 5 times more affected than girls. Classic autism is the most common diagnosis among ASDs, and is associated with comorbid conditions like seizures (Jeste & Tuchman, 2015). The wide range of symptoms of ASD may be explained by distinct etiologies encompassing genetic, environmental, and/or immune factors that are not well understood (Mandy & Lai, 2016; Martínez-Cerdeño et al., 2016; Schaefer, 2016), and these are most likely associated with functional abnormalities in neural networks (Rubenstein & Merzenich, 2003). Among various causes, an altered cytoarchitecture in specific areas of the cerebral cortex may be at the basis of functional abnormalities in neural networks. Cytoarchitectonic abnormalities in neural networks have predominantly been reported in prefrontal and temporal cerebral cortices, hippocampus, amygdala, striatum and cerebellum (Barnea-Goraly et al., 2004; Casanova, 2004; Courchesne & Pierce, 2005; Herbert et al., 2004; Just et al., 2007; Wegiel et al., 2014). Within the prefrontal cortex (PFC), three main Brodmann areas (BAs) have been strongly linked to ASD: dorsolateral PFC BA9 and BA46 - responsible for working and spatial memory, verbal fluency, auditory and verbal attention, and involved in attention; and ventrolateral PFC BA47 - implicated in oral and sign language processing (Dixon & Christoff, 2014; Dumontheil, 2014; Dumontheil et al., 2008; Jeon, 2014; Romanski, 2007; Shalom, 2009; Teffer & Semendeferi, 2012). Circuit disruption can result, among other causes, from an altered number of specific cell types in the cortex. Accordingly, data from animal models and genomic studies in postmortem human brain, suggest that the prenatal pattern of cortical neurogenesis and gliogenesis may be altered. This concept is supported by mutations in *Rab39b* and *CHD8* genes, linked to autism, that lead to an altered switch in stem cell progression, neural proliferation, and neuronal output (Gompers et al., 2017; Zhang et al., 2020). And by many other genes with roles during development that are also altered in autism, as is for example the gene *NOTCH2NL*, a human-specific gene involved in the promotion of clonal expansion of human cortical neuronal progenitors and that when duplicated induces autism (Fiddes et al., 2018)(Suzuki et al., 2018). In addition, gene co-expression network analysis comparing autistic and control brains showed that regional patterns that usually distinguish frontal and temporal cortices are altered in the ASD, pointing also to abnormalities in cortical patterning (Voineagu et al., 2011). Further

whole genome studies mapped the expression of several ASD risk genes in early gestation, likely affecting the production of neurons (Parikshak et al., 2013; Satterstrom et al., 2020; Willsey et al., 2013). A few studies have investigated whether and how cytoarchitecture and altered cell number in cortical areas may underlie ASD phenotype and disrupted neural networks. A seminal study by Wegiel et al. reported that the presence of dysplasia and heterotopias is common in the cortex and other brain regions in autism (Wegiel et al., 2010). Additional studies reported an increase or decrease in cell number or density (Camacho et al., 2014; Casanova et al., 2002, 2010; Courchesne et al., 2011; Kim et al., 2015; Mukaetova-Ladinska et al., 2004; Uppal et al., 2014; van Kooten et al., 2008), or a lack of changes (Kennedy et al., 2007) for neurons, pyramidal neurons, and von Economo neurons in prefrontal, temporal, and fusiform areas. We have previously described a decrease in the number of chandelier interneurons in prefrontal cortex (Ariza et al., 2018; Hashemi et al., 2017). It has also been reported that the number of astrocytes is increased in the primary olfactory cortex in autism (Menassa et al., 2017), but unaffected in the white matter of dorsolateral PFC (Lee et al., 2017) in ASD. Each cortical layer plays a defined function and therefore determining whether there are layer-specific alterations in the number of specific neural cell types in autism is crucial to understand the basis of the excitation/inhibition imbalance in ASD. To the best of our knowledge, there are no studies that quantified cell types within specific cortical layers of the cerebral cortex in ASD.

METHODS

Samples

Postmortem human tissue samples from 19 subjects with ASD and 19 age-matched control subjects (Table 1 and Table 2) were obtained from the Autism Tissue Program (currently Autism BrainNet), the NIH Neurobiobank, and the UCD Medical Center. The diagnosis of autism was confirmed by standard postmortem use of the autism diagnostic interview-revised (ADI-R) in all cases. ADI-R data included sociability, verbal/nonverbal communication, and repetition scores. The presence of intellectual disability and seizures was also reported (Table 2). Control subjects were free of neurological disorders, based on medical records obtained at the moment of death. Of the 19 cases obtained to be included in each group, only 10 ASD and 10 age-matched controls were analyzed for each cortical area because not all prefrontal areas analyzed in this study were available from each case or of sufficient quality to quantify cell number (Table 1). For each BA, different groups of 10 ASD and 10 age-matched controls were analyzed. The average age of control and ASD subjects was 19.2 ± 4.3 years for BA9 (ctr: 15.2 ± 4.2 and ASD: 23.7 ± 4.0 years), 27.7 ± 4.9 years for BA46 (ctr: 22.2 ± 4.7 and ASD: 33.3 ± 4.6 years), and 27.1 ± 4.6 years for BA47 (ctr: 29.3 ± 4.7 and 25.0 ± 4.8 years), with a range of 2 to 50 in BA9, 2 to 56 years in BA46, and 7 to 56 years in BA47. The number of male subjects was higher than females, with a ratio of males:females of 13:6 for BA9 (ctr: 5:5 and ASD: 8:1), 15:5 or BA46 (ctr: 6:4 and ASD: 9:1), and 18:2 for BA47 (ctr: 8:2 and ASD: 10:0). The average postmortem interval (PMI) was 30.3 ± 6.2 hours. No difference in PMI was observed between control and ASD group ($p = 0.34$). Causes of death are listed in Table 1.

Tissue Processing

After removal of the brain from the skull, brain tissue was immersed in 10% buffered formalin for at least 8 weeks. 3 cm^3 blocks of the prefrontal cortex in BA9, 46 and 47 were dissected based on Brodmann anatomy as previously described (Hashemi et al., 2017). Tissue blocks were post-fixed in 4 % PFA, cryoprotected in a 30% sucrose solution in 0.1 M phosphate buffered saline (PBS) with 0.1% sodium azide, embedded in Optimum Cutting Temperature (OCT) compound, and frozen at −80°C. Tissues were sectioned on a Leica CM 1950 cryostat to obtain 14 μm – thick slide-mounted sections, and stored at −80 °C until use. Tissue sections were stained with cresyl violet (Nissl staining), dehydrated through sequential immersion in 50%, 70%, 90%, 96%, and 100% EtOH for 3 minutes each, cleared in Xylene for 6 minutes and mounted on glass slide, and coverslipped with Permount mounting medium (Martínez-Cerdeño et al., 2003; Martínez-Cerdeño & Clascá, 2002). Cortical areas were confirmed based on von Economo histology as previously described (Hashemi et al., 2017) (Figure 1 A, B).

Quantification

Small blocks of tissue were obtained from each case included a portion of BA9, BA46, and BA47, but did not encompass the entire Brodmann area. We quantified the total cell number in one 1 mm-wide bin for each of the select regions of each BA that encompassed the entire thickness of the cortical gray matter. We identified neurons, astrocytes and oligodendrocytes based on their morphological characteristics (Figure 1 C–F) that present specific unique morphology in Nissl (Pelvig et al., 2008). Two blinded investigators quantified cells and obtained similar results.

Statistics

Statistical analyses compared the percentage of each cell type over the total number of cells, per each cortical layer in each specific BA, between ASD and control cases. $N = 10$ control and $N = 10$ age matched ASD cases were analyzed for each BA (except for BA9 where $N = 10$ control and $N = 9$ ASD cases were analyzed). Different groups of samples were analyzed for each BA, from a pool of $N = 19$ control and ASD cases. Results were compared between ASD and control using ANOVA and t-test with significance set at $\alpha < 0.05$. The effect of multiple factors (age, sex and PMI) on the cell ratios of any BA and any cortical layer, were analyzed by ANCOVA. No significant correlation was observed ($p > 0.05$).

Community involvement

Community involvement is not applicable.

RESULTS

We collected postmortem human tissue samples from subjects with ASD and age-matched control (ctr) subjects. Age, sex, PMI, and cause of death are provided in Table 1. We obtained blocks containing prefrontal cortex from 19 ASD and 19 control cases. Because not all prefrontal areas analyzed in this study were available from each case or of sufficient quality to quantify cell number, a total of 10 ASD and 10 age-matched controls where analyzed for each cortical area (Table 1). We performed Nissl staining on cryosections

obtained from blocks of prefrontal cortex that included BA9, 46 and 47 (isolated based on Brodmann anatomy, Figure 1A), and outlined specific cortical layers based on von Economo layer histology, (Figure 1B). We quantified the number of three cell types (neurons, astrocytes and oligodendrocytes) within each layer of each area of interest in the Nissl-stained prefrontal sections from ASD and age and sex-matched controls. Nissl staining clearly allowed us to distinguish among cellular types (García-Cabezas et al., 2016; Pelvig et al., 2008; Pilati et al., 2008). Nissl labels both cytoplasm and nuclei of neurons, while only the nucleus of glial cells. Cells were classified as neurons based on a pale chromatin pattern in a triangular/rounded nucleus, with a large nucleolus free of surrounding heterochromatin, and a visible surrounding cytoplasm (Berry M, 1997; Duffy PE, 1983). Cells were identified as oligodendrocytes based on a small rounded or oval nucleus with dense chromatin structure. Astrocytes were characterized by a round or oval pale nucleus, with the heterochromatin condensed in granules (Baumann & Pham-Dinh, 2001; Fuller GN, 1997). Endothelial cells and microglial cells were identified by their association with capillaries and their very characteristic elongated nuclei (Ludwig & M Das, 2020) and were not quantified. We selected and quantified the three cell types in a single 1 mm-wide bin that encompassed the entire thickness of the cortical gray matter for each BA region (Figure 1B). We analyzed the data following two different paradigms. First, we compared the total number of each cell type in ASD and the control cases, and second, to avoid the confounding effect of a potential differential stretch factor among different samples of the human brain tissue, we calculated the ratio of each cell type as a percentage with reference to the total number of quantified cells. We analyzed both absolute cell number and cell ratio within the bins that encompassed the entire thickness of the cortex, and within each cortical layer. A graphical representation of cell type quantification of a representative case of ASD and control is depicted in Figure 2.

We compared the total number of each specific cell type within the defined 1 mm-wide bin that encompassed layers II to VI and compared numbers between ASD and control cases but did not find a difference. Next we performed a layer-specific analysis and found some differences between ASD and control cases. In BA46, the total neuron number was increased in layer II (+58.2%, $p<0.03$), while the number of astrocytes and oligodendrocytes was decreased (+51.2%, $p < 0.004$ and −33.9%, $p<0.05$, respectively), (Figure 3 A–C). In BA47, neuron number was increased in layer II (+73.3%, $p < 0.01$) and astrocyte number was decreased in layer III (−35.3%, $p < 0.03$) in ASD vs control (Figure 3 D,E). No further differences were found in any other cortical layer in BA46 and BA47, nor in any layer in BA9 (Figure 3).

We next calculated the percentage of each cell type with reference to the total number of cells per bin and compared data between ASD and control cases. We found a significant increase in the percentage of neurons per bin and a mild but significant decrease in the percentage of astrocytes per bin in BA47 (+7.9%, $p < 0.04$ and −2.7%, $p < 0.03$, for neurons and astrocyte, respectively) (Figure 4 C). No difference was observed for these variables in BA9 and BA46 (Figure 4 A,B).

Next we analyzed the percentage of specific cell types within each cortical layer. Analysis showed layer-specific changes in the percentage of each cell type in ASD compared to

control cases (Figures 5–7). Specifically, BA9 presented a significant 11% increase in neurons in layer II (+11.2%, $p < 0.02$; Figures 5 A) and a decrease in the percentage of both astrocytes and oligodendrocytes in layer II (−3.8%, $p < 0.05$ and −7.4%, $p < 0.05$, respectively; Figure 5 B,C). Similarly, in BA46 we found a 21% increase in the percentage of neurons in layer II and an 11% decrease in the percentage of astrocytes in layer II (+21.6%, $p < 0.001$, and −11.6%, $p < 0.002$ for neurons and astrocytes, respectively; Figure 6 A,B). In BA47 we noted a significant difference in the percentage of neurons, astrocytes, and oligodendrocytes in multiple cortical layers. Analyses showed an 18% increase in the percentage of neurons in layer II (layer II: +18%, $p < 0.003$), an 11% increase in the percentage of neurons in layer IV($p < 0.01$), and an 8.8% increase in the percentage of neurons in layer V ($p < 0.03$) (Figure 7 A). There was also a decrease in the percentage of glial cells across multiple layers in BA47. In layer II the percentage of astrocytes was decreased by 6%, ($p < 0.03$), and the percentage of oligodendrocytes was decreased by 12.8%, ($p < 0.02$). In Layer III there was a significant decrease in the percentage of astrocytes (29.1%, $p < 0.02$) and oligodendrocytes (79.8%, $p < 0.03$) in ASD compared to controls (Figure 7 B,C) and no change in neurons (Figure 7 A). In layer IV the percentage of oligodendrocytes was decreased by 9%, ($p < 0.03$). In layer V the percentage of astrocytes was decreased by 2.3%, ($p < 0.04$; Figure 7 B,C).

We did not find a significant correlation between age and cell number or cell percentage for any cortical area or cortical layer analyzed ($p > 0.05$ for all correlations). Assessing the effect of sex on the cell numbers and percentage was not possible due to the mostly male composition of our cohort.

Overall, we observed a consistent increase in the number of neurons, and a decrease in the number of astrocytes number in layer II of all the analyzed prefrontal cortical areas. The percentage of neurons, astrocytes was most strongly affected in BA47, where we observed cell specific changes across multiple layers.

DISCUSSION

We here provide a comprehensive analysis of the number of specific cell types across cortical layers of the prefrontal cortex in ASD postmortem human brains compared to age-matched control cases. We found that the number of neurons was increased, and the number of astrocytes decreased in layer II in prefrontal areas BA9, BA46, and BA47. In addition, we found an increased number of neurons and a decreased number of astrocytes in multiple cortical layers in BA47.

Neuron number is increased in cortical layer II in the prefrontal cortex in autism

The number of neurons and other cell types have been investigated in several areas of the brain in autism. We present here for the first time an analysis of the number of neurons on a layer by layer basis in the prefrontal cortex in ASD. The seminal work by Courchesne at al. reported a significant increase in the number of neurons in the prefrontal cortex of ASD (Courchesne et al., 2011). They quantified the number of neurons in the postmortem dorsolateral cortex (lateral part of BA9 and BA46) and mesial prefrontal cortex (BA10, BA12 and BA32) (Brodmann, 1909; Knutson et al., 2003) from 7 autistic and 6 control male

children aged 2 to 16 years. They found that children with autism had 67% more neurons in the PFC compared with control children, including 79% more in dorsolateral PFC and 29% more in mesial PFC. No subsequent studies examining neuron numbers in the prefrontal cortex have been performed, but Karst and Hutsler examined cell density in Nissl sections of 9 ASD and 8 control cases and provided evidence for an increase in total cell density in layer II, but not in layer III of BA9, BA21 and BA7 in ASD (Karst & Hutsler, 2016). Our data is partially in agreement with that from Courchesne at al. since we detected a significant increase in the number of neurons. However, the increase in neuronal number we found was more modest and was not detected when examined the cortex as a whole, with the exception of a mild but significant increase in the percentage of neurons in BA47. Our data may also be consistent with that from Karst and Hutsler since we only found a consistent increased number of neurons in layer II, and they reported an increase in the density of cells in layer II but not in layer III in BA9. As far as we know, no other cortical layers were studied, and the magnitude of such increase was not reported in their publication.

In our study, neuronal number in layer II was the only layer affected regarding cell type numbers in ASD in the three cortical areas investigated. Layer II has a mixed neuronal composition and has been correlated to higher cognitive functions. Pyramidal cells in supragranular layers (II-III) make local connections over short distances with the supragranular cells of other cortical areas, and pyramidal cells layer II/III of the medial prefrontal cortex have been reported to play a role in stress-induced behavioral disorders such as depression (Shrestha et al., 2015). Layer II interneurons in BA46 represent a powerful inhibitory system that regulates prefrontal activity during working memory (Arteaga et al., 2015). Our result that neuron number is increased in layer II is consistent with MRI studies that indicate an increase in thin axons that communicate over short distances locally in the cortex in ASD, specifically in BA46 (Egaas et al., 1995; Fingher et al., 2017; Herbert et al., 2004; Kana et al., 2017; Keown et al., 2013; Uddin et al., 2011; Wilkinson et al., 2016; Zikopoulos & Barbas, 2013). The increase in neurons we found in layer II is also consistent with rodent studies. Increasing the number of pyramidal neurons in the upper neocortical layers by using the small molecule XAV939 to expand the intermediate progenitor population in mice, resulted in perturbations of behavior resembling those of autism, together with phenotypic changes and dysregulated excitatory and inhibitory synaptic balance typical of autism (Fang et al., 2014). Overall, the results presented here is in agreement with previous data.

Astrocyte number is decreased in cortical layer II in the prefrontal cortex ASD

We provide here for the first-time layer-specific data on astrocyte number in the prefrontal cortex in ASD. Astrocytes, together with neurons, are actively involved in information processing (Agulhon et al., 2010; Araque & Navarrete, 2010; Hamilton & Attwell, 2010) by participating in synaptic plasticity (Araque & Navarrete, 2010). Detecting an alteration in the number of astrocytes in the cerebral cortex in ASD could provide crucial information on signal processing and synaptic plasticity disruption. We found a mild but significant decrease in the number of astrocytes in layer II of BA9, BA46 and BA47 and in layer III and V of BA47 (see Figure 7 B).

Previous studies by Lee et al. reported unchanged density of $GFAP^+$ astrocytes within the white matter of dorsolateral prefrontal cortex (Lee et al., 2017) in ASD, and unchanged total glial number identified with Nissl (Courchesne et al., 2011), respectively. However, these data are not comparable to the data we report here for the following reasons. GFAP does not label all astrocytes, but only a fraction of astrocytes that retain the capacity to proliferate under specific circumstances (Sofroniew & Vinters, 2010). Several studies have reported an increase in total levels of GFAP protein, implying a potential increase in astrocyte number (Edmonson et al., 2014; Liddelow & Barres, 2017; Serrano-Pozo et al., 2013; Sofroniew, 2009; Sofroniew & Vinters, 2010). However, increased GFAP protein expression may result from an increase in GFAP protein synthesis by activated astrocytes and not necessarily from an increase in the astrocyte number.

BA47 is the most prominently affected area in ASD prefrontal cortex

Previous neuropathological studies suggest that the cytoarchitectural changes in ASD are region-specific. For example, alterations in the columnar organization of the cortex have been described in the dorsal and orbital frontal cortex in ASD, but not in the primary visual cortex (Buxhoeveden et al., 2006; Casanova, 2004; Casanova et al., 2002, 2010). Brain region-specific changes associated with ASD have also been found in mitochondria-related gene expression (Anitha et al., 2012), in the response to oxidative stress, and in neurotrophin levels (Sajdel-Sulkowska et al., 2011). Our data also points to a region-specific anatomical alteration in ASD. Here we report that while all three BAs of interest present alterations in layer II, BA47 is the only area with alterations in both neuron and glial cell number across multiple layers (II, III and V). In a previous study we examined pyramidal cell number in supragranular and infragranular layers of the superior temporal cortex (BA 41, 42, 22), but found no differences (32). All together, these data point to a region-specific alterations of cellular density and/or distribution in ASD.

Relatively few studies have investigated cortical layer-specific alterations in ASD. Among studies that have analyzed layer specific alterations in ASD, evidence presented supports the concept of region-specific anatomical alterations in ASD. For example, it has been reported that neuron density in layer I is unchanged in the temporal cortex (Kim et al., 2015), that spine density on apical dendrites is increased in layer II of the temporal cortex (Hutsler & Zhang, 2010), that fewer MAP2+ dendrites are present in the prefrontal cortex in ASD (Mukaetova-Ladinska et al., 2004), and that glial cell number is increased in layer II/III of the primary olfactory cortex (Menassa et al., 2017).

Radial glial cells may fail to shift daughter cell production from neurons to astrocytes during prenatal development in ASD

The alteration in the number of cortical specific cell types reported here may result from altered programs of prenatal and/or postnatal stem cell proliferation and cell migration, and/or cell survival and death. During cortical development, individual radial glial cells (RGCs) produce both neurons and astrocytes (Noctor et al., 2001, 2004). During early stages of cortical formation new-born neurons are generated by RGCs and migrate to their laminar destination in the cortex following an inside-out pattern (Noctor et al., 2001, 2004, 2008). Later RGCs lose their apical contact and their cell bodies translocate into

the SVZ where they are known as outer RGCs (oRGCs), (Hansen et al., 2010). During this translocation, oRGCs continue producing neurons but also give rise to glial daughter cells and eventually transform into astrocytes (Holst et al., 2019; Howard et al., 2008). Mechanisms regulating astrocyte development have been extensively studied in rodent models, while few studies have been performed on human astrogenesis. In human astrocytes are generated late in development mainly from oRGCs and they keep proliferating locally at their final destination. It has been suggested that human cortical astrocytes first populate the upper cortical marginal zone (layer I) and the lower subplate, and then they populate the cortical plate in between these two regions later in development (Marín-Padilla, 1995; deAzevedo et al., 2003; de Majo et al., 2020). We reasoned that the increased number of neurons we detected in ASD could be explained, among other mechanisms, by a delay in the switch of RGC daughter cell production from neurons to astrocytes. Consistent with this notion, several ASD risk genes (including *PTEN*, *MECP2*, *CHD8*, *ARID1B*, *ERBIN*, and the 16p11.2 locus) are involved in neural/glia proliferation, differentiation and migration (Courchesne et al., 2019, 2020). Large-scale analyses of ASD-linked genes show that the highest enrichment of autism-linked genes is present early in the developing brain (Grove et al., 2019; Parikshak et al., 2013; Satterstrom et al., 2020). Grove et al. show high enrichment of ASD-linked genes specifically in prenatal corticogenesis (Grove et al., 2019). A switch in stem cell progression and a dysregulation of cortical development have been reported before in ASD animal models. For example, in a autism-like maternal reactive autoimmune model, autism-specific autoantibodies directed against fetal brain proteins shift radial glia cell proliferation from the ventricular zone to the subventricular zone (SVZ) of the embryonic neocortex resulting in increased brain size, spine abnormalities, and behavioral abnormalities (Ariza et al., 2018; Camacho et al., 2014; Martínez-Cerdeño et al., 2016). A study on the effects of mutations in the small GTPase gene *RAB39b*, associated with ASD and other developmental disorders showed increased proliferation and delayed differentiation of outer SVZ neuronal progenitor cells, resulting in neuronal overproduction in Rab39b knockout (KO) mice and RAB39b-deleted human cerebral organoids (Zhang et al., 2020). Another study showed that a heterozygous mutation in the chromatin remodeling gene *CHD8,* a key gene in neurodevelopmental gene networks involved in autism, results in an increased neuronal proliferation and developmental splicing perturbation, among other disrupted pathways in early brain development (Gompers et al., 2017). The changes in neuron number we observed were primarily localized to layer II, suggesting that if these modifications resulted from altered cell production this cell fate shift would have taken place during late stages of neurogenesis when layer II cells are generated (Figure 8). Cell migration, and/or cell survival and death may be also involved in this process.

Limitations

19 ASD and 19 controls cases were initially included in this study. However, the quality of tissue varied across cases and by cortical areas within cases. As a result, some cases did not have high enough quality tissue for reliable cell number quantification in each of the three cortical areas examined in this study. We were not able to quantify cell number from each of the areas in all cases and we used tissue from 10 ASD and 10 CT cases for cell number quantification. This may be a limitation, so caution is needed concerning the potential for quantitative differences across areas. On the other hand, use of tissue from different cases

has the benefit of informing us that the increase in layer II neuron we found in each area did not result from large differences in a small subset of cases that may have a unique pathological phenotype, but rather the difference in cell number may result from a broadly observed change in neuron numbers across autism cases.

We did not find any significant effect of age on the cell ratios in any BA nor any cortical layer. ASD cases age ranged from 7 to 56 and control case age ranged from 2 to 56 years. The majority of cases were adults, adolescents and preadolescents, and only 4 control and 3 ASD subjects were children. The limited number of cases at very young ages limited analyses of possible ASD age-related events. Due to the mostly male composition of our cohort, we could not assess the effect of sex on the cell numbers and percentage.

We did not apply stereological methods since it is not applicable to anatomical structures where defined borders cannot be delimited. This is the case of specific Broadman areas and of specific anatomical regions such as the prefrontal cortex. Based on the accepted definition of stereology (Boyce et al., 2010; Peterson, 2010), true stereological methods can only be applied to the cortex as a whole, for which one can clearly delimit boundaries, but only possible if the entire brain is available for study. Since we could not employ stereological methods, and to avoid the confounding effect of differential stretching factor for each human brain, we reasoned that the best solution for obtaining reliable results was quantification of ratio among cell types. This approach allowed us to obtain a reliable representation of potential changes in cell populations.

Nissl staining was used for cell recognition. A limitation of this approach may be that cell nuclei morphology and size may be specifically affected in ASD (Wegiel et al., 2015). Also, neuronal microstructural alteration in the actin cytoskeleton has been associated to ASD (Joensuu et al., 2018; Falougy et al., 2019). Glial cell cytoplasm are not labled qith Nissl, thus the use of specific nuclear characteristics for glial type recognition may lead to some error. Another way to identify cell types would be using immunohistochemical methods that we routinely use in our laboratory, for example to identify chandelier interneurons (Hashemi et al., 2017; Ariza et al., 2018). However, there is not a universal marker that labels all astrocytes. While GFAP is widely used as an astrocyte marker, it only labels reactive astrocytes, astrocytes with proliferative capacity, astrocytes located in the SVZ and layer I, and astrocytes localized to patches throughout the cortical plate (Köhler et al., 2019; Lundgaard et al., 2014; Sun & Jakobs, 2012). Other commonly used markers for astrocytes are S100β, Aldh1l1 and Aldolase C that are not specific to astrocytes or do not label all the astrocyte population (Fujita et al., 2014; Garwood et al., 2017; Steiner et al., 2007). NeuN is a definitive marker of cortical neurons, but we obtained inconsistence labeling with NeuN in postmortem human tissue. In contrast, Nissl staining also allowed us to quantify all cell types of interest in the same section, rather than in sequential sections, and therefore the cell type ratios are not an estimation but represent the number of cells in tissue samples. Whilw this study focused on three regions in the prefrontal cortex known to be affected in ASD, we do not know if other cortical regions are similarly affected in ASD and this will be the subject of a follow-up study.

Conclusion

We found a consistent and significant increase in neuron number and a decrease in astrocytes in layer II of the prefrontal cortex (BA46, BA4, BA9), and in most layers in BA47 (layers III, IV and V). These data suggest a potential alteration in stem cell proliferation during the prenatal neurogenic and gliogenic periods in the prefrontal cortex in ASD. A combination of increased neuronal number together with decreased astrocyte number within specific layers in each cortical area analyzed may represent a crucial cytoarchitectural alteration that could contribute to cortical circuit disfunction in ASD. This finding provides an important insight on the cellular basis of ASD pathogenesis and should be the subject of a follow-up studies.

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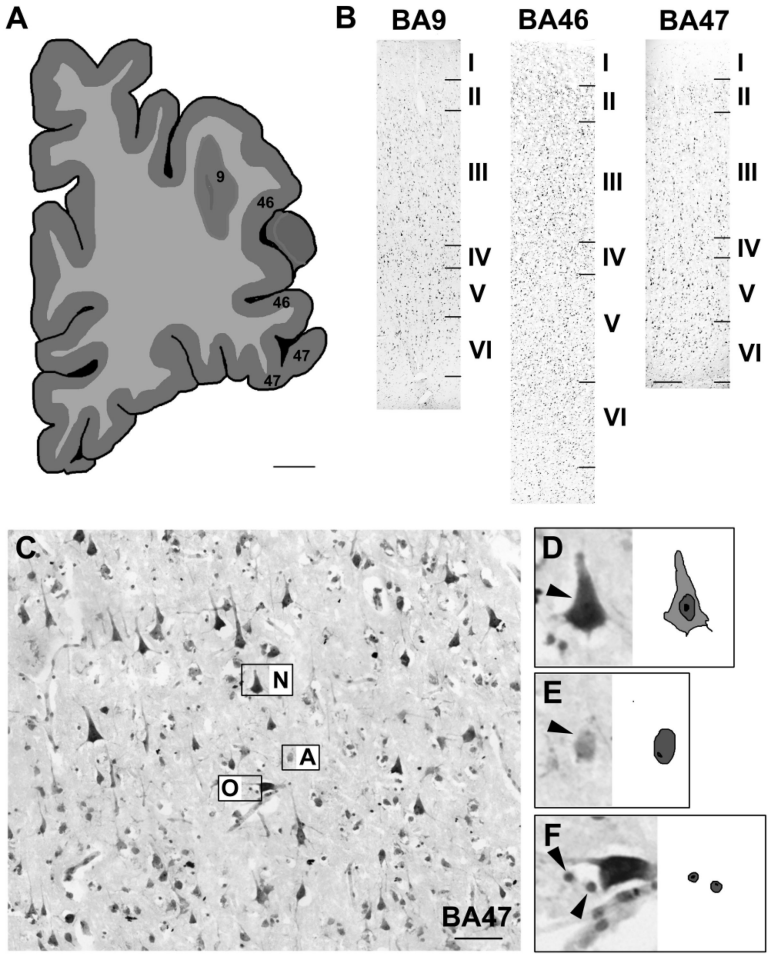

Figure 1. Cortical areas and cell types.

(A) Coronal section of prefrontal cortex from a left hemisphere. Blocks containing BA9, BA46, and BA47 (labeled in A) were isolated based on Brodmann and von Economo anatomy. (**B**) Nissl-stained sections of BA9, BA46, and BA47. (**C-D**) Representative Nissl stained layer III of BA47 cortex. N = neuron: pale chromatin pattern in a triangular/ rounded nucleus, with a large nucleolus free of surrounding heterochromatin, and a visible surrounding cytoplasm, A = astrocyte: round or oval pale nucleus, with the heterochromatin condensed in granules, O = oligodendrocytes: small rounded or oval nucleus with dense chromatin structure. Scale bar in A: 0.5cm, B: 200 μm, C: 60 μm.

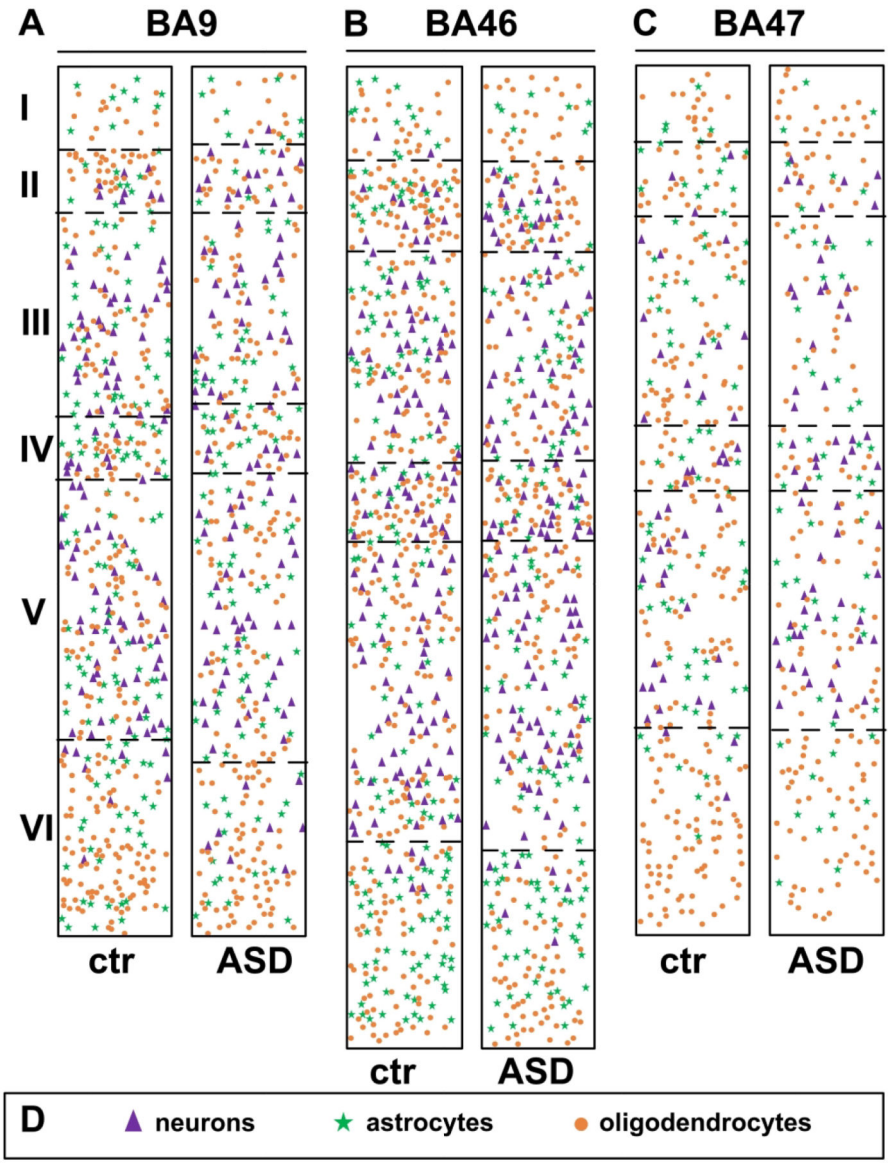

Figure 2.

(A-C) Representation of neuron, astrocyte, and oligodendrocyte distribution within cortical layer of BA9 (A), BA46 (B) and BA47 (C), in ctr and ASD brains in a control and ASD cases. (D) purple triangle = neurons, green stars = astrocytes, orange circle= oligodendrocytes.

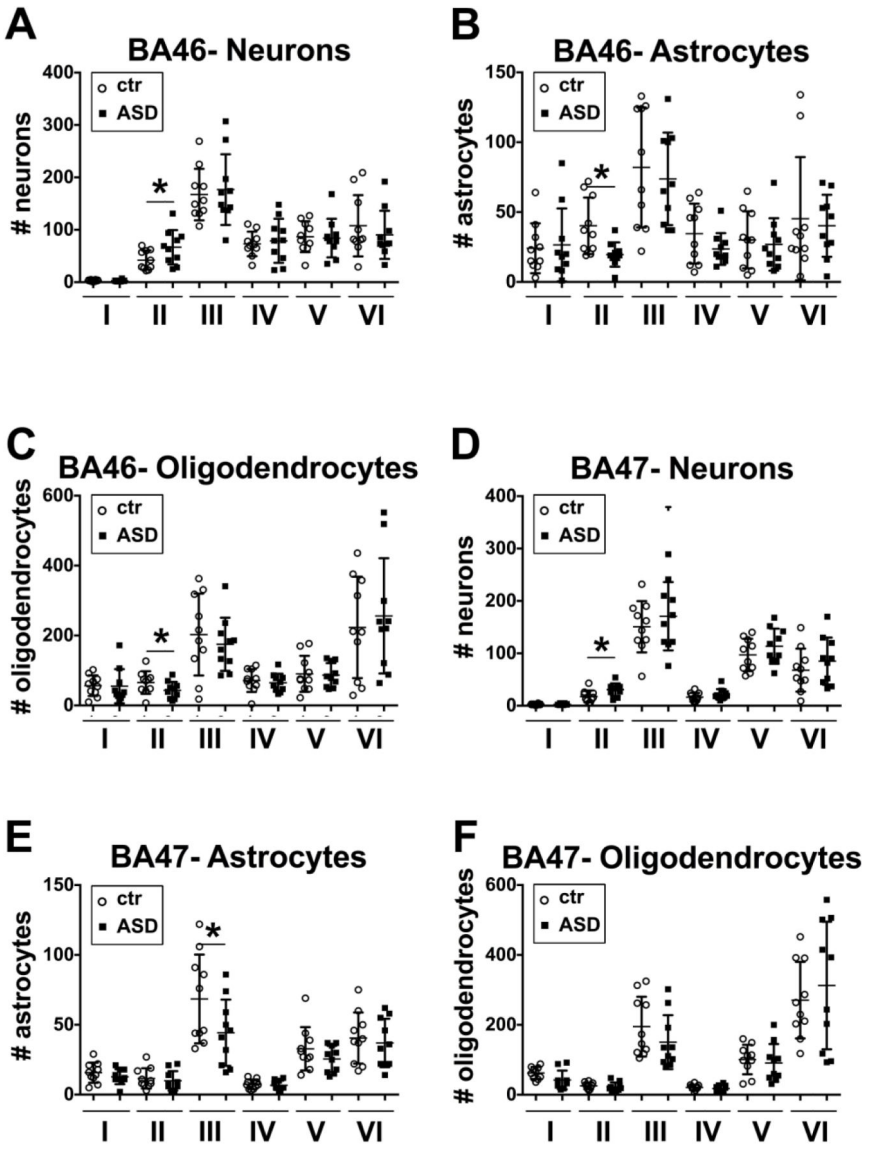

Figure 3. Cell types number in BA46 and BA47 in ASD and control cases (ctr).

(**A**-**C**) Layer-specific number of neurons (A), astrocytes (B), and oligodendrocytes (C) in BA46 in ASD and control cases (ctr). (**D**-**F**) Layer-specific numbers of neurons (D), astrocytes (E), and oligodendrocytes (F) in BA47 in ASD and control cases (ctr). Circles = ctr; Squares = ASD. Asterisks indicate $p < 0.05$.

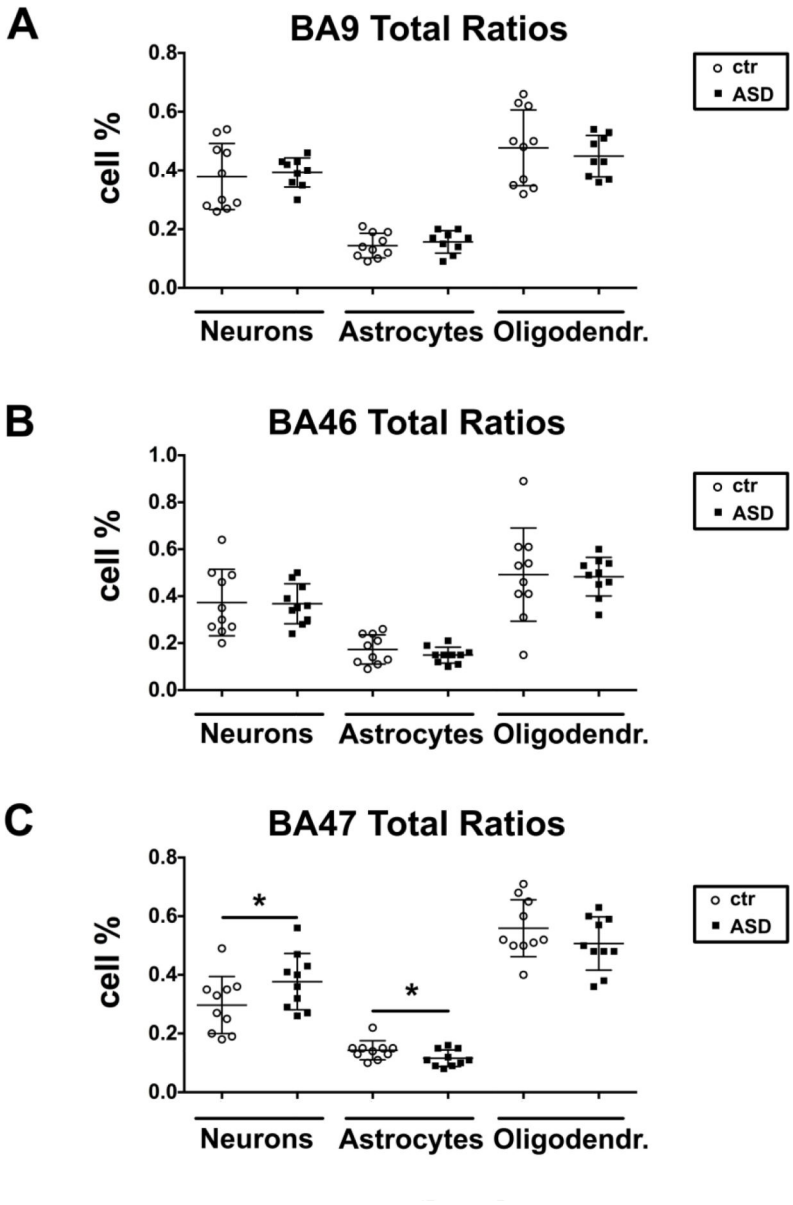

Figure 4. Cell type percentage in BA9, BA46 and BA47 in the whole cortex of ASD and control cases (ctr).

(**A**-**C**) Layer-specific percentages of neurons, astrocytes, and oligodendrocytes in (A) BA9, (B) BA46, and (C) BA47 in ASD and control cases (ctr). Circles = ctr; Squares = ASD. Asterisks indicates $p < 0.05$.

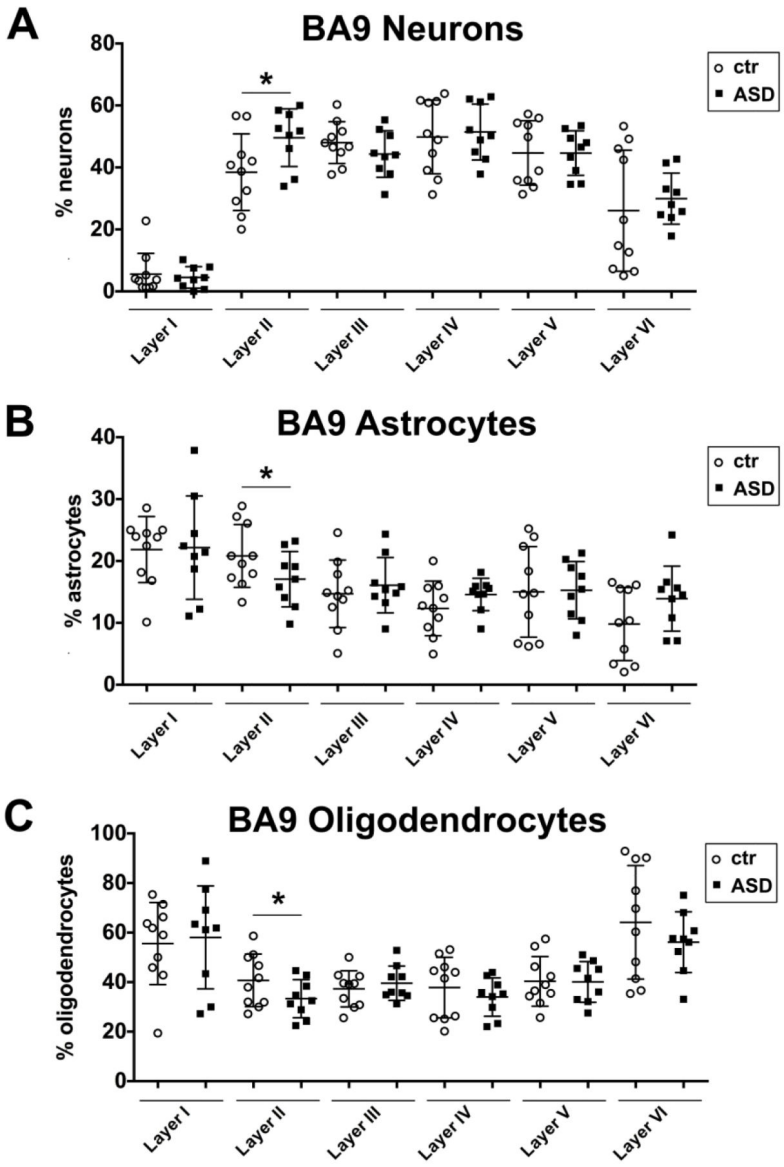

Figure 5. Cell type percentages in BA9 by cortical layers in ASD and control cases (ctr).

(A-C) Layer-specific percentages in neuron (A), astrocytes (B), and oligodendrocytes (C) in BA9 in ASD and control cases (ctr). Circles = ctr; Squares = ASD. Asterisks indicate $p < 0.05$.

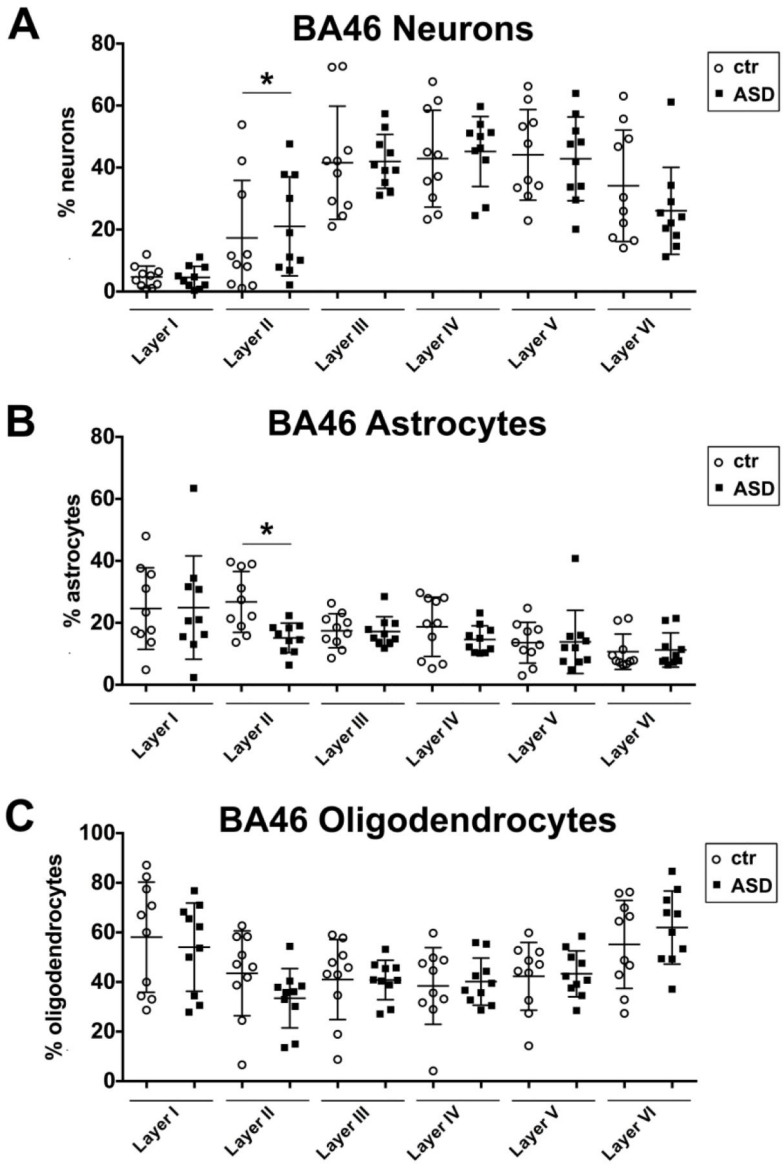

Figure 6. Cell type percentages in BA46 by cortical layers in ASD and control cases (ctr).

(A-C) Layer-specific percentages in neuron (A), astrocytes (B), and oligodendrocytes (C) in BA46 in ASD compared to controls (ctr). Empty circle= ctr; full square= ASD. Asterisks indicates a $p < 0.05$. Specific p values are in the Results.

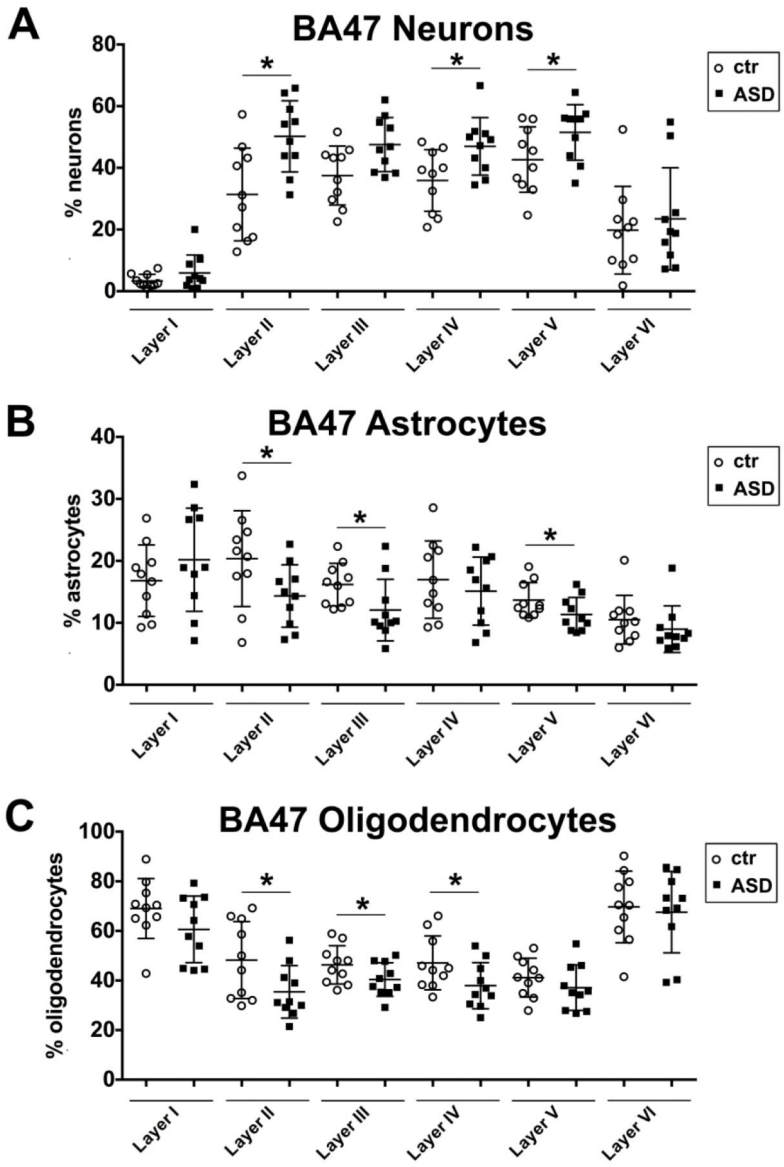

Figure 7. Cell type percentages in BA47 by cortical layers in ASD and control cases (ctr).

(A-C) Layer-specific changes in neuron (A), astrocytes (B), and oligodendrocytes (C) in BA47 in ASD compared to controls (ctr). Empty circle= ctr; full square= ASD. Asterisks indicates a $p < 0.05$. Specific p values are in the Results.

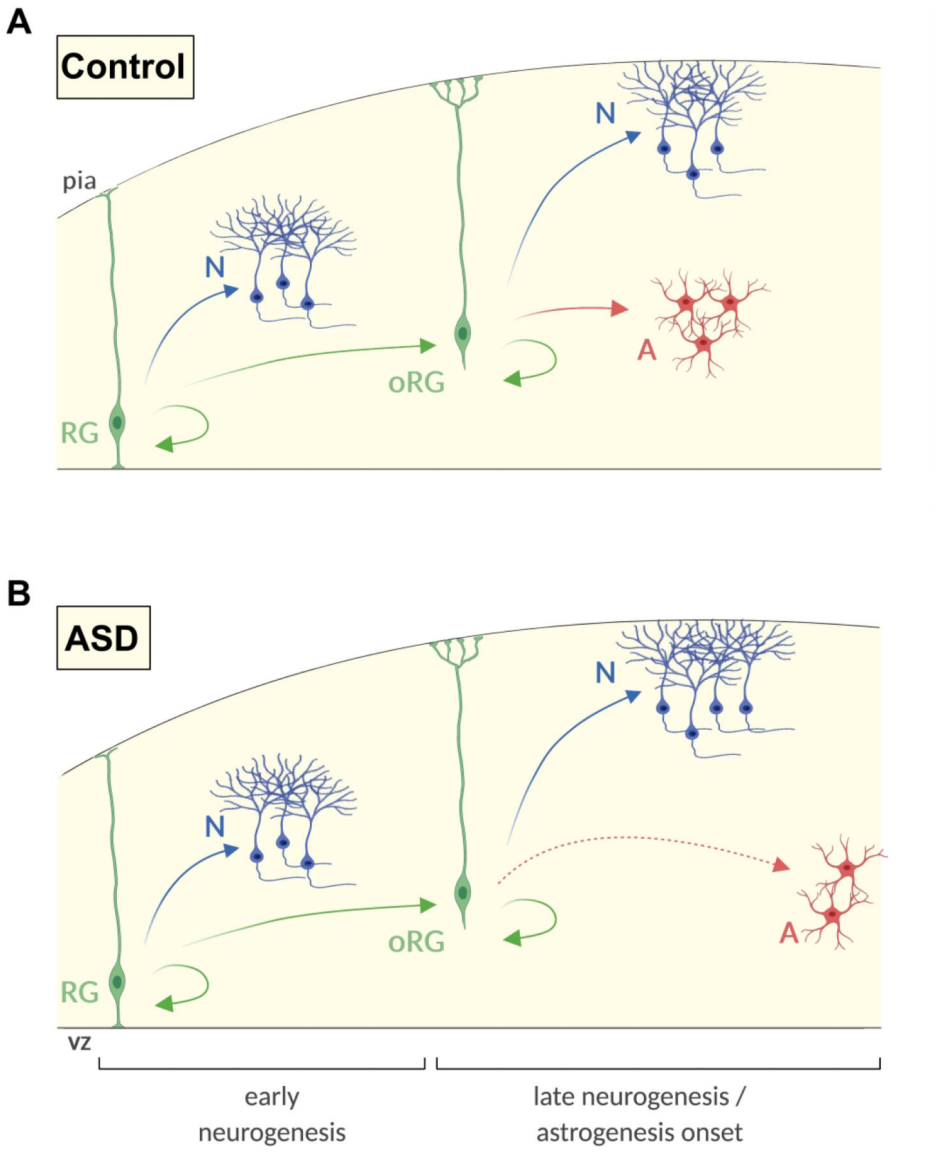

Figure 8. Hypothesis: Radial glial cells may fail to shift daughter cell production from neurons to astrocytes during prenatal development in ASD.

(A) ctr and **(B)** ASD hypothesis. RG = radial glia cell; oRG = outer radial glia cell; N = neurons; A = astrocytes.

Table 1

Clinical characteristic of ASD and control patients: Diagnosis, Sex, Age, PMI, cause of death, and BA analyzed. Brodmann areas included in the analysis.

Case ID	Diagnosis	Sex	Age (years)	PMI (hours)	Cause of death	BAs analyzed
1791	ctr	F	2	12	Drowning	9, 46
4327	ctr	F	5	24	Respiratory failure	9, 46
4337	ctr	M	8	16	Blunt force	9
738	ctr	F	8	12	Cardiac Arrhythmia	9, 46
5834	ctr	M	14	38	Cardiac arrhythmia	9, 47
5309	ctr	F	14	8	Streptococcal Toxic Shock Syndrome	9, 47
4638	ctr	F	15	5	Chest injury	9
AN07444	ctr	M	17	30.7	Asphyxia	47
AN00544	ctr	M	17	28	NK	9
5893	ctr	M	19	11	Dilated cardiomegaly	9, 46, 47
5646	ctr	F	20	23	Reactive airway disease	46
5958	ctr	M	22	24	Dilated cardiomegaly	46
UCD-16-02	ctr	M	26	35.7	Burn/ Respiratory failure	46, 47
UCD-15-05	ctr	M	26	224	E coli sepsis, end-stage renal disease	47
AN12137	ctr	M	31	32	Asphyxia	46
AN15566	ctr	F	32	28	NK	47
AN05475	ctr	M	39	NK	Cardiac arrest	46, 47
AN19442	ctr	M	50	20	NK	9, 46, 47
AN13295	ctr	M	56	22	NK	47
5144	ASD	M	7	3	Cancer	9
AN03407	ASD	M	7	41.83	Drowning	47
AN01293	ASD	M	9	3.75	Cardiac arrest	46, 47
4305	ASD	M	12	13	Serotonin Syndrome	9
AN00394	ASD	M	14	10.3	Cardiac arrest	47
4899	ASD	M	14	9	Drowning	9
5403	ASD	M	16	35	Cardiac arrhythmia	9,47
4269	ASD	M	19	45	Meningitis	47
AN00764	ASD	M	20	23.66	Car Accident/Trauma	46
5176	ASD	M	22	18	Subdural hemorrhage	9, 47
5574	ASD	M	23	14	Pneumonia	9, 46
AN00493	ASD	M	27	8.3	Drowning	9, 46
AN18892	ASD	M	31	99	Gun shot	9, 46, 47
AN09901	ASD	M	32	28.7	Heat Stress / Pulmonary Congestion	46
5027	ASD	M	37	26	Bowel obstruction	47
1575	ASD	F	40	24	Complications of diabetes	9, 46
AN06746	ASD	M	44	30.75	Cardiac arrest	9, 46, 47

Case ID	Diagnosis	Sex	Age (years)	PMI (hours)	Cause of death	BAs analyzed
5137	ASD	M	51	72	Pneumonia	46, 47
AN01093	ASD	M	56	19.48	aspiration/anoxic encephalopathy	46

Table 2

Additional clinical information of ASD cases

Case ID	Diagnosis	PM-ADI-R	CNS co-morbidities
5144	ASD	Yes	-
AN03407	ASD	N/A	N/A
AN01293	ASD	Yes	Pervasive developmental disorder
4305	ASD	Yes	Pervasive developmental disorder
AN00394	ASD	Yes	-
4899	ASD	No (clinical diagnosis)	-
5403	ASD	Yes	Functional constipation
4269	ASD	Yes	Intellectual disability, intermittent explosive disorder
AN00764	ASD	Yes	-
5176	ASD	Yes	Intellectual disability
5574	ASD	Yes	Intellectual disability
AN00493	ASD	Yes	-
AN18892	ASD	Yes	Major depressive disorder, borderline personality
AN09901	ASD	N/A	-
5027	ASD	No (clinical diagnosis)	Pervasive developmental disorder
1575	ASD	Yes	-
AN06746	ASD	Yes	-
5137	ASD	Yes	-
AN01093	ASD	N/A	Seizures