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Original Article

Increased number of excitatory synapsis and decreased number of inhibitory synapsis in the prefrontal cortex in autism

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Previous studies in autism spectrum disorder demonstrated an increased number of excitatory pyramidal cells and a decreased number of inhibitory parvalbumin+ chandelier interneurons in the prefrontal cortex of postmortem brains. How these changes in cellular composition affect the overall abundance of excitatory and inhibitory synapses in the cortex is not known. Herein, we quantified the number of excitatory and inhibitory synapses in the prefrontal cortex of 10 postmortem autism spectrum disorder brains and 10 control cases. To identify excitatory synapses, we used VGlut1 as a marker of the presynaptic component and postsynaptic density protein-95 as marker of the postsynaptic component. To identify inhibitory synapses, we used the vesicular gamma-aminobutyric acid transporter as a marker of the presynaptic component and gephyrin as a marker of the postsynaptic component. We used Puncta Analyzer to quantify the number of co-localized pre- and postsynaptic synaptic components in each area of interest. We found an increase in the number of excitatory synapses in upper cortical layers and a decrease in inhibitory synapses in all cortical layers in autism spectrum disorder brains compared with control cases. The alteration in the number of excitatory and inhibitory synapses could lead to neuronal dysfunction and disturbed network connectivity in the prefrontal cortex in autism spectrum disorder.

Key words: autism; synapsis; excitatory; inhibitory; postmortem; human.

Introduction

Autism spectrum disorder (ASD) is a heterogeneous neurodevelopmental disorder. The core symptoms of ASD are social-communication deficits and repetitive behaviors (Lord et al. 2018). However, we know that there is an excitation/inhibition imbalance in the cerebral cortex in ASD (Rubenstein and Merzenich 2003; Said et al. 2013; Rosenberg et al. 2015; Trakoshis et al. 2020). Glutamate is a excitatory neurotransmitter and has a key role in neurodevelopment and higher cognitive functions (Nisar et al. 2022). There is evidence indicating abnormalities in the expression of glutamate and its receptor and transporters in ASD (Purcell et al. 2001; Bourgeron 2015; Nisar et al. 2022). Excitatory neurotransmission via glutamate receptors that regulates learning and memory are impaired in ASD (Choudhury et al. 2012). Gamma-aminobutyric acid (GABA) is the neurotransmitter in charge of cortical synaptic inhibition (Obata 2013), and changes in GABAergic system in ASD, including a reduction in GABA release and GABA receptor expression, have been also reported (Coghlan et al. 2012; Cellot and Cherubini 2014).

Excitatory synapses are asymmetric. At the presynaptic site, the vesicular glutamate transporters (VGlut1 and VGlut2) pack glutamate neurotransmitter into vesicles. At the postsynaptic site, the postsynaptic density protein-95 (PSD95) anchors glutamate receptors onto the surface of the postsynaptic neuron (Gatto and Broadie 2010; Risher et al. 2014; Gao and Penzes 2015). Inhibitory synapses are symmetrical. At the presynaptic site, the vesicular GABA transporter (VGAT) packs GABA neurotransmitter, whereas at the postsynaptic site, gephyrin clusters GABA receptors onto the surface of postsynaptic neuron (Gatto and Broadie 2010; Risher et al. 2014; Gao and Penzes 2015). Data from our laboratory showed an increase in the number of pyramidal cells in layer II (L2) and a decrease in parvalbumin (PV)+ GABAergic interneurons in all layers of the dorsolateral prefrontal cortex (PFC) and ventral PFC in ASD (Hashemi et al. 2017; Amina et al. 2021). Others also detected an increase in pyramidal cell numbers (Courchesne et al. 2007) in the PFC. In addition, an elevation in dendritic spine density has been described in ASD, suggesting a disturbance in neuronal connections within the cerebral cortex in ASD (Hutsler and Zhang 2010). Herein, we quantified the number of excitatory and inhibitory synapses in Brodmann area (BA)9 and BA46 from the dorsolateral PFC that are responsible for attention and working memory, and BA47 from the ventral PFC that is in charge of language processing (Zhang et al. 2003; Friederici 2011). We examined 10 ASD cases and compared them with 10 age- and sex-matched control (CT) cases.

Materials and methods

Samples

Human brains were obtained from the NIH NeuroBioBank, the ASD Tissue Program (current ASD BrainNet). ASD diagnoses were confirmed through clinical records and next-of-kin interviews.

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Table 1. Clinical features of the control (CT) and ASD cases, including sex, age, postmortem interval (PMI), and cause of death. NK, not known.

Case ID	Diagnosis	Sex	Age (years)	PMI (hours)	Cause of death
4203	CT	M	7	24	Respiratory insufficiency
AN00544	CT	M	17	28	NK
AN17868	CT	M	46	18.8	Cardiac arrest
AN19760	CT	M	28	23	NK
AN15566	CT	F	32	28	NK
AN01891	CT	M	24	35	NK
5958	CT	M	22	24	Dilated cardiomegaly
5834	CT	M	14	38	Cardiac arrhythmia
AN07444	CT	M	17	30.8	Asphyxia
5893	CT	M	19	19	Dilated cardiomegaly
AN00764	ASD	M	7	23.7	Accident
5403	ASD	M	16	35	Cardiac arrhythmia
AN06746	ASD	M	44	30.8	Cardiac arrest
AN09412	ASD	M	29	38	NK
1575	ASD	F	40	24	Complications of diabetes
AN00493	ASD	M	27	8.3	Drowning
5176	ASD	M	22	18	Subdural hemorrhage
4305	ASD	M	12	13	Serotonin syndrome
AN00394	ASD	M	14	10.3	Cardiac arrest
4269	ASD	M	19	45	Meningitis

Care was taken to ensure that all diagnoses followed the DSM-5 guidelines, and that only ASD was diagnosed, with no additional clinically diagnoseable disorders such as Fragile X. Ten CT brains free of neuropathology were age- and sex-matched with 10 ASD brains. Of the 10, 9 males and 1 female in each group, with an average age of 22.6 (a range between 7 and 46 years old) for the CT group and an average age of 23 (a range between 7 and 44 years old) for the ASD cases. No significant difference was observed in the postmortem interval (PMI) between the CT and ASD groups. Additional details regarding the age, PMI, and cause of death are in Table 1. Limitations of these studies include a sample size of only 10 cases per group, wide age range, and reduced numbers of females.

Tissue processing

Blocks of the PFC BA9, BA46, and BA47 were isolated using a human brain atlas (Mai et al. 2015) to dissect the target areas of interest in the coronal plane. Tissues were thereafter fixed in 10% buffered formalin, cryoprotected in a 30% sucrose solution in 0.1 M phosphate-buffered saline (PBS) with 0.1% sodium azide. Tissue was embedded in Optimum Cutting Temperature compound and frozen at −80°C. A cryostat (Leica CM 1950) was used to cut 14-μm-thick sections. These were immediately mounted onto coated slides and stored at −80°C until they were ready for processing.

IF staining

We used 2 sequential sections for staining. Primary antibodies used for the excitatory synapses were rabbit anti-VGlut1 (1:50, Abcam), mouse anti-PSD95 (1:100, Antibodies Inc.), and rat anti-NeuN (1:100, Abcam). Secondary antibodies including donkey anti-rabbit Alexa Fluor 488 (1:400, Thermo Fisher), donkey anti-mouse Alexa Fluor 594 (1:300, Thermo Fisher), and goat anti-rat Alexa Fluor 647 (1:300, Abcam). Primary antibodies used for the inhibitory synapses were chicken anti-VGAT (1:300, Synaptic systems), mouse anti-gephyrin (1:50, Invitrogen), and rat anti-NeuN (1:100, Abcam). Secondary antibodies were donkey anti-chicken Alexa Fluor 488 (1:400, Jackson), donkey anti-mouse Alexa Fluor 594 (1:300, Thermo Fisher), and goat anti-rat Alexa Fluor 647 (1:300, Abcam). After rinsing with PBS, sections were incubated with 2X TrueBlack Plus Lipofuscin Autofluorescence Quencher (Biotium) for 10 min to remove auto-fluorescence and rinsed again with PBS. Slides were cover-slipped with Mowiol (Millipore Sigma) and stored at 4°C.

Imaging and quantification of the synapses

We took images with a Nikon A1 confocal laser-scanning microscope at ×20 first to localize the layer of interest and at ×100 later to image synapses. We quantified pre- and postsynaptic-colocalized puncta in 12 areas (10,000 μm^2) within 2 sections (6 per section, 3 within L2, and 3 within layer V (L5)). We reported the final data point as the average number of synapses per layer and per area. We quantified the number of colocalized excitatory (VGlut1 and PSD95) and inhibitory (VGAT and gephyrin) puncta using the plugin Puncta Analyzer in ImageJ.

Statistical analysis

The numbers of synapses were compared between CT and ASD for each area (BA9, BA46, or BA47), layer (L2 or L5), and synapse type (excitatory or inhibitory) using a linear mixed effects model. The model included fixed effects for ASD/CT, area, layer, type, all 2-, 3-, and 4-way interactions among these variables, age, and sex, as well as a random intercept for subject. The number of synapses was log transformed prior to analysis in order to better satisfy model assumptions. Analyses were conducted using R version 4.2.1 (2022 June 23). Linear mixed effects modeling was conducted using the R package nlme, version 3.1–159.

Results

We obtained tissue from 10 ASD subjects and 10 age- and sex-matched CT cases. We isolated BA9, BA46 and BA47 blocks and cut 14-μm-thick cryosections. We applied NeuN antibody to label neurons and confirm the area of interest based on von Economo

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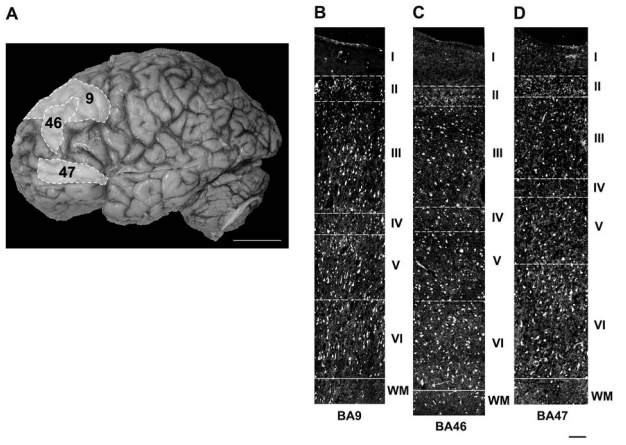


Fig. 1. Prefrontal BA9, BA46, and BA47. A) Coronal sections from left hemisphere. B–D) NeuN immunofluorescence staining of BA9 B), area BA46 C), and BA47 D). Scale bar in a: 3 cm; d b–d): 200 μm.

histology (Fig. 1), and to delineate L2 and L5. We labeled excitatory synapses with VGlut1 and PSD95 proteins (Fig. 2A–D), and inhibitory synapses with VGAT and gephyrin proteins. We found a significant increase in the number of excitatory synapses in L2 in BA9 and BA47 in ASD compared with the CT cases. We also found a significant decrease in the number of inhibitory synapses in L2 and L5 in all the BAs of interest in ASD compared with CT cases.

The number of excitatory synapses is increased in L2 of the PFC in ASD

We found a 4.7-fold increase in the number of excitatory synapses in L2 of BA9 ($P = 0.009$) and 2.5-fold increase in BA47 ($P = 0.01$) in ASD cases compared with CT subjects. We found an increase of 1.9-fold in the number of excitatory synapses in L2 of BA46 but this was not significant (CT: BA9 = 162, BA46 = 454, BA47 = 187; ASD: BA9 = 766, BA46 = 878, BA47 = 473; Fig. 2E).

We did not find any change in the number of excitatory synapses in L5 of any of the areas of interest (CT: BA9 = 102, BA46 = 319, BA47 = 169; ASD: BA9 = 238, BA6 = 244, BA47 = 197; Fig. 2F).

The number of inhibitory synapses is decreased in the PFC in ASD

We found a 3.7-fold decrease in the number of inhibitory synapses in L2 in BA9 ($P < 0.001$), 3.2-fold decrease in BA46 ($P = 0.03$), and 3.3 decrease in BA47 ($P = 0.01$) of the ASD group compared with CT cases (CT: BA9 = 457, BA46 = 382, BA47 = 307; ASD: BA9 = 122, BA46 = 116, BA47 = 92; Fig. 3E).

We found a 3.8-fold decrease in the number of inhibitory synapses in L5 of BA9 ($P = 0.004$), 2-fold decrease in BA46 ($P = 0.04$), and 3.7 decrease in BA47 ($P = 0.02$) of ASD compared with the CT cases (CT: BA9 = 225, BA46 = 320, BA47 = 374; ASD: BA9 = 59, BA46 = 154, BA47 = 101; Fig. 3F).

We compared synapse density between areas and found that excitatory synapsis in L2 of area BA47 in CT cases was higher than in other areas (BA47 and BA9). There were not any other differences. There was not a difference in values compared with age. There were not enough females to compare sexes. Excitatory and inhibitory synapse density is not significantly associated.

Overall, our data indicate an increase in the number of excitatory synapses in L2 and a decrease in the number of inhibitory synapses in L2 and L5 in ASD when compared with CT cases.

Discussion

We found a increase in the number of excitatory synapses in L2 in BA9 and BA47 in the prefrontal cortex. The increase in the excitatory synapsis in BA9 was higher compared with BA47 (±5-fold in BA9 vs. ±2 in BA46 and BA47).

These findings agree with our previous publication demonstrating an increase in excitatory pyramidal cells in L2 of the PFC, but not in other layers (Falcone et al. 2021). Our findings are also in line with the short-range fiber hyperconnectivity previously reported. More fibers mean more cells, and more cells may translate into more synapses. Short-range-myelinated fiber hyperconnectivity was found in the superficial white matter below the anterior cingulate cortex (ACC) of the PFC in 5 ASD postmortem cases compared with 4 CT cases (Zikopoulos and Barbas 2010). Connectional changes were also reported by a meta-analysis from studies using magnetic resonance imaging showing local hyperconnectivity within the frontal lobe that is excessive and hyperactive (Courchesne and Pierce 2005). An excessive and disorganized connectivity within the frontal cortex could produce inability to integrate information and disturb the frontal cortex function in the ASD brain (Courchesne and Pierce 2005; Redcay and Courchesne 2005).

Cortical excitatory synapses secrete the glutamate neurotransmitter. Gene studies reported elevated mRNA levels of excitatory amino acid transporter 1 and glutamate receptor (AMPA-1) gene in the cerebellum of 10 postmortem ASD cases (Purcell et al. 2001). Increased levels of metabotropic glutamate receptor 5 with a reduction in $GABA_A$ receptor protein were also reported in the cerebellum of 11 ASD children (Fatemi et al. 2011). A mutation in the SHANK3 gene in an ASD mouse model showed excitatory synapse dysfunction in the pyramidal neurons of the ACC (Durand et al. 2012; Guo et al. 2019). Alterations in synaptic activity are also in line with the increased dendritic spine density that was reported on cortical pyramidal cells in L2 and L5 of temporal (BA21), and L2 of frontal (BA9) and parietal lobes (BA7) in 10 ASD postmortem cases compared with sex- and age-matched CT cases. Increased number of spines in the superficial cortical L2 could be because of the prolonged maturation of the upper cortical layers during the postnatal period (Hutsler and Zhang 2010). It has also been proposed that ASD patients might present with altered synapse elimination (Courchesne 2004; Frith 2004).

We also found a significant decrease in the number of inhibitory synapses in L2 and L5 of the PFC in the areas of interest. This decrease was homogeneous among areas (±3-fold). This agrees with our previous finding demonstrating a decrease of PV+ chandelier GABAergic interneurons in all layers of the PFC in ASD (Hashemi et al. 2017; Ariza et al. 2018; Amina et al. 2021). Other postmortem and animal studies identified a dysregulation in the inhibitory system in ASD pathology including disruption of inhibitory biomarkers and mutation in genes related to the maturation of inhibitory cortical neurons and synaptic communication (Chao et al. 2010; Blatt and Fatemi 2011). Our human studies showed that $GABA_AR\alpha 2$ subunit is decreased in the proximal axon of pyramidal cells where GABAergic chandelier interneurons synapse (Hong et al. 2020). A decrease in $GABA_A$ receptor subunits has also been reported in the cerebellum, BA9, and BA40 in postmortem ASD cases compared with the CT cases indicating GABAergic dysfunction in ASD brain (Fatemi et al. 2009). The number of $GABA_A$ receptors and their density also decreased by 46.8% in layers I–III and 20.2% in layers V–VI in the ACC of adult ASD cases (Oblak et al. 2009). The $GABA_A$ receptor subunit expression is regulated during development

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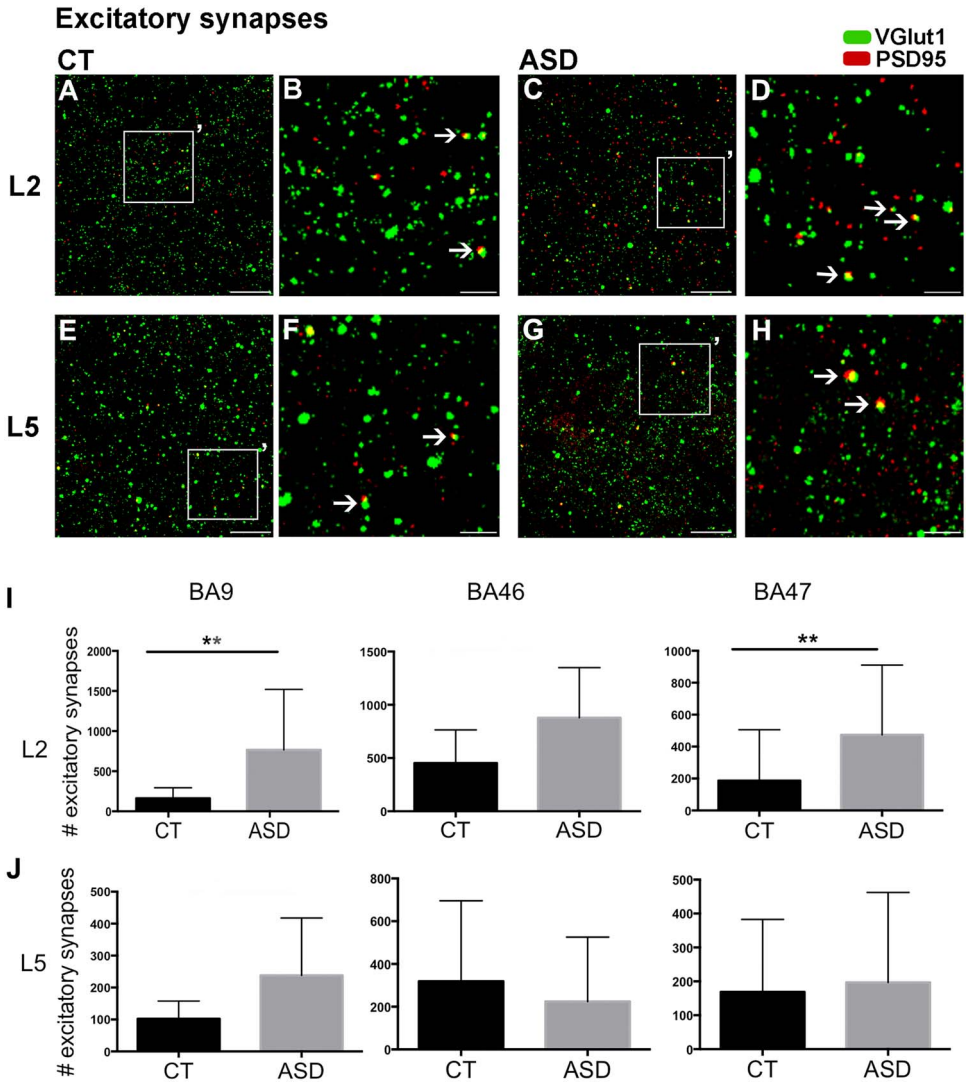


Fig. 2. PFC stained with antibodies against VGlut1 and PSD95 depicting pre- and postsynaptic excitatory synaptic components in L2 (A–D) and L5 (E–H). I) Number of excitatory synapses in L2 of BA9, BA46, and BA47 in CT and ASD cases. There was a significant increase in the number of excitatory synapses in L2 in ASD cases compared with CT cases in BA9 and BA47 (**$P \leq 0.01$). J) Number of excitatory synapses in L5 of BA9, BA46, and BA47 in CT and ASD cases. There was not a significant increase in the number of excitatory synapses in L5 of the areas of interest in ASD cases compared with CT cases ($P \geq 0.05$). Scale bar in A, C, E, G: 5 μm; B, D, F, H: 2 μm.

and is required for the formation and maturation of GABAergic synapses. Therefore, alteration in $GABA_A$ receptor subunit expression could have an impact on inhibitory synaptogenesis and synaptic function (Heinen et al. 2004; Ren et al. 2015). Protein levels of glutamic acid decarboxylase-65 and -67 and the number of Purkinje cells and their size were decreased in the cerebellum of ASD brains suggesting dysregulation in GABA production and cell atrophy in ASD pathology (Fatemi et al. 2002; Yip et al. 2007). GABA receptors are reduced in hippocampus, cerebellum, BA9, and BA40 of ASD brains (Blatt and Fatemi 2011).

Mutation in genes that encode synaptic scaffolding proteins such as NRXNs and NLGNs have also been reported in ASD patients (Moessner et al. 2007; Zoghbi and Bear 2012). Polymorphisms and epigenetic factors disturb neurite growth, synaptic formation, and transmission in both excitatory and inhibitory circuits in ASD brains (Samaco et al. 2005; Gilman et al. 2011; Hussman et al. 2011; Voineagu et al. 2011). Changes in synapse and synaptic components have also been detected in other neurodevelopmental disorders. In *15q dup* mice, while a reduction in the number of inhibitory synapses was reported, there was an increase in the number of glutamatergic synapses in L5 of the PFC (Saitow et al. 2020). In Fragile X syndrome, the mutation of the Fmr1 gene that encodes Fragile X Messenger Ribonucleoprotein 1 (FMRP) leads to an alteration in the number and structure of synapses resulting in synaptic dysfunction. FMRP has a major role in synapse elimination, development, and therefore overall plasticity in the brain (Pfeiffer and Huber 2009). Rett syndrome is a synaptic disorder caused by mutations in MECP2 resulting in abnormalities in synaptic plasticity that, in turn, disrupts normal synaptic function (Banerjee et al. 2019). Loss of MeCP2 that is involved in protein synthesis and degradation presents with an alteration of axonal arborization and synaptic transmission and a decreased number of synapses (Belichenko et al. 2009; Chao et al. 2007). GABAergic neurons lacking the Mecp2 gene in mice induce ASD symptoms such as repetitive behaviors, indicating MeCP2 protein is essential for correct functioning of the GABAergic system (Chao et al. 2010). Moreover, loss of MeCP2 in cortical excitatory neurons leads to decrease GABAergic

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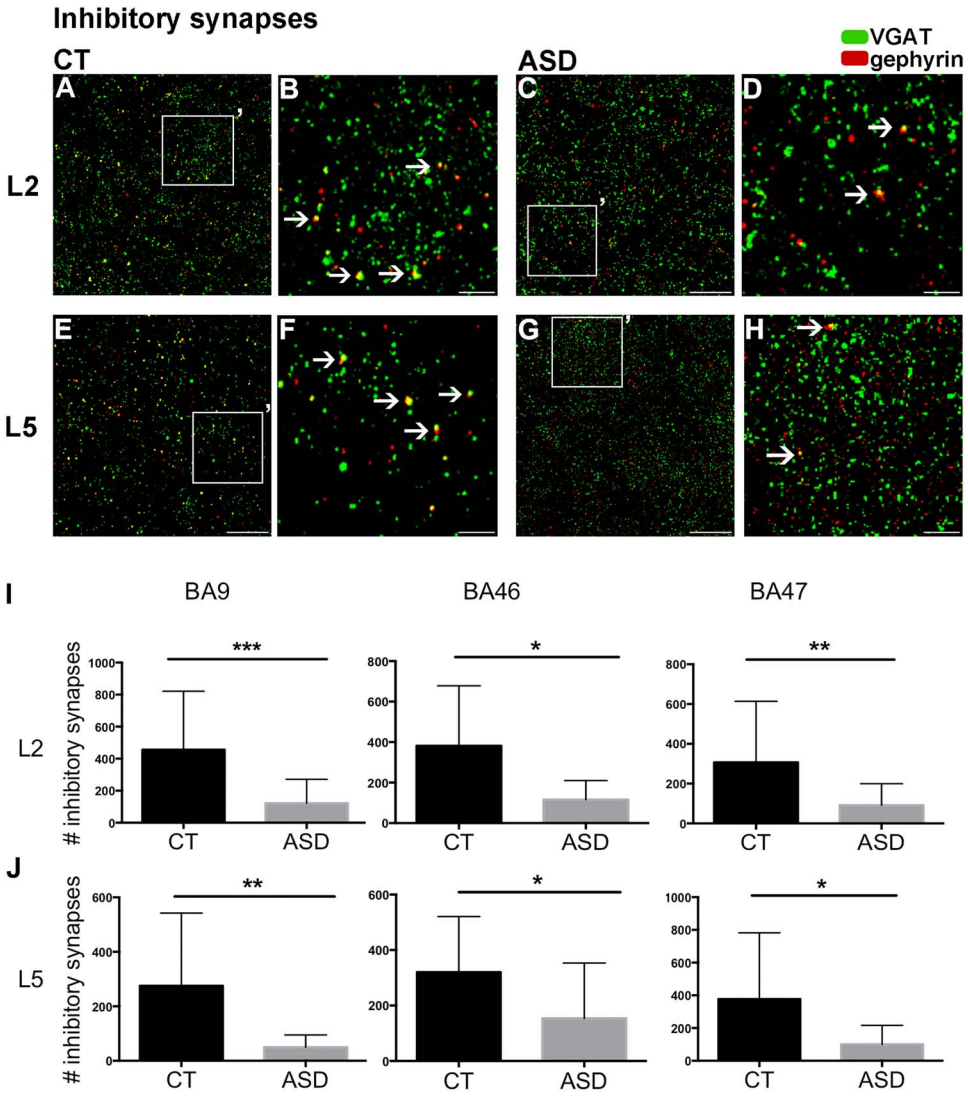


Fig. 3. PFC stained with antibodies against VGAT and gephyrin depicting pre- and postsynaptic inhibitory synaptic components in L2 (A–D) and L5 (E–H). I) Number of inhibitory synapses in L2 of BA9, BA46, and BA47 in CT and ASD cases. There was a significant decrease in the number of inhibitory synapses in L2 in ASD cases compared with CT cases in BA9, BA46, and BA47 (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$). J) Number of inhibitory synapses in L5 of BA9, BA46, and BA47 in CT and ASD cases. There was a significant decrease in the number of inhibitory synapses in L5 of BA9, BA46, and BA47 in ASD cases compared with CT cases (*$P \leq 0.05$; **$P \leq 0.01$). Scale bar in A, C, E, G: 5 μm; B, D, F, H: 2 μm.

neurotransmission by reducing the number of GABAergic synapses and increase the excitability in L5 pyramidal neurons in mice (Zhang et al. 2014). In schizophrenia (SCZ), defects in synaptic formation, stability, and elimination with altered apoptotic regulatory proteins and DNA fragmentation patterns have also been reported (Glantz et al. 2006; Berdenis van Berlekom et al. 2020). Also, a reduction in postsynaptic proteins in pyramidal neurons observed in layer III of the PFC in SCZ can result in a decrease in excitatory synapses (Melchitzky et al. 1998). A decreased spine density was also reported in layer III of 61 SCZ patients associated with a reduction in macroscale connectivity in the brain (van den Heuvel et al. 2016). Slow atrophy of terminal dendritic ramification, impairment of dendritic spines, and loss of excitatory synapses also occur throughout the cerebral cortex in Down syndrome (DS; Sarnat and Flores-Sarnat 2021). Moreover, cells derived from the patients with DS showed normal growth of neurons but with less dendritic spine turnover and network activity (Real et al. 2018).

Taken together, our data show an increase in the number of excitatory synapses (not in all areas) and a reduction in the number of inhibitory synapses. Our findings are in line with previous studies that report ASD as a neurodevelopmental synaptic disorder with an imbalance in glutamatergic and GABAergic synapses and abnormal synaptic homeostasis (El-Ansary and AlAyadhi 2014). An increased prevalence of seizures in ASD patients highlights the dysfunction of excitatory and inhibitory neuronal network activity (Montanari et al. 2022). Our finding provides evidence for E/I imbalance in the PFC of ASD individuals that can lead to the disruption of normal signal transduction within the cortex and between cortical and subcortical areas of the brain.

Author contributions

Gelareh Vakilzadeh (Conceptualization, Formal analysis, Investigation, Methodology, Writing—original draft), Busisiwe C.

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