

GABA_AR α 2 is Decreased in the Axon Initial Segment of Pyramidal Cells in Specific Areas of the Prefrontal Cortex in Autism

Tiffany Hong,^a Carmen Falcone,^a Brett Dufour,^a Sarwat Amina,^{a,b} Rosalia Perez Castro,^a Jade Regalado,^a Weston Pearson,^a Stephen C. Noctor^{b,c} and Verónica Martínez-Cerdeño^{a,b,*}

^a Department of Pathology and Laboratory Medicine, UC Davis School of Medicine, Institute for Pediatric Regenerative Medicine and Shriners Hospitals for Children of Northern California, Sacramento, CA, United States

^b MIND Institute, UC Davis Medical Center, Sacramento, CA, United States

^c Department of Psychiatry and Behavioral Sciences, UC Davis School of Medicine, Sacramento, CA, United States

Abstract—Some forms of Autism Spectrum Disorder, a neurodevelopmental syndrome characterized by impaired communication and social skills as well as repetitive behaviors, are purportedly associated with dysregulation of the excitation/inhibition balance in the cerebral cortex. Through human postmortem tissue analysis, we previously found a significant decrease in the number of a gamma-aminobutyric acid (GABA)ergic interneuron subtype, the chandelier (Ch) cell, in the prefrontal cortex of subjects with autism. Ch cells exclusively target the axon initial segment (AIS) of excitatory pyramidal (Pyr) neurons, and a single Ch cell forms synapses on hundreds of Pyr cells, indicating a possible role in maintaining electrical balance. Thus, we herein investigated this crucial link between Ch and Pyr cells in the anatomy of autism neuropathology by examining GABA receptor protein expression in the Pyr cell AIS in subjects with autism. We collected tissue from the prefrontal cortex (Brodmann Areas (BA) 9, 46, and 47) of 20 subjects with autism and 20 age- and sex-matched control subjects. Immunohistochemical staining with antibodies against the GABA_A receptor subunit α 2 (GABA_AR α 2) – the subunit most prevalent in the Pyr cell AIS – revealed a significantly decreased GABA_AR α 2 protein in the Pyr cell AIS in supra-granular layers of prefrontal cortical areas BA9 and BA47 in autism. Downregulated GABA_AR α 2 protein in the Pyr cell AIS may result from decreased GABA synthesis in the prefrontal cortex of subjects with autism, and thereby contribute to an excitation/inhibition imbalance. Our findings support the potential for GABA receptor agonists as a therapeutic tool for autism. © 2020 Published by Elsevier Ltd on behalf of IBRO.

Key words: GABA, GABA receptor, chandelier cell, pyramidal cell, autism, axon initial segment.

INTRODUCTION

Autism Spectrum Disorder is a neurodevelopmental syndrome characterized by impaired communication and social skills, as well as repetitive behaviors (American Psychiatric Association, 2013; Lord et al., 2020). The disorder affects about 1% of children in the US and is more common in males than in females with a range of reported ratios commonly estimated at 4:1 (Brugha et al., 2016;

Loomes et al., 2017). In 2003, Rubenstein and Merzenich postulated that the mechanism of autism is related to an imbalance between excitation and inhibition in areas of the cerebral cortex concerned with cognitive, language, and social communication functions, and that cortical overexcitation could potentially enhance vulnerability to, and thus explain the high comorbidity with, seizures (Rubenstein and Merzenich, 2003). Additionally, abnormal cortical gamma-wave activity has been observed in children with autism during stimulus-related electroencephalogram (EEG) (Wilson et al., 2007). We previously investigated anatomical abnormalities that could contribute to an electrical imbalance in autism neuropathology, and found that the chandelier (Ch) cell, a gamma-aminobutyric acid (GABA)ergic interneuron subtype expressing the calcium-buffering protein parvalbumin (PV), is decreased in number in Brodmann Areas (BA) 9, 46 and 47 in subjects with autism (Hashemi et al., 2017; Ariza et al., 2018). Indeed, optogenetic studies in mice have demonstrated not only that PV+ GABAergic

*Correspondence to: V. Martínez-Cerdeño, 2425 Stockton Boulevard, Sacramento, CA 95817, United States. Fax: +1-(916)-453-2288.

E-mail address: vmartinezcerdeno@ucdavis.edu (V. Martínez-Cerdeño).

Abbreviations: ADI-R, Autism diagnostic interview-revised; AIS, axon initial segment; AU, autism; BA, Brodmann area; Ch Cell, chandelier; CT, control; DSM-5, 5th Edition of the Diagnostic and Statistical Manual of Mental Disorders; EEG, electroencephalogram; GABA, gamma-aminobutyric acid; GABA_AR α 2, GABA_A receptor α 2 subunit; GAD65 and GAD67, glutamic acid decarboxylase 65kDa and 67kDa; OCT Compound, optimum cutting temperature; PMI, postmortem interval; PV, parvalbumin; Pyr Cell, Pyramidal; SOM, somatostatin.

interneurons, which include Ch and basket cells, regulate gamma rhythms and promote cognitive flexibility (Cardin et al., 2009; Sohal et al., 2009), but also that stimulation of Pyr cells in the prefrontal cortex induces social deficits associated with abnormal gamma oscillations while coactivation of PV+ cells and Pyr cells does not (Yizhar et al., 2011). These studies support the hypothesis that irregular PV+ interneuron function in the prefrontal cortex can result in aberrant gamma oscillation regulation and contribute to autistic-like behaviors (Lee et al., 2017).

Ch cells are a fast-spiking subset of GABAergic interneurons (Szentagothai and Arbib, 1974; Jones, 1975) characterized by axon terminals called cartridges that are arranged perpendicularly to the cortical surface and lined with synaptic boutons (Defelipe et al., 1985). Ch cells are axo-axonic; their cartridges synapse on the axon initial segment (AIS) of excitatory Pyr cells. Each Pyr cell receives input from only one or a few Ch cells (Defelipe et al., 1985; Inan et al., 2013), but a single Ch cell can innervate hundreds of Pyr cells, suggesting its importance in cortical circuit regulation (Kawaguchi, 1997; Douglas and Martin, 2004; Markram et al., 2004) and that the loss or alteration of even a few Ch cells can potentially have widespread effects on the excitation/inhibition balance. Furthermore, GABA is the most abundant inhibitory neurotransmitter in the mammalian brain, and a significant downregulation of protein and mRNA of glutamic acid decarboxylase 65 kDa and 67 kDa (GAD65 and GAD67) – enzymes involved in GABA synthesis – has been described in homogenate from the cortex and cerebellum of subjects with autism (Fatemi et al., 2002; Yip et al., 2007, 2009). BA9 and BA46 from the dorsolateral prefrontal cortex and BA47 from the ventrolateral prefrontal cortex were selected for our previous and current studies in autism neuropathology due to the involvement of BA9 and BA46 in modulating attention and behavior (Alexander and Stuss, 2000), and the role of BA47 in language processing (Ardila et al., 2017); systems known to be affected in autism.

The AIS of Pyr cells, the location of Ch cell synaptic targets, contains ionotropic GABA class A receptors (GABA_AR), ligand-gated ion channels primarily permeable to chloride ions. The two general classes of GABA receptors – GABA_AR and GABA_BR – differ in composition, pharmacology, and action. There are 19 known subunits that comprise a GABA_AR heteropentamer, with subunit $\alpha 2$ being most prevalent in the AIS (Loup et al., 1998). Decreased protein and mRNA for various GABA_AR subunits in homogenate from the superior frontal cortex (BA9) and parietal cortex (BA40) in postmortem tissue from subjects with autism has been reported (Fatemi et al., 2009b, 2014). In addition, receptor binding studies have found downregulated GABA_AR in the hippocampus (Blatt et al., 2001) and downregulated GABA_AR and GABA_BR subunit density in the fusiform gyrus and cingulate cortex in subjects with autism (Oblak et al., 2009, 2010, 2011). Currently available data thus indicates a downregulation of the GABA system in autism, but cellular localization of these alterations is not yet clear.

To further understand the possible role of Ch cells and alterations to the GABA system in the anatomy of autism neuropathology, we analyzed postmortem human tissue from patients with autism age- and sex-matched to subjects without neurological disorders (Table 1), and quantified levels of GABA_AR $\alpha 2$ protein in the Pyr cell AIS in prefrontal cortex BA9, BA46 and BA47 (Fig. 1).

EXPERIMENTAL PROCEDURES

Samples

Postmortem human tissue samples were obtained from the Autism Tissue Program (ATP) (predecessor to Autism BrainNet), the NIH NeuroBioBank, and the UC Davis Medical Center. All autism diagnoses were confirmed by respective brain banks through standard postmortem use of the Autism Diagnostic Interview-Revised (ADI-R) or through record review, and diagnoses conform with the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) diagnosis of autism (AU). Control (CT) subjects were defined as free of neurological disorders, including autism, based on medical records and information gathered at the time of death from next of kin. Twenty subjects with autism and twenty age- and sex-matched control subjects were included in this study, consisting of thirty-eight males ($n = 19\text{CT} + 19\text{AU}$) and two females ($n = 1\text{CT} + 1\text{AU}$) (Table 1); a pair-matched design was utilized for analysis. One subject with autism presented with seizures, while no other control or autism subject had a history of seizures or epilepsy (Table 1). Details including age, postmortem interval (PMI), time in formalin, and causes of death are listed in Table 1. The average age of control subjects was 24.9 years, with a range of 7–56 years, and the average age of subjects with autism was 25.6 years, with a range of 6 to 56 years. Blocks of prefrontal cortex Brodmann areas BA9, BA46 and BA47 were isolated based on Brodmann anatomy as previously described (Hashemi et al., 2017). Tissue blocks were 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 (OCT) compound and frozen at -80°C . A cryostat (Leica CM 1950) was used to cut $14\text{ }\mu\text{m}$ -thick slide-mounted sections, stored at -80°C until use. Occasionally, tissue from a BA for a given case was highly degraded or otherwise unsuitable for analysis, including: BA9 from subject UCD13AP86; BA46 from subjects 210, 4899 and 4999; and BA47 from subjects 4337, 210, 4899 and 5574. One section per block was Nissl-stained and used to confirm cortical areas based on von Economo histology as previously described (Fig. 1) (Hashemi et al., 2017); adjacent sections were used for immunostaining.

Immunostaining

Slide mounted tissue sections were enzymatically immunostained with antibodies against both GABA_AR $\alpha 2$ and nonphosphorylated neurofilament H (SMI-32). Our

Table 1. Subjects included in this study. Columns include: subject ID, diagnosis, sex, age, postmortem interval (PMI), time in formalin, and cause of death. CT = Control. AU = Autism. NK = not known. One subject (ID: 4305) presented with seizures. Control subjects were defined as free of neurological disorders, including autism, based on medical records and information gathered at the time of death from next of kin

Case ID	Diagnosis	Sex	Age (years)	PMI (h)	Time in formalin (months)	Cause of death
UCD13AP86	CT	M	6	44.3	64	NK
4203	CT	M	7	24	164	Respiratory insufficiency
4337	CT	M	8	16	97	Blunt force
210	CT	M	10	18	278	Myocarditis
5834	CT	M	14	38	26	Cardiac arrhythmia
AN07444	CT	M	17	30.8	74	Asphyxia
AN00544	CT	M	17	28.9	NK	NK
5893	CT	M	19	19	21	Dilated cardiomegaly
5958	CT	M	22	24	13	Dilated cardiomegaly
AN01891	CT	M	24	35	86	NK
UCD1602	CT	M	26	35.7	28	NK
UCD1505	CT	M	26	> 72	47	Renal disease
AN19760	CT	M	28	23.3	NK	NK
AN12137	CT	M	31	32.9	NK	Asphyxia
AN15566	CT	F	32	28.9	NK	NK
UCD1510	CT	M	35	> 72	39	NK
AN05475	CT	M	39	NK	123	Cardiac arrest
AN17868	CT	M	46	18.8	NK	Cardiac arrest
AN19442	CT	M	50	20.4	NK	NK
AN13295	CT	M	56	22.1	NK	NK
AN03221	AU	M	7	11.4	123	Drowning
5144	AU	M	7	3	109	Cancer
AN01293	AU	M	9	4.4	120	Cardiac arrest
4305	AU	M	12	13	119	Serotonin syndrome
4899	AU	M	14	9	128	Drowning
AN00394	AU	M	14	10.3	197	Cardiac arrest
5403	AU	M	16	35	82	Cardiac arrhythmia
4269	AU	M	19	45	135	Meningitis
4999	AU	M	20	14	111	Cardiac arrhythmia
AN00764	AU	M	20	23.7	167	Accident
5176	AU	M	22	18	106	Subdural hemorrhage
5574	AU	M	23	14	56	Pneumonia
AN00493	AU	M	27	8.3	171	Drowning
AN09412	AU	M	29	38	42	NK
AN18892	AU	M	31	> 72	177	Gun shot
5027	AU	M	37	26	119	Bowel obstruction
1575	AU	F	40	24	136	Complications of diabetes
AN06746	AU	M	44	30.8	216	Cardiac arrest
5137	AU	M	51	72	107	Pneumonia
AN01093	AU	M	56	NK	190	NK

use of enzymatic immunostaining was on the grounds that the non-perfused, long-PMI characteristics of postmortem human tissue often resulted in increased autofluorescence, and fluorescent immunolabeling has a limited lifespan that would not allow preservation of our staining for future analysis. Briefly, tissue was treated with chloroform: 100% ethanol at 1:1 followed by sequential immersion in 100%, 96%, 90%, 70% and 50% EtOH, then diH₂O. Antigen retrieval was performed by exposing the tissue to 110 °C for 8 min, and slides were washed (TBS twice followed by TBS + 0.05% tween once). Endogenous peroxidase blocking was performed with 3% H₂O₂. The slides were incubated with 10 drops of BLOXALL (Vector Labs) for 10 min in a dark humidified box, washed with TBS, blocked with TBS + 10% NDS + 0.3% triton for 1 h at room temperature, and then treated with an avidin–biotin blocking kit (Vector Labs). Primary antibody solution

(rabbit anti-GABA(A) α 2 receptor antibody (Alomone, 1:200) and mouse anti-neurofilament (SMI-32) antibody (Covance, 1:400)) was added to each slide for 24 h at 4 °C with Parafilm coverslips in a dark humidified box. Slides were then washed and incubated with the first secondary antibody (biotinylated donkey anti-rabbit IgG (Jackson, 1:150)) for 1 h, washed, and incubated with ABC solution (Vector Labs) for 2 h. After washing, slides were developed with DAB substrate (brown; Vector Labs) and washed again. Avidin-biotin blocking sequence (Vector Labs) was repeated as above, followed by a quick TBS rinse and application of the second secondary antibody (biotinylated donkey anti-mouse IgG (Jackson, 1:150)) for 1 h, then washed and incubated with ABC solution (Vector Labs) for 2 h in a dark, humidified box. After a wash, slides were developed with VIP substrate (purple; Vector Labs), then washed again. Tissue was dehydrated through

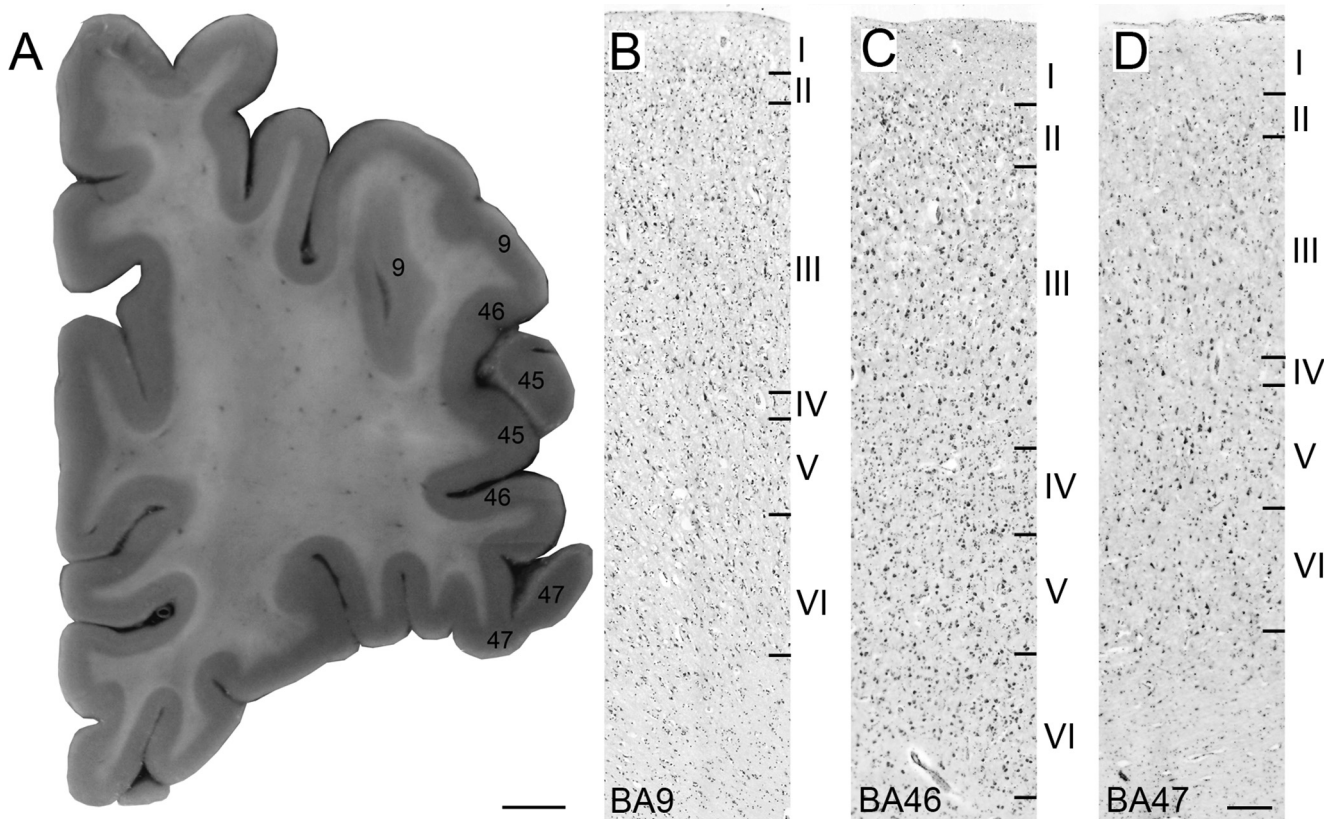


Fig. 1. Cortical areas investigated in this study. Blocks of prefrontal cortical tissue containing BA9, BA46, and BA47 were isolated based on Brodmann and von Economo anatomy. **(A)** Coronal section of cerebral cortex from a left hemisphere, marked with Brodmann areas of interest (BA9, BA46, and BA47). Adjacent area BA45 is marked for reference. **(B–D)** Nissl-stained sections of **(B)** area BA9, **(C)** area BA46, and **(D)** area BA47. Short horizontal lines in **(B–D)** denote layer boundaries. Scale bar in A: 0.5 cm, scale bar in D **(B–D)**: 200 μ m.

sequential immersion in 50%, 70%, 90%, 96%, and 100% EtOH for 3 min each, then cleared in Xylene for 6 min and coverslipped with Permount mounting medium.

Antibody characterization

Anti-GABA_A α 2 Receptor: *Immunogen*: Peptide (C) TPEPNKKPENKPA, corresponding to amino acid residues 393–405 of rat GABA_AR α 2 (Accession P23576) 2nd intracellular loop; *Homology*: Mouse, human, bovine – identical; *Host*: Rabbit; *Isotype* IgG.; *Antibody type*: polyclonal; *Manufacturer and Catalogue#*: Alomone Labs Ltd. #AGA-002; *Concentration*: 1:200; *Characterization reference*: Immunohistochemistry on mouse brain sections (Skilbeck et al., 2018); *Additional information*: It has been designed to recognize GABA_AR α 2 from human, rat and mouse samples; *RRID*: AB_2039864.

Anti-Neurofilament H (NF-H), Nonphosphorylated Antibody (SMI-32R, clone SMI-32): *Immunogen*: Homogenized hypothalami from Fischer 344 rat brain; *Host*: Mouse; *Antibody type*: monoclonal; *Manufacturer and Catalogue#*: Covance #SMI-32R; *Concentration*: 1:400; *Characterization reference*: Immunohistochemistry in mouse and rat brains (Broms et al., 2015); *Additional information*: This antibody recognizes a non-phosphorylated epitope on the neurofilament heavy polypeptide in human and monkey brain lysate

(Campbell and Morrison, 1989). The antibody has previously been used to differentiate between different subnuclei in the rat and mouse LHb (Geisler et al., 2003; Wagner et al., 2014); *RRID*: AB_509998.

Imaging, quantification, and statistics

As enzymatic immunostaining necessitates brightfield examination with overlapping chromogens, our initial simultaneous labeling with antibodies against GABA_AR α 2 as well as the AIS-specific marker Ankyrin G (AnkG) precluded clear AIS identification. Pyr neurons labeled against SMI-32, however, exhibited staining in the cell body, axon, and apical and basal dendrites, and provided a landscape for more apparent morphology-based brightfield AIS identification beneath the GABA_AR α 2 staining, and the AIS could be distinguished based on its origin from the axon hillock (Fig. 2D–I). The distal end of the AIS could also be determined by thickness and darker staining compared with the thinner continuation of the axon (Fig. 2D–I). SMI-32 is an intermediate filament present in some neurons, including Pyr neuron subpopulations located in the human cerebral cortex (Campbell and Morrison, 1989). Expression of SMI-32 in Pyr cells varies by cortical area; in our cases, immunoreactivity across prefrontal areas BA9, BA46 and BA47 was located primarily in Pyr cells of deep layer III and the superficial part of layer V

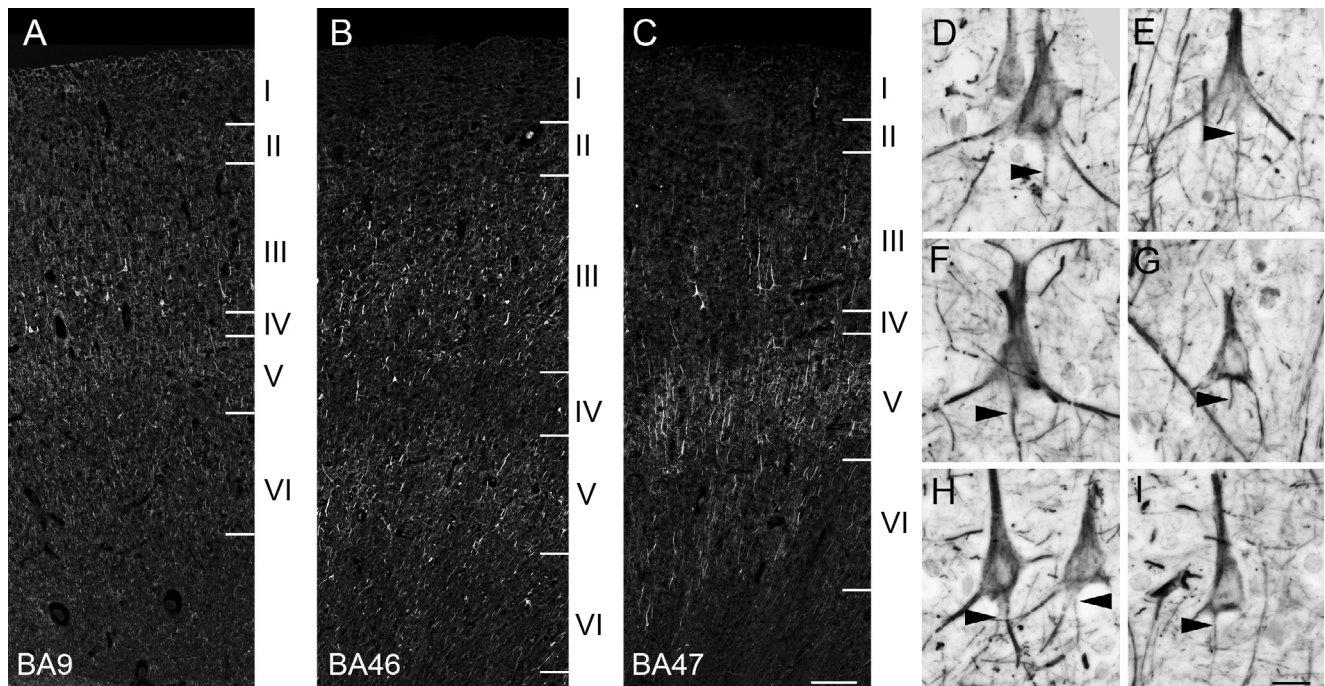


Fig. 2. Prefrontal cerebral cortical sections immunostained with an antibody against neurofilament protein SMI-32, showing pyramidal (Pyr) cell cytoarchitecture including axon initial segment. (A–C) SMI-32-labeled sections of (A) area BA9 (B) area BA46, and (C) area BA47. (A–C) colors have been inverted to emphasize Pyr cells (white), and short horizontal lines denote cortical layers. (D–I) High-magnification images of SMI-32-labeled Pyr cells, with arrowheads marking axon initial segments. Scale bar in C (A–C) = 150 μ m, scale bar in I (D–I) = 10 μ m.

(Fig. 2A–C), as previously described in several dorsolateral prefrontal cortex areas (Law and Harrison, 2003). The SMI-32 staining pattern was consistent across our cases, and qualitative analysis did not reveal a noticeable difference in the Pyr cell expression of SMI-32 between autism and control subjects.

We captured 100 \times brightfield images using an Olympus microscope (BX61) – cells were chosen based on their Pyr soma morphology, the presence of a complete AIS, and clear immunostaining (Fig. 3). The AIS was identified as emerging from the pyramidal cell base opposite to the very thick apical dendrite and identified as complete if a faint thin axonal process was visible beyond the AIS distal end. We then measured the percent area of GABA_AR α 2 protein labeling in the Pyr cell AIS. This was done using ImageJ by first outlining the AIS, then quantifying the percent of the AIS area occupied by GABA_AR α 2 protein. Specifically, with the ImageJ Threshold Colour tool, we sampled regions of the AIS containing GABA_AR α 2 protein from a subset of images, and from that sample created a hue/saturation/brightness threshold by which the GABA_AR α 2 protein color would be extracted across all of the images. We then used a Protein Expression Macro in ImageJ with our thresholded values to determine the percent area of AIS occupied by the thresholded color in each image. During imaging and quantification, all experimenters were blinded to all diagnoses. Data collected from the supragranular (layer III) and infragranular (layer V) layers (3 Pyr cells per layer) were analyzed for each BA included in this study (BA9, BA46, BA47); data for a given layer of a given case was

comprised of the average values from all Pyr cells analyzed within that layer of that case. The goal of the statistical analysis was to compare percent area of GABA_AR α 2 protein labeling in the Pyr cell AIS between autism and control cases, and to assess the relationship between anatomical parameters and other patient/sample characteristics (such as age, PMI, and time in formalin). Each variable was compared between autism and control cases using t-tests. The joint influence of autism and patient/sample characteristics on variables was assessed with an ANCOVA. SPSS 26 (IBM) was used for statistical analyses, and graphs were generated with Prism 6 (GraphPad).

RESULTS

In samples from subjects with autism, we found a significant decreased percent area of GABA_AR α 2 protein labeling in the AIS of Pyr cells in the supragranular layers of BA9 and BA47 (Fig. 4); layer III of BA9 displayed a 61% decrease ($n = 19\text{CT} + 20\text{AU}$, $p < .01$), and layer III of BA47 displayed a 54% decrease ($n = 18\text{CT} + 18\text{AU}$, $p < .05$), when compared with controls. We did not find any significant difference in GABA_AR α 2 protein levels in supragranular or infragranular layers of BA46 or in the infragranular layers of BA9 or BA47, when compared to controls. A pair-matched design was utilized for comparison between age-matched autism and control group samples, and ANCOVA analysis showed no statistically significant influence of covariates including age, PMI, or time in formalin on GABA_AR α 2 protein levels in any BA

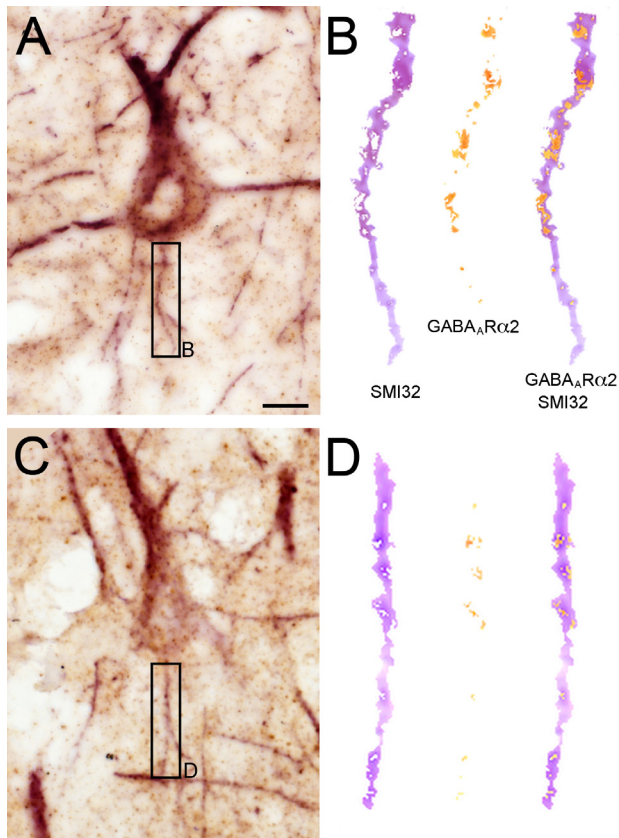


Fig. 3. ImageJ reconstructions of the axon initial segment (AIS) from prefrontal cortex pyramidal (Pyr) cells from layer III of Brodmann Area (BA) 9, double-immunostained with antibodies against GABA_AR α 2 (brown) and SMI-32 (purple). (A) and (C) High-magnification images of double-immunostained Pyr cells from (A) a control subject and (C) a subject with autism. (B) and (D) ImageJ digital reconstructions of the AIS boxed in (A) and (C), respectively; leftmost = SMI-32 (purple) only, center = GABA_AR α 2 (brown) only, and rightmost = both colors overlaid. Scale bar in A (A, C) = 10 μ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

or any cortical layer analyzed ($p > .05$ for all). Overall, we found a decreased percent area of GABA_AR α 2 protein in the Pyr cell AIS in supragranular layers of two of the three prefrontal areas investigated in this study.

DISCUSSION

Imbalance in excitation/inhibition in autism

An imbalance in excitation/inhibition in subjects with autism could result from a number of factors such as an altered number and/or function of excitatory cells, inhibitory cells, or both. Indeed, there is compelling data suggesting that both the excitatory glutamatergic and the inhibitory GABAergic main neurotransmitter systems in the cerebral cortex are involved (Fatemi et al., 2009a, b, 2014). Various research has produced a spectrum of results including increased (Courchesne et al., 2011), decreased (van Kooten et al., 2008), or unchanged (Mukaetova-Ladinska et al., 2004; Uppal et al., 2014; Kim et al., 2015) excitatory Pyr cell numbers in the cerebral cortex of cases with autism. In addition, some studies

have found a higher ratio of excitatory spindle cells to Pyr cells in layer V of the frontoinsular cortex (Santos et al., 2011), while others found an unchanged number of spindle cells (Kennedy et al., 2007). With regard to inhibitory cell number, a scarce inhibitory cell type in layer I, known as the Cajal-Retzius cell, was shown to be unchanged in the superior temporal cortex in autism (Camacho et al., 2014). On the other hand, we previously described a decrease in the number of a GABAergic interneuron subtype, PV+ cells, in BA46, BA47, and BA9 of the prefrontal cortex in autism (by 70%) (Hashemi et al., 2017; Ariza et al., 2018). Furthermore, we determined that the decrease in PV+ neurons was mainly due to a decrease in PV+ Ch cells (Ariza et al., 2018). Importantly, Pyr cell axons primarily receive innervation from Ch cells whereas other inhibitory interneurons synapse onto the dendritic arbor or, in the case of basket cells, the soma of Pyr cells. This quality affords Ch cells a critical modulatory role in the final output of Pyr cells, regulating them through GABA release from cartridges in close apposition to the Pyr cells' AIS.

GABA in autism

Postmortem studies measuring mRNA and total protein have previously shown alterations of the GABA system in specific brain areas in cases with autism. Previous work in postmortem human tissue obtained from the cerebral cortex and cerebellum of patients diagnosed with autism indicates that there is a reduction in the levels of the enzymes that synthesize GABA: GAD65 and GAD67. Fatemi's group reported a downregulation in the cerebellar cortex ($n = 8CT + 5AU$), finding a 50% reduction of GAD65 protein and a 51% reduction of GAD67 protein (Fatemi et al., 2002). In the same vein, Yip et al. reported a GAD67 mRNA reduction of 40% in the neurons in the cerebellar dentate nuclei and Purkinje cells ($n = 8CT + 8AU$) (Yip et al., 2007), and in a follow-up study also reported a 51% reduction of GAD65 mRNA in the large cells of the cerebellar dentate nucleus in subjects with autism ($n = 5CT + 5AU$) (Yip et al., 2009). These data suggest reduced Purkinje cell GABA input to the cerebellar nuclei that potentially disrupts cerebellar output to the cerebral cortex. The only data on GAD65/67 in the cerebral cortex are those provided by Fatemi et al., who reported a 48% reduction of GAD65 protein and a 61% reduction of GAD67 protein in the parietal cortex of the autistic brain ($n = 4CT + 5AU$) (Fatemi et al., 2002). Overall, these data may indicate a decrease in the amount of GABA neurotransmitter in the cerebellum and cortex in subjects with autism. To our knowledge, no data is available on levels of GAD proteins in cortical areas that are strongly associated with autism, such as the prefrontal and temporal cortices.

GABA receptors in autism

GABA_A receptor α 2 subunit labeling presented as punctate staining outlining cell bodies, dendrites, and axons (Fig. 3). Similarly, GABA_AR α 2 has been described on the Pyr cell soma, dendrites, and axon initial segment in rodents (Fritschy and Mohler, 1995;

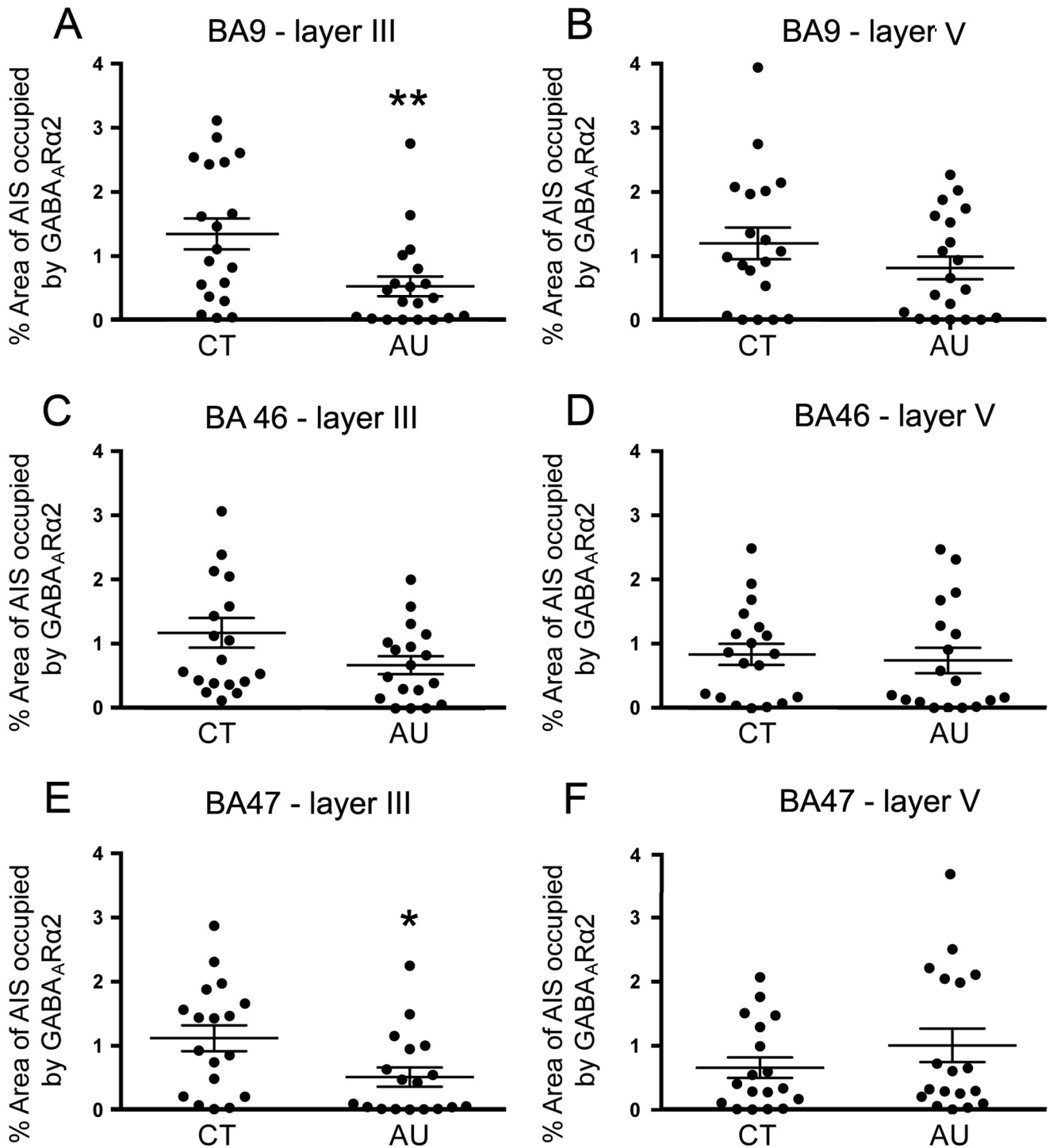


Fig. 4. The percent area of GABA_ARα2 protein labeling in the Pyr cell AIS was significantly lower in supragranular layers of BA9 and BA47 in subjects with autism (AU) when compared with controls (CT). Protein levels were decreased in **(A)** layer III of BA9 ($n = 19\text{CT} + 20\text{AU}$, $p < .01$) and **(E)** layer III of BA47 ($n = 18\text{CT} + 18\text{AU}$, $p < .05$), when compared to control subjects. No significant difference was seen in **(B, F)** layer V of BA9 or BA47, or **(C, D)** in either layer of BA46. The degree of significance is indicated with asterisks: * $p < .05$, ** $p < .01$.

Fagiolini, 2004), and expression on the Pyr AIS has been previously shown in the rhesus macaque prefrontal cortex (Cruz et al., 2003). In the marmoset forebrain, GABA_ARα2 expression is widespread prenatally, but by adulthood remains mostly in the prefrontal cortex supragranular layers as well as the hippocampus and striatum, elsewhere

becoming largely replaced by the α1 subunit (Hornijng and Fritschy, 1996). In humans, GABA_AR expression has been identified throughout the basal ganglia (Faull and Villiger, 1988; Zezula et al., 1988; Glass et al., 2000), with highest levels of α2 seen mainly in the dorsal striatum (Waldvogel and Faull, 2015). In addition, other

GABA_AR subunits known to be associated with GABA_AR α 2 have been described in macaque and human (Hendry, 1994).

As the Pyr cell AIS is normally enriched with α 2 subunits, the decreased amount of GABA_AR α 2 protein reported here suggests a reduced capacity of Pyr cells to respond to Ch cell GABA signaling in autism, potentially resulting in hyper-excitation of the circuit. Inhibitory synapse dysfunction has been seen in mouse models of neurodevelopmental or epileptic disorders including autism, Angelman, Rett and Dravet syndromes (Ali Rodriguez et al., 2018). Mice with abnormalities in cell adhesion molecules – membrane proteins that function both pre- and post-synaptically – exhibit autism-like behavioral dysfunction. Specifically, neuroligin knockout mice show reduced social behavior and vocalization (Radyushkin et al., 2009), and homozygous knockout of a member of the neurexin family causes reduced social and increased repetitive behaviors as well as increased epilepsy, along with decreased cortical PV-positive GABAergic interneuron signaling (Peñagarikano et al., 2011; Karayannis et al., 2014). A mouse model with a point mutation altering the surface stability of GABA_AR β 3 exhibits autism-like features and increased seizures (Vien et al., 2015). In addition, deleting MeCP2, the transcriptional repressor gene that is mutated in human Rett syndrome, from GABAergic inhibitory neurons in mice, replicates the autism-like features seen in the human disease, and in the ventrolateral medulla, reduced GABA release is accompanied by reduced levels of GABA_AR α 2 and GABA_AR α 4 (Medrihan et al., 2008). Furthermore, in mouse models of the epilepsy-associated Dravet syndrome, PV-positive and somatostatin (SOM)-positive interneurons have reduced excitability leading to reduced GABAergic inhibition (Tai et al., 2014), and RNA-Seq has revealed significant alterations in total gene expression of GABA_AR α 2 (Hawkins et al., 2016).

To study the altered amounts of GABA receptor subunits in subjects with autism, Fatemi et al. analyzed the amount of GABA receptor protein in the cerebellum ($n = 7\text{CT} + 5\text{AU}$), finding that GABA_AR α 1 and GABA_AR β 3 were significantly altered (Fatemi et al., 2009a). They also found a 52% reduction of GABA_AR α 1, a 39% reduction of GABA_AR α 2, a 57% reduction of GABA_AR α 3, and a 38% reduction of GABA_AR β 3 in the parietal cortex ($n = 5\text{CT} + 6\text{AU}$), as well as a 65% reduction of GABA_AR α 1 in the superior frontal cortex (BA9) ($n = 3\text{CT} + 4\text{AU}$) (Fatemi et al., 2009b). In addition, they also found a reduction of mRNA for GABA_AR α 2, GABA_AR α 3, and GABA_AR β 3 in BA9 (Fatemi et al., 2009b). When they measured GABA_AR β 3 levels in the vermis, they found a significant 37% reduction in adults with autism when compared with healthy controls ($n = 11\text{CT} + 16\text{AU}$) (Fatemi et al., 2011). Through qRT-PCR and western blot, Fatemi's group also found a protein reduction for subunits α 6, β 2, δ , ϵ , γ 2, and ρ 2 in BA9 in autism, as well as variable changes in the levels of mRNA for subunits α 2, α 3, α 6, β 2, β 3, γ 2, γ 3, and θ in parietal and prefrontal cortices and in the cerebellum, including the significant decrease in mRNA for α 2 in BA9 (Fatemi et al., 2014). The authors further suggest

that the variable change in mRNA species may be a compensatory response to decreased protein expression. The substantial body of work by the Fatemi group indicates that the cortical areas included in their studies present with receptor subunit changes, mainly reductions, and that more subunits were significantly decreased in the prefrontal cortex than in other brain areas, suggesting a greater involvement of the GABA system in the prefrontal cortex in the pathology of autism. In addition, the Blatt laboratory used single-concentration binding assays to demonstrate a significant reduction in GABA_BR density in the cingulate and fusiform gyri ($n = 19\text{CT} + 15\text{AU}$) (Oblak et al., 2010). They also used multiple-concentration binding assays to find significant decreases in the density of GABA_AR in the supragranular (46.8%) and infragranular (20.2%) layers of the anterior cingulate cortex in the autism group ($n = 10\text{CT} + 7\text{AU}$) (Oblak et al., 2009). In addition, with ligand binding autoradiography they demonstrated a reduction in the number of GABA_AR and benzodiazepine binding sites in the cingulate ($n = 7\text{CT} + 7\text{AU}$) and fusiform ($n = 10\text{CT} + 8\text{AU}$) gyri (Oblak et al., 2011). Overall, the work by the Blatt group implies that the reported decrease in GABA receptor subunits is greater in the supragranular than in the infragranular layers of the cortex.

Decrease in GABA_AR α 2 protein in the pyramidal AIS in supragranular pyramidal cells in the prefrontal cortex in autism

We quantified the levels of GABA_AR α 2 protein in the AIS of Pyr cells – the portion of the axon on which Ch cells form synapses – in the prefrontal cortex in postmortem tissue from subjects with autism. In the AIS of supragranular Pyr cells in BA9 and BA47 of the prefrontal cortex from subjects with autism, we found a decreased percent area of GABA_AR α 2 protein labeling when compared with controls. Our data exhibiting a 61% decrease in BA9 is consistent with that of the Fatemi group, which demonstrated a 60% decrease in mRNA of the α 2 subunit in BA9 from subjects with autism (Fatemi et al., 2014). Our results showing reductions localized to supragranular layers also agrees with data by the Blatt group (Oblak et al., 2009), which demonstrated that the reduction in the density of GABA_AR is more prominent in supragranular than in infragranular layers of the cortex. The decreased levels of GABA_AR α 2 protein we reported may have many implications in the function of the cerebral cortex and the cortical and sub-cortical circuitry in autism. This reduction could be responsible, in part, for the excitation/inhibition imbalance present in the cortex in autism.

Pyr cells in supragranular layers (II–III) mostly make local connections with the supragranular cells of other cortical areas. The use of brain imaging tools such as MRI have generated a body of data demonstrating that, in autism, there is a decrease in axons that communicate over long distances to subcortical areas, and, as shown for prefrontal BA46 (Zikopoulos and Barbas, 2013), an increase in thin axons that communicate over shorter distances with other cortical regions (Egaas et al., 1995; Herbert, 2004; Uddin et al., 2011;

Keown et al., 2013; Wilkinson et al., 2016; Fingher et al., 2017; Kana et al., 2017). It is therefore possible that due to an increased number of thin short-range axons in autism, a decrease in GABA_AR α 2 could be a consequence of negative feedback due to the overabundance of fibers reported in layers II–III in the prefrontal cortex.

Decreased GABA_AR α 2 protein in the AIS may be a compensatory mechanism

Based on our data and previous data which consistently demonstrated a downregulation of GABA synthesis enzymes across many areas of the brain in subjects with autism (Fatemi et al., 2002; Yip et al., 2007, 2009), it is likely that a downregulation of GABA_AR α 2 protein in the Pyr cell AIS is a response to a decrease in the presence of GABA at the AIS. In addition, our previous finding of a decrease in the number of Ch cells in the prefrontal cortex suggests overall fewer synapses formed on each Pyr cell AIS, which can also lead to the postsynaptic decrease in GABA_AR α 2 protein.

It is also possible that decreased GABA_AR α 2 protein in the AIS may be in response to changes in GABA release from remaining Ch cells or changes in connectivity within the cortex. With a decreased number of PV+ Ch cells in the prefrontal cortex of subjects with autism, it is possible that reduced Ch cell availability results in a compensatory increase in GABA secretion or decreased reuptake of GABA from the remaining Ch cells, thus resulting in downregulated expression of GABA_AR α 2 in the target Pyr cell AIS.

Reduced levels of GABA receptors could have genetic causes as well. For example, Ma et al. demonstrated that polymorphisms in the genes for the α 4 and β 1 GABA_A receptor subunits have been associated with autism, potentially through interaction with each other, and GABA_A receptor α 2 has been linked as well (Ma et al., 2005). In addition, genetic abnormalities in synaptic organizers like gephyrin, the postsynaptic scaffolding protein that interacts with GABA_AR α 2, can affect proper receptor localization and contribute to autistic or epileptic symptoms (Lionel et al., 2013; Chen et al., 2014; Dejanovic et al., 2014).

A reduction in GABA_AR α 2 and other GABA receptors suggest that GABA receptor agonists may be potential targets for the treatment of patients with autism. For example, the anticonvulsant and mood stabilizer drug valproate, which increases the effects of GABA in the brain, and acamprosate, which has shown indirect effects on GABA_A receptors, are being examined as possible treatments for autism (Brondino et al., 2016). In addition, baclofen functions as a selective GABA_BR agonist, and in a study of 64 children with varying autism behavioral symptoms, the children given baclofen in conjunction with risperidone over 10 weeks showed significant improvement in many behaviors typical of autism (Mahdaviniasab et al., 2019).

Reduced GABA_AR α 2 protein in the Pyr cell AIS in the supragranular layers of prefrontal cortex areas BA9 and BA47 may be a response to the decreased GABA synthesis in the prefrontal cortex, or to a decreased number of Ch cell synapses in autism. Downregulated

GABA_AR α 2 protein could also be a compensatory response to modified activity among remaining Ch cells, including increased GABA release or decreased reuptake. Overall, reduced levels of GABA_AR α 2 protein in the AIS of Pyr cells may contribute to an excitation/inhibition imbalance in the prefrontal cortex of subjects with autism, and our findings support the potential for GABA receptor agonists as a therapeutic tool for autism.

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AUTHOR CONTRIBUTIONS

Tiffany Hong performed data collection and data analysis and contributed to study design, manuscript writing, and figure preparation. Carmen Falcone contributed to sample preparation, proofreading the manuscript and editing. Brett Dufour contributed to sample preparation and manuscript editing. Sarwat Amina and Rosalia Perez Castro contributed to sample preparation. Jade Regalado and Weston Pearson contributed to data collection and analysis. Stephen C. Noctor edited and proofread the manuscript. Verónica Martínez-Cerdeño designed the study, wrote the manuscript and built figures.

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GLOSSARY

Pyramidal cell: The main excitatory neuron in the mammalian prefrontal cortex.
Chandelier cell: A fast-spiking parvalbumin-positive GABAergic interneuron.
Cartridge: Chandelier cell axonal structure containing synaptic terminal boutons.
Axon initial segment: Proximal segment of the pyramidal cell axon and the site of chandelier cell synapses.