GABAARα2 is Decreased in the Axon Initial Segment of Pyramidal Cells in Specific Areas of the Prefrontal Cortex in Autism

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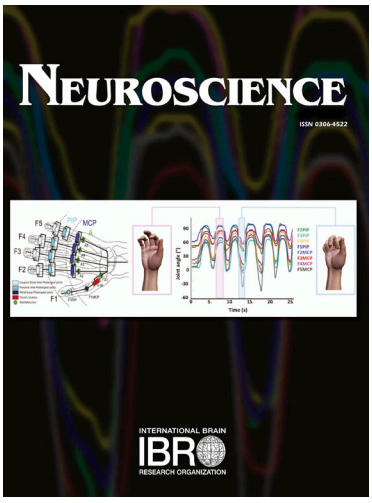

Research Article

$GABA_AR\alpha2$ is decreased in the axon initial segment of pyramidal cells in specific areas of the prefrontal cortex in autism

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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 α2 ($GABA_AR\alpha2$) – the subunit most prevalent in the Pyr cell AIS – revealed a significantly decreased percent area of $GABA_AR\alpha2$ protein labeling in the Pyr cell AIS in supragranular layers of prefrontal cortex areas BA9 and BA47 in autism. Downregulated $GABA_AR\alpha2$ 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.

Keywords: GABA, GABA receptor, chandelier cell, pyramidal cell, autism, axon initial segment

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

Abbreviations: Autism (AU), Control (CT), Brodmann Area (BA), Electroencephalogram (EEG), Parvalbumin (PV), Chandelier (Ch) Cell, Pyramidal (Pyr) Cell, Axon Initial Segment (AIS), gamma-aminobutyric Acid (GABA), $GABA_A$ Receptor α2 Subunit ($GABA_AR\alpha2$), Glutamic Acid Decarboxylase 65kDa and 67kDa (GAD65 and GAD67), Autism Diagnostic Interview-Revised (ADI-R), 5th Edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), Postmortem Interval (PMI), Optimum Cutting Temperature (OCT) Compound

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). To investigate anatomical abnormalities that could contribute to an electrical imbalance in autism neuropathology, we previously 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 (Ariza et al., 2018; Hashemi et al., 2017). Indeed, optogenetic studies in mice have demonstrated not only that PV+ GABAergic 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+ 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 (Jones, 1975; Szentagothai and Arbib, 1974) 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 65kDa and 67kDa (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 α2 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 $GABA_AR\alpha2$ protein in the Pyr cell AIS in prefrontal cortex BA9, BA46 and BA47 (Figure 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=19CT+19AU) and two females (n=1CT+1AU) (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 to 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 0.1M phosphate-buffered saline (PBS) with 0.1% sodium azide. Tissue was embedded in Optimum Cutting Temperature (OCT) compound and frozen at -80ºC. A cryostat (Leica CM 1950) was used to cut 14µm-thick slide-mounted sections, stored at -80ºC 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 (Figure 1) (Hashemi et al., 2017); adjacent sections were used for immunostaining.

IMMUNOSTAINING

Slide mounted tissue sections were enzymatically immunostained with antibodies against both $GABA_AR\alpha2$ and nonphosphorylated neurofilament H (SMI-32). Our 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 diH_20. Antigen retrieval was performed by exposing the tissue to 110ºC for 8 min., and slides were washed (TBS twice followed by TBS + 0.05% tween once). Endogenous peroxidase blocking was performed with 3% H_2O_2. The slides were incubated with 10 drops of BLOXALL (Vector Labs) for 10 minutes in a dark humidified box, washed with TBS, blocked with TBS + 10% NDS + 0.3% triton for 1 hour at room temperature, and then treated with an avidin-biotin blocking kit (Vector Labs). Primary antibody solution (rabbit anti-GABA(A) α2 receptor antibody (Alomone, 1:200) and mouse anti-neurofilament (SMI-32) antibody (Covance, 1:400)) was added to each slide for 24 hours 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 hour, washed, and

incubated with ABC solution (Vector Labs) for 2 hours. 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 hour, then washed and incubated with ABC solution (Vector Labs) for 2 hours in a dark, humidified box. After a wash, slides were developed with VIP substrate (purple; Vector Labs), then washed again. Tissue was dehydrated through sequential immersion in 50%, 70%, 90%, 96%, and 100% EtOH for 3 minutes each, then cleared in Xylene for 6 minutes and coverslipped with Permount mounting medium.

ANTIBODY CHARACTERIZATION

Anti-$GABA_A$ α2 Receptor: *Immunogen*: Peptide (C)TPEPNKKPENKPA, corresponding to amino acid residues 393-405 of rat $GABA_AR$α2 (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 $GABA_AR$α2 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 $GABA_AR$α2 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 $GABA_AR$α2 staining, and the

AIS could be distinguished based on its origin from the axon hillock (Figure 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 (Figure 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 (Figure 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 100x 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 (Figure 3). The AIS was identified as emerging from the pyramidal cell base opposite 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 $GABA_AR\alpha2$ 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 $GABA_AR\alpha2$ protein. Specifically, with the ImageJ Threshold Colour tool, we sampled regions of the AIS containing $GABA_AR\alpha2$ protein from a subset of images, and from that sample created a hue/saturation/brightness threshold by which the $GABA_AR\alpha2$ 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 $GABA_AR\alpha2$ 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 significantly decreased percent area of $GABA_AR\alpha2$ protein labeling in the AIS of Pyr cells in the supragranular layers of BA9 and BA47 (Figure 4); layer III of BA9 displayed a 61% decrease (n=19CT+20AU, $p<.01$), and layer III of BA47 displayed a 54% decrease (n=18CT+18AU, $p<.05$), when compared with controls. We did not find any significant difference in $GABA_AR\alpha2$ 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 $GABA_AR\alpha2$ protein levels in any BA or any cortical layer analyzed ($p>.05$ for all). Overall, we found a decreased percent area of $GABA_AR\alpha2$ 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, 2009b, 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%) (Ariza et al., 2018; Hashemi et al., 2017). 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 α2 subunit labeling presented as punctate staining outlining cell bodies, dendrites, and axons (Figure 3). Similarly, $GABA_AR$α2 has been described on the Pyr cell soma, dendrites, and axon initial segment in rodents (Fritschy and Mohler, 1995; 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, $GABA_AR$α2 expression is widespread prenatally, but by adulthood remains mostly in the supragranular prefrontal cortex 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 $GABA_AR\alpha2$ have been described in macaque and human (Hendry, 1994).

As the Pyr cell AIS is normally enriched with α2 subunits, the decreased amount of $GABA_AR\alpha2$ 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 $GABA_AR\beta3$ 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 $GABA_AR\alpha2$ and $GABA_AR\alpha4$ (Medrihan et al., 2008). Furthermore, in mouse models of the epilepsy-associated Dravet syndrome, PV-positive and 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 $GABA_AR\alpha2$ (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=7CT+5AU), finding that $GABA_AR\alpha1$ and $GABA_AR\beta3$ were significantly altered (Fatemi et al., 2009a). They also found a 52% reduction of $GABA_AR\alpha1$, a 39% reduction of $GABA_AR\alpha2$, a 57% reduction of $GABA_AR\alpha3$, and a 38% reduction of $GABA_AR\beta3$ in the parietal cortex (n=5CT+6AU), as well as a 65% reduction of $GABA_AR\alpha1$ in the superior frontal cortex (BA9) (n=3CT+4AU) (Fatemi et al., 2009b). In addition, they also found a reduction of mRNA for $GABA_AR\alpha2$, $GABA_AR\alpha3$, and $GABA_AR\beta3$ in BA9 (Fatemi et al., 2009b). When they measured $GABA_AR\beta3$ levels in vermis, they found a significant 37% reduction in adults with autism when compared with healthy controls (n=11CT+16AU) (Fatemi et al., 2011). Through qRT-PCR and western blot, Fatemi's group also found a protein reduction for subunits α6, β2, δ, ε, γ2, and ρ2 in BA9 in autism, as well as variable changes in the levels of mRNA for subunits α2, α3, α6, β2, β3, γ2, γ3, and θ in parietal and prefrontal cortices and in the cerebellum, including the significant decrease in

mRNA for α2 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=19CT+15AU) (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=10CT+7AU) (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=7CT+7AU) and fusiform (n=10CT+8AU) 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 $GABA_AR\alpha2$ PROTEIN IN THE PYRAMIDAL AIS IN SUPRAGRANULAR PYRAMIDAL CELLS IN THE PREFRONTAL CORTEX IN AUTISM

We herein quantified the levels of $GABA_AR\alpha2$ 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 $GABA_AR\alpha2$ 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 α2 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 $GABA_AR\alpha2$ protein we reported may have many implications in the function of the cerebral cortex and the cortical and subcortical 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 $GABA_AR\alpha2$ could be a consequence of negative feedback due to the overabundance of fibers reported in layers II-III in the prefrontal cortex.

DECREASED $GABA_AR\alpha2$ PROTEIN IN THE AIS MAY BE A COMPENSATORY MECHANISM

Based on 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 $GABA_AR\alpha2$ 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 $GABA_AR\alpha2$ protein.

It is also possible that decreased $GABA_AR\alpha2$ 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 $GABA_AR\alpha2$ 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 α4 and β1 $GABA_A$ receptor subunits have been associated with autism, potentially through interaction with each other, and $GABA_A$ receptor α2 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 $GABA_AR\alpha2$, 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 $GABA_AR\alpha2$ 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 symptomatic of autism (Mahdavinasab et al., 2019).

CONCLUSION

Reduced $GABA_AR\alpha2$ 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 $GABA_AR\alpha2$ 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 $GABA_AR\alpha2$ 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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Ethical standards: This study complies with ethical standards.

Table 1

Case ID	Diagnosis	Sex	Age (years)	PMI (hours)	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

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.

Figure 1

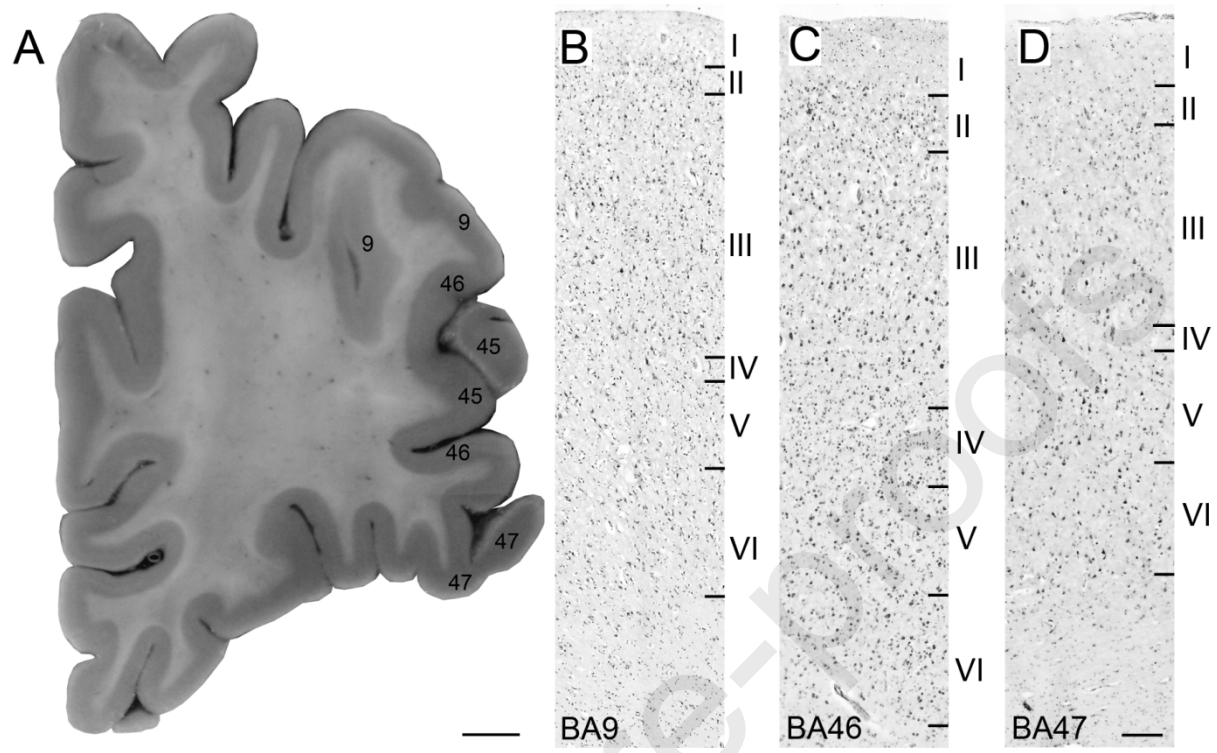

Fig. 1 Cortical areas investigated in this study. Blocks of prefrontal cortical tissue containing BA9, BA46, and BA47 were isolated based on Brodmann and von Economo analysis. A shows a coronal section of cerebral cortex from a left hemisphere, marked with Brodmann areas of interest (BA9, BA46, and BA47). Adjacent area BA45 is marked for reference. B-D show 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: 0.5cm, scale bar in D (B-D): 200μm.

Figure 2

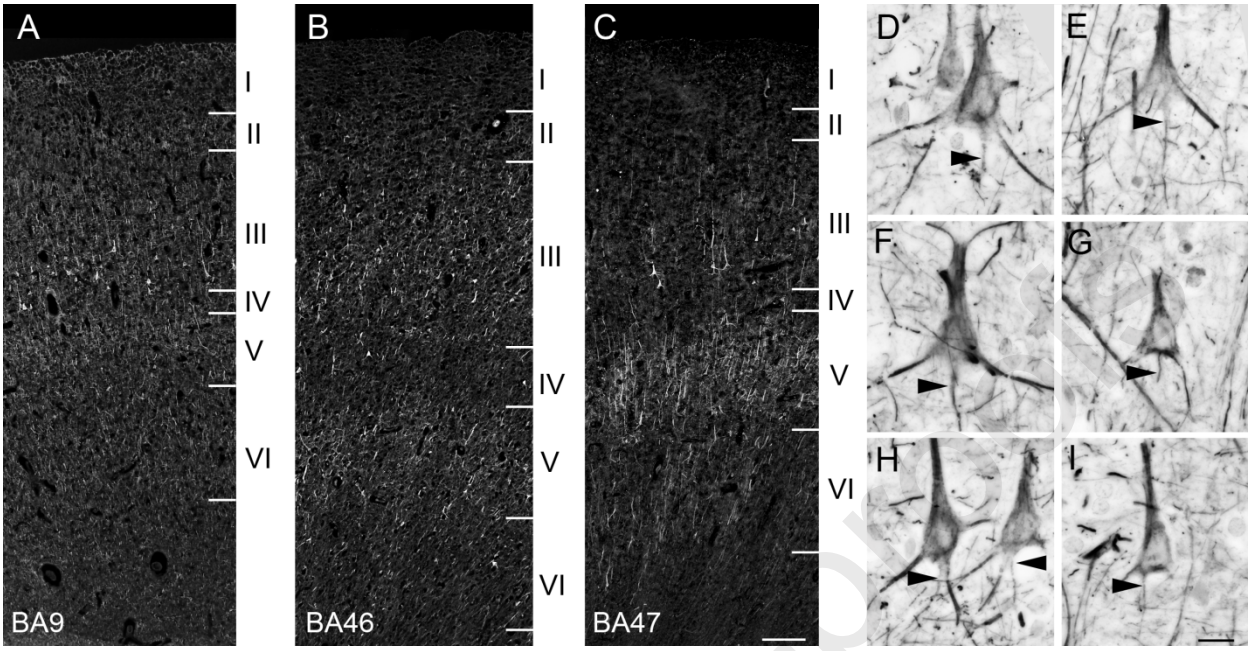

Fig. 2 Prefrontal cerebral cortical sections immunostained with an antibody against neurofilament protein SMI-32, showing pyramidal (Pyr) cell cytoarchitecture including axon initial segment. A-C show SMI-32-labeled sections of (A) area BA9 (B) area BA46, and (C) area BA47. A-C colors have been inverted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

Figure 3

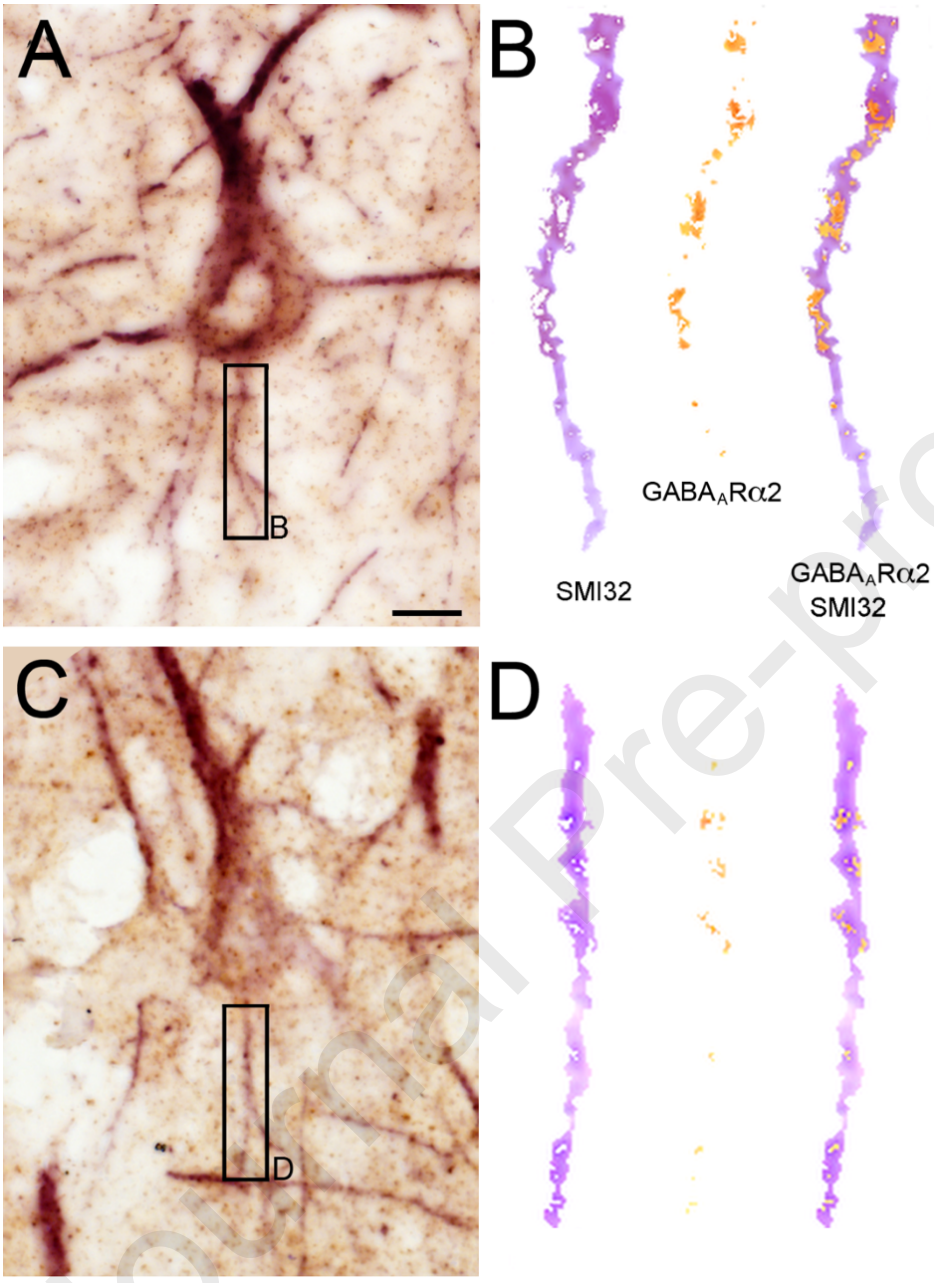

Fig. 3 ImageJ reconstructions of the axon initial segment (AIS) from prefrontal cortex pyramidal (Pyr) cells from layer III of Brodmann Area (BA) 9, double-immunostained with antibodies against $GABA_AR\alpha2$ (brown) and SMI-32 (purple). A and C show high-magnification images of double-immunostained Pyr cells from (A) a control subject and (C) a subject with autism. B and D show ImageJ digital reconstructions of the AIS boxed in (A) and (C), respectively; leftmost = SMI-32 (purple) only, center = $GABA_AR\alpha2$ (brown) only, and rightmost = both colors overlaid. Scale bar in A (A,C) = 10μm.

Figure 4

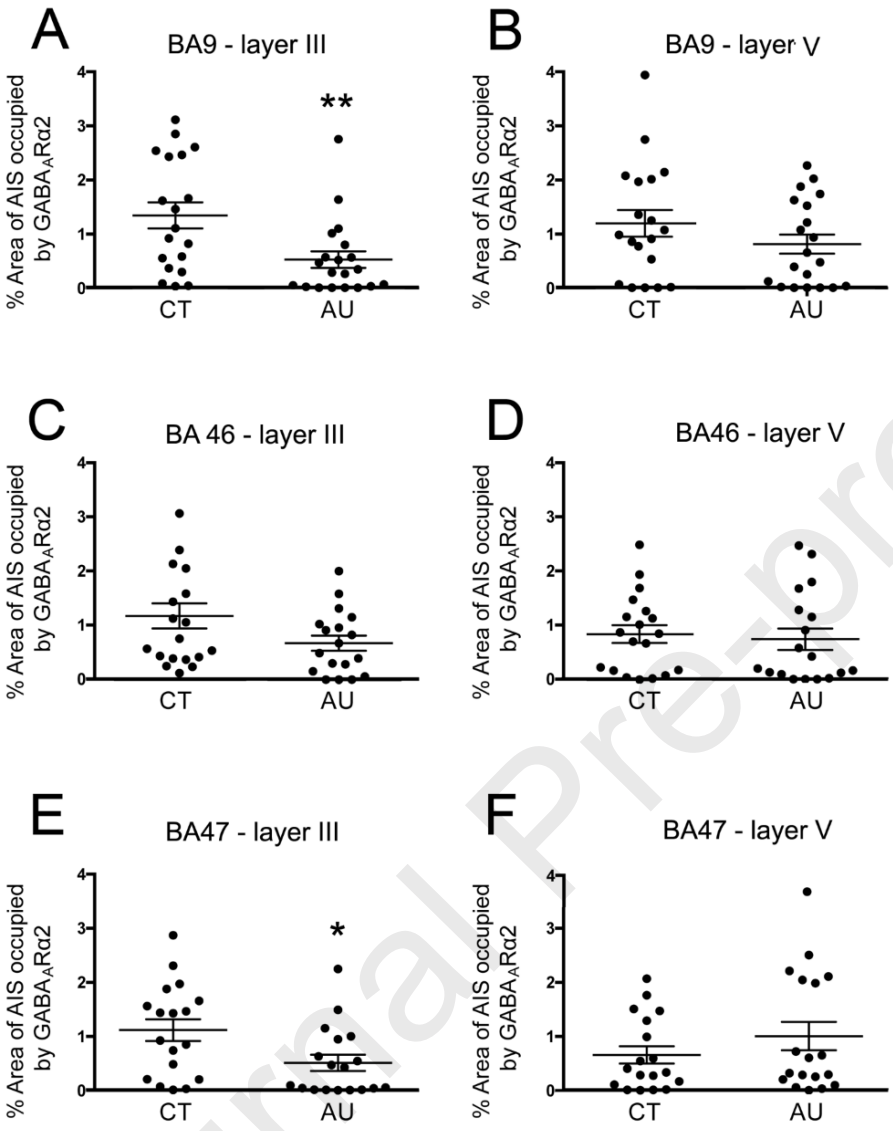

Fig. 4 The percent area of GABA$_A$Rα2 protein labeling in the Pyr cell AIS was significantly lower in supragranular layers of BA9 and BA47 in subjects with autism (AU) when compared with controls (CT). Protein levels were decreased in (A) layer III of BA9 (n=19CT+20AU, p<.01) and (E) layer III of B47 (n=18CT+18AU, 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.

- $GABA_AR\alpha2$ protein is reduced in the axon initial segment of pyramidal cells in the prefrontal cortex in autism
- $GABA_AR\alpha2$ protein reduction in the pyramidal cell AIS is localized to supragranular cortical layers in autism
- Reduced Ch cell availability in autism may lead to increased GABA release or decreased reuptake from remaining Ch cells