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Research Report

Reduced $GABA_A$ receptors and benzodiazepine binding sites in the posterior cingulate cortex and fusiform gyrus in autism

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ABSTRACT

Individuals with autism display deficits in the social domain including the proper recognition of faces and interpretations of facial expressions. There is an extensive network of brain regions involved in face processing including the fusiform gyrus (FFG) and posterior cingulate cortex (PCC). Functional imaging studies have found that controls have increased activity in the PCC and FFG during face recognition tasks, and the FFG has differential responsiveness in autism when viewing faces. Multiple lines of evidence have suggested that the GABAergic system is disrupted in the brains of individuals with autism and it is likely that altered inhibition within the network influences the ability to perceive emotional expressions. On-the-slide ligand binding autoradiography was used to determine if there were alterations in $GABA_A$ and/or benzodiazepine binding sites in the brain in autism. Using ^{3}H-muscimol and ^{3}H-flunitrazepam we could determine whether the number (B_{max}), binding affinity (K_d), and/or distribution of $GABA_A$ receptors and benzodiazepine binding sites (BZD) differed from controls in the FFG and PCC. Significant reductions were found in the number of $GABA_A$ receptors and BZD binding sites in the superficial layers of the PCC and FFG, and in the number of BZD binding sites in the deep layers of the FFG. In addition, the autism group had a higher binding affinity in the superficial layers of the $GABA_A$ study. Taken together, these findings suggest that the disruption in inhibitory control in the cortex may contribute to the core disturbances of socio-emotional behaviors in autism.

1. Introduction

Autism spectrum disorder (ASD) is a developmental syndrome observed before three years of age. Children and adults with ASD are markedly inattentive to faces, show poorer facial identity discrimination and fixate less to the eye region (Sigman et al., 1986; Klin et al., 1999). Although individuals with autism are not incapable of attending to the face, when they do, they may process the information in much the same manner as inanimate objects (Hobson et al., 1988; Schultz et al., 2000).

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Faces are multi-dimensional stimuli conveying complex social and motivational signals simultaneously. Functional brain-imaging studies have delineated an extensive network implicated in face processing in humans, including the fusiform gyrus, and several limbic system regions such as the amygdala, orbitofrontal cortex, and posterior cingulate cortex (Kanwisher et al., 1997; Haxby et al., 2000; Ishai et al., 2000). Regions within this network are associated with functions related to socio-emotional processing. For example, the posterior cingulate cortex (PCC) is active during simple emotions driven by faces (Fink et al., 1996; Shah et al., 2001). Interactions between emotion and face perception may provide insights into more general mechanisms underlying reciprocal links between emotion and cognitive processes and the dysfunction of this network in autism.

The etiology of autism is poorly defined both at the cellular and the molecular level. Despite considerable effort toward the identification of chromosome regions affected in autism, only a few genes have been reproducibly shown to display specific mutations (e.g. Schroer et al., 1998; Nakatani et al., 2009). A number of genetic studies have implicated subunits of the $GABA_A$ receptor, suggesting that GABA changes can influence the developmental trajectory of the brain in autism (Schroer et al., 1998; Fatemi et al., 2009). Glutamic acid decarboxylase 65 and 67 (GAD65, GAD67), key enzymes for GABA synthesis, is decreased in the cortex and altered in the cerebellum (Fatemi et al., 2002, Yip et al., 2007, 2008, 2009). Furthermore, $GABA_A$ receptors and benzodiazepine binding sites are decreased in limbic regions (Blatt et al., 2001; Guptill et al., 2007; Oblak et al., 2009). Additionally, protein levels for $GABA_A$ receptor subunits are reduced in multiple cortical areas (Fatemi et al., 2009). Together, these findings suggest that alterations in GABA system, can potentially affect cortical circuit formation.

The $GABA_A$ receptor contains a combination of five subunits and has binding sites for many modulators including benzodiazepines (BZDs). Benzodiazepine binding sites are allosteric modulatory sites on $GABA_A$ receptors and benzodiazepines have been used as anxiolytics, muscle relaxants, sedatives, and anticonvulsants (Sieghart, 1992). Co-morbid seizure disorder is relatively common in autism and has been estimated to occur in 25–33% of individuals (Olsson et al., 1988; Volkmar and Nelson, 1990). The present study examined two regions within the socio-emotional processing network, the fusiform gyrus and posterior cingulate cortex to determine whether a common deficit in $GABA_A$ receptors and its benzodiazepine binding sites occurs in the autism group relative to controls. A discussion of how results might contribute to the underlying face-processing and social behavior deficits in autism follows.

2. Results

Overall there were significant reductions in the number of $GABA_A$ receptors and benzodiazepine binding sites in both areas in the group of patients with autism when compared to the controls. Furthermore, the K_d in the patients with autism was significantly lower than controls in the superficial layers of $GABA_A$ receptors indicating an increased binding affinity. Individual case information for B_{max} and K_d values for both $GABA_A$ and BZD binding is available in the Supplementary data (Tables 1–3).

2.1. $GABA_A$ receptor binding

2.1.1. Posterior cingulate cortex

Fig. 1 is a pseudocolored image of a control and autism cases demonstrating reduced ^{3}H-muscimol labeled $GABA_A$ receptor binding. Multiple concentration binding experiments revealed that all autistic cases fell below the mean B_{max} value for control cases in the superficial layers of the posterior cingulate cortex. The results from the seven concentration binding studies indicate a significant reduction of $GABA_A$ receptors (B_{max}) in the superficial layers ($p=4.0\times10^{-6}$) of the posterior cingulate cortex in the group of patients with autism (n=7) when compared to age- and post-mortem interval (PMI) matched controls (n=6; Fig. 2). The B_{max} values demonstrate that there was a 49.0% reduction in $GABA_A$ receptors.

2.1.2. Fusiform gyrus

Fig. 1 is a pseudocolored image demonstrating the reduced ^{3}H-muscimol labeled $GABA_A$ receptors in the FFG. Similar to the PCC results, all autism cases fell below the mean of the control group in the superficial layers. The multiple concentration binding experiments in the fusiform gyrus demonstrated significant reductions in individuals with autism (n=8) for the number of $GABA_A$ receptors (B_{max} value) in the superficial layers ($p=5.20\times10^{-6}$) when compared to controls (n=10; Fig. 3). The B_{max} values indicated that there was a 31.2% reduction in the number of $GABA_A$ receptors. Similar to

Table 1 – Posterior cingulate cortex case information. Note: Cases with an asterisk (*) had a history of at least one seizure.

Case	Diagnosis	Age (years)	PMI (hours)	Cause of death	Gender
1401	Autism	21	20.6	Sepsis	Female
1484*	Autism	19	15	Burns	Male
2825*▲	Autism	19	9.5	Heart attack	Male
3845*‡	Autism	30	28.4	Cancer	Male
3924	Autism	16	9	Seizure	Female
4099	Autism	19	3	Conj. heart failure	Male
5754	Autism	20	29.98	Unknown	Male
Mean		21.4	17.3		
4103	Control	43	23	Heart attack/ disease	Male
4104	Control	24	5	Gun shot	Male
4267	Control	26	20	Accidental	Male
4268	Control	30	22	Heart attack/ disease	Male
4271	Control	19	21	Epiglotitus	Male
4275	Control	20	16	Accidental	Male
4364	Control	27	27	Motor vehicle accident	Male
Mean		25.9	19.0		

The following symbols indicate medication history:
▲ Klonopin, Mysoline, Phenobarbitol, Thorazine.
‡ Dilantin, Mellaril, Phenobarbital.

Table 2 – Fusiform gyrus case information. Note: Cases with one asterisk (*) had a history of at least one seizure.

Case	Diagnosis	Age (years)	PMI (hours)	Cause of death	Gender
1664	Autism	20	15	Unknown	Male
4899	Autism	14	9	Drowning	Male
5027	Autism	37	26	Bowel obstruction	Male
5000	Autism	27	8.3	Drowning	Male
5144	Autism	20	23.7	Auto trauma	Male
5173*◊	Autism	30	20.3	GI bleeding	Male
6337*	Autism	22	25	Choked	Male
6756*	Autism	16	22	Myocardial infarction	Male
6677*	Autism	30	16	Congestive heart failure	Male
Mean		24.0	18.4		
602	Control	27	15	Accident	Male
1026	Control	28	6	Congenital heart disease	Male
1365	Control	28	17	Multiple injuries	Male
4605	Control	29	18.3	Renal failure	Male
4642	Control	28	13	Cardiac arrythmia	Male
4916	Control	19	5	Drowning	Male
5873	Control	28	23.3	Unknown	Male
6004	Control	36	18	Unknown	Female
6207	Control	16	26.2	Heart attack	Male
6221	Control	22	24.2	Unknown	Male
Mean		26.1	16.6		

The following symbol indicates medication history:
◊ Cisapride, Clorazepate, Depakote, Dilantin, Mysoline, Phenobarbital.

the PCC results, the number of $GABA_A$ receptors did not differ from the controls in the deep layers. Age and post-mortem interval were similar in the cases in both groups.

2.2. *Benzodiazepine binding sites*

2.2.1. *Posterior cingulate cortex*

The results from the seven concentration binding studies indicated a significant decrease in the B_{max} values in the superficial layers ($p=4.00\times10^{-6}$) of the posterior cingulate cortex in autism (Fig. 3). The multiple concentration binding study revealed a 35.0% reduction in benzodiazepine binding sites in the superficial layers in individuals with autism, but equivalent values for the deep layers. Fig. 2 is a pseudocolored image that demonstrates the reduced BZD binding density. Again, all patients with autism fell below the mean of the control cases. There was no significant difference observed in binding affinity (Fig. 3).

2.2.2. *Fusiform gyrus*

The multiple concentration binding assays demonstrated significant reductions in the number of BZD binding sites in the fusiform gyrus in patients with autism ($p=0.0052$; $n=8$) when compared to controls ($n=10$; Figs. 2 and 4), and values for all individuals with autism were below the mean of the control group in the superficial layers only. The reduction in the number of binding sites was calculated to be 27.2% in autism. In contrast to the results in the PCC, there was a significant 21.5% reduction in the number of BZD binding sites in the deep layers of the FFG in the individuals with autism ($p=0.017$). No significant difference in binding affinity was found in this study (Fig. 4).

2.3. *Effect of seizure on $GABA_A$ receptor and BZD binding site number*

In the posterior cingulate cortex, four of the cases had a reported history of at least one seizure. A non-parametric Mann–Whitney U test was completed to determine if the history of seizure in the autism group had any effect on the number of $GABA_A$ receptors or BZD binding sites. No significant difference between the patients with autism with a history of seizure and the patients with autism with no history of seizure was found in either the $GABA_A$ or BZD study. In the fusiform gyrus study, four of the cases had a reported history of seizure. Again, using a Mann–Whitney U test, there was no difference between the autism cases with a history of seizure and the autism cases with no history of seizure in either of the $GABA_A$ or BZD studies.

There was a significant difference in the binding affinity in the superficial layers of both the PCC and FFG in the ^{3}H-muscimol study. The individuals with autism demonstrated a significantly lower K_d, similar to that found in the posterior cingulate cortex and fusiform gyrus (Figs. 3 and 4). There was no difference in binding affinity between the autism group with a history of seizure when compared to the autism group with no seizure.

3. Discussion

Each region within the extensive network of face and emotional processing may underlie separate and functionally specific processes (Adolphs et al., 2003). Autism may be characterized by multiple disruptions to individual processes or deficits could arise from a single impairment with cascading deleterious effects on the downstream processing of specific social cues. The downstream effects could occur directly, via disruptions in typical patterns of information flow or could occur indirectly, via disrupting the normal

Table 3 – Multiple concentration binding conditions for $GABA_A$ receptors and benzodiazepine binding sites in the posterior cingulate cortex and fusiform gyrus.

Ligand	Target	Concentration (nM)	Displacer	Exposure time
[^{3}H]-muscimol	$GABA_A$ receptor	[0.14, 1.84, 2.77, 5.26, 13.6, 17.5, 149]	100 mM GABA	6–390 days
[^{3}H]-flunitrazepam	Benzodiazepine binding site	[0.16, 0.95, 2.90, 4.11, 16.7, 37.0, 123]	100 mM Clonazepam	10–230 days

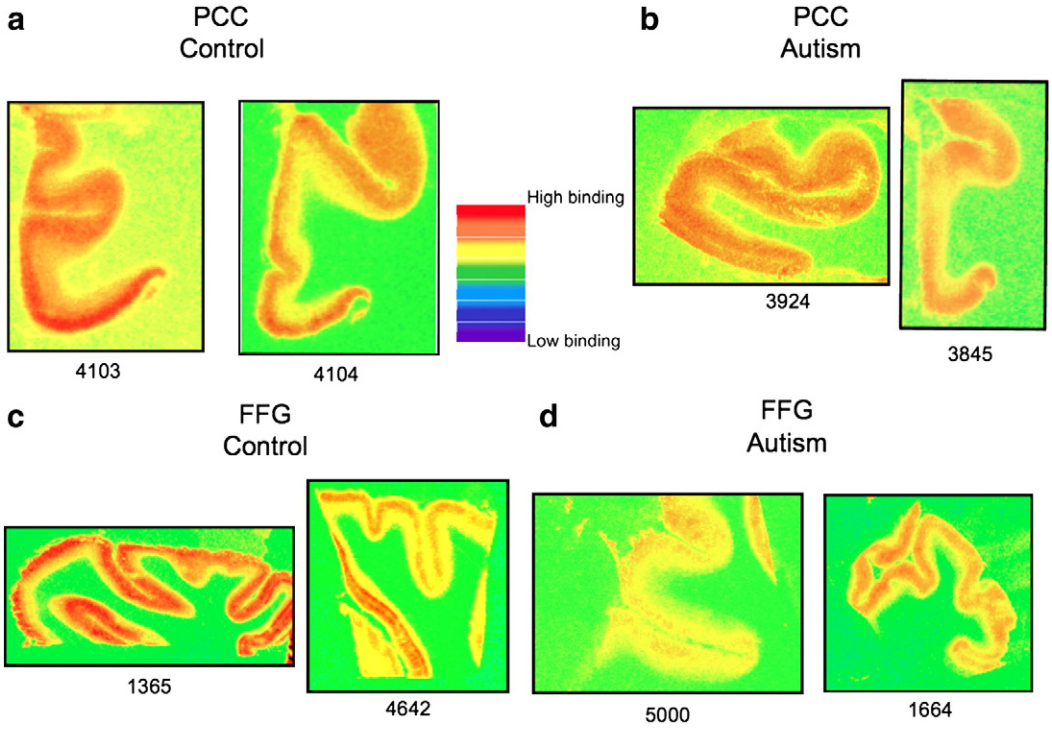

Fig. 1 – ^{3}H-muscimol labeled GABA$_A$ receptor binding density in the PCC (a,b) and FFG (c,d) from two patients with autism and two controls. Case numbers are under each image. Images are taken from ^{3}H sensitive film (concentration 15 nM). Note the reduced binding density in the patients with autism (b,d) compared to the control (a,c) cases. Also note the variability in binding that occurs within groups.

accumulation of socially relevant experiences (Schultz, 2005). In this study, we investigated the GABA$_A$ receptor system in the posterior cingulate cortex and fusiform gyrus and discuss how altered inhibitory modulation within the network might affect the ability to properly process socio-emotional behaviors.

3.1. *GABA in development and dysfunction in autism*

Most theories regarding amino acid neurotransmitters in autism suggest that the GABAergic system is suppressed, resulting in excessive stimulation of the glutamate system (e.g. Rubenstein and Merzenich, 2003; Hussman, 2001). This can be attributed to several findings. First, researchers found abnormalities in cellular development in the limbic system and cerebellum postmortem (Kemper and Bauman, 1993; Raymond et al., 1996). Secondly, glutamate activity peaks during the second year of life (Kornhuber et al., 1989), a time when symptoms of autism emerge. A hyperfunctional system could result in increased release of GABA from interneurons, resulting in a compensatory downregulation of receptors.

GABA$_A$ receptor activation can influence DNA synthesis in proliferative cells (LoTurco et al., 1995), cell motility and morphological development in early postmitotic neurons (Barbin et al., 1993; Behar et al., 1996; Marty et al. 1996). There are a number of studies that report that the development of the cerebral cortex is abnormal in autism. Bailey et al. (1998) found irregular laminar patterning in the frontal cortex, with abnormally oriented pyramidal cells, increased white matter neurons, and increased neurons in layer I. Irregular laminar patterning has been found in the anterior cingulate cortex in autism (Simms et al., 2009). The frontal cortex and anterior cingulate cortex has previously been found to exhibit decreased protein levels for GABA$_A$ receptor subunits (Fatemi et al., 2009) in autism as well as reduced GABA$_A$ receptors and BZD binding sites (Oblak et al., 2009).

3.2. *Face-processing abnormalities in autism: the role of the FFG and PCC*

There is converging evidence in non-human primates and humans that eyes are viewed more frequently than any other region of the face when viewed in an upright manner (Hirata et al., 2010; Farroni et al., 2006). However, little is known about the specific visual properties of faces that activate the FFG, what neural interactions within the FFG underlie face processing, and how accumulated experience and other brain regions may shape the development and dysfunction of the FFG in autism. An important component of face recognition is emotion processing. The recognition of facial expressions of emotion is thought to develop slowly during the first two years of life, a time frame when the symptoms of autism become apparent, and continue to mature into adolescence.

The posterior cingulate cortex appears preferentially involved in visuospatial cognition (Olson and Musil, 1992; Olson et al., 1996), and is part of a network recruited when

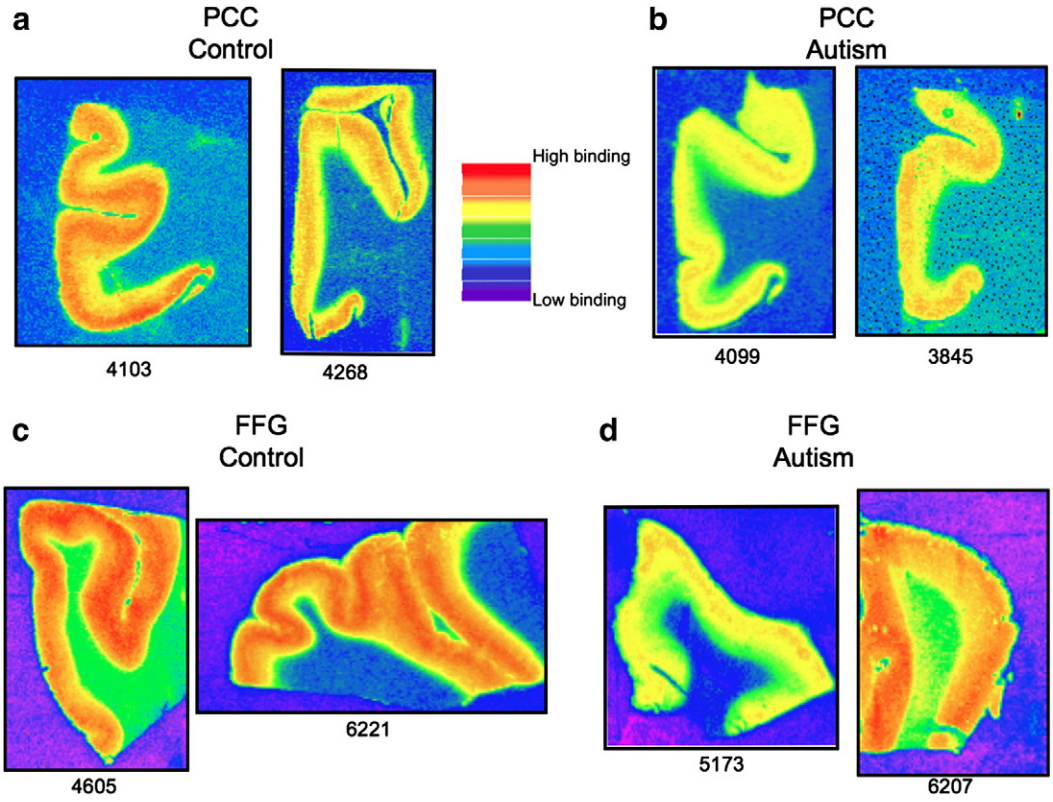

Fig. 2 – ^{3}H-flunitrazepam labeled benzodiazepine binding site density in the PCC (a,b) and FFG (c, d) from two patients with autism and two control cases. Case numbers are below each image. Images are taken from ^{3}H sensitive film (concentration 5 nM). There is reduced binding density in individuals with autism (b,d) when compared to the control (a,c) cases in both the PCC and FFG. Note the variability in binding between cases within a group.

typically developing subjects see the faces or hear the voices of emotionally significant people in their lives (Maddock et al., 2001) and modulates emotion by responding to emotional scripts and faces (Mayberg et al., 1999). Individuals with autism are known to have difficulties in the perception of faces and direction of eye gaze, lack of eye contact and impairments in face recognition abilities, failing to use eye gaze and facial expression to regulate social interaction (Braverman et al., 1989; Davies et al., 1994; Joseph and Tanaka, 2003).

Early studies of adults with ASD compared to controls found evidence for reduced FFG activation and greater object–area activation by faces, even though measures of attention to the faces were similar (Schultz et al., 2000; Pierce et al., 2001; Hubl et al., 2003). The apparent failure of individuals with ASD to develop normal cortical face specialization in the fusiform gyrus and "expertise" in face recognition may be the accumulated effect of reduced social interest and lack of motivation to view faces (Schultz et al., 2000; Pierce et al., 2001; Hubl et al., 2003). However, other studies found FFG activation by faces that was modulated by personal familiarity in ASD (Pierce et al., 2004), or FFG activation that was indistinguishable from controls. Using eye-tracking, a recent study suggested that this disparity of findings might be explained by differences in fixation behavior (Dalton et al., 2005).

The current study found significant reductions in the number of $GABA_A$ receptors and BZD binding sites in the superficial layers of the PCC, the layers that actively modulate information that arrives from other cortical and/or thalamic regions within the circuit. It is likely that synaptic information entering the superficial layers of the PCC is being modulated in a different manner than controls. This information is then passed on to the deep layers and sent to higher order cortical regions to be processed. The modulation in the PCC at this level seems to be intact as there were no differences in $GABA_A$ receptor or BZD binding site number. Significant reductions in the number of $GABA_A$ receptors and BZD binding sites in the superficial layers and a reduction in BZD binding sites in the deep layers of the FFG were also demonstrated. This suggests that, similar to the PCC findings, information about emotion from faces and eye gaze, entering the FFG from cortical regions could also be abnormal in autism.

In contrast, the deep layers of the FFG in the autism group have a normal number of $GABA_A$ receptors. Thus, the information is being sent further downstream with the same $GABA_A$ modulation as controls, but the information that is received from the superficial layers is modulated with less inhibitory control. The resultant output in the autistic brain could be substantially different thereby leading to reduced face and emotion processing. Therefore, relevant neurochemical or neuroanatomical events may occur relatively early in

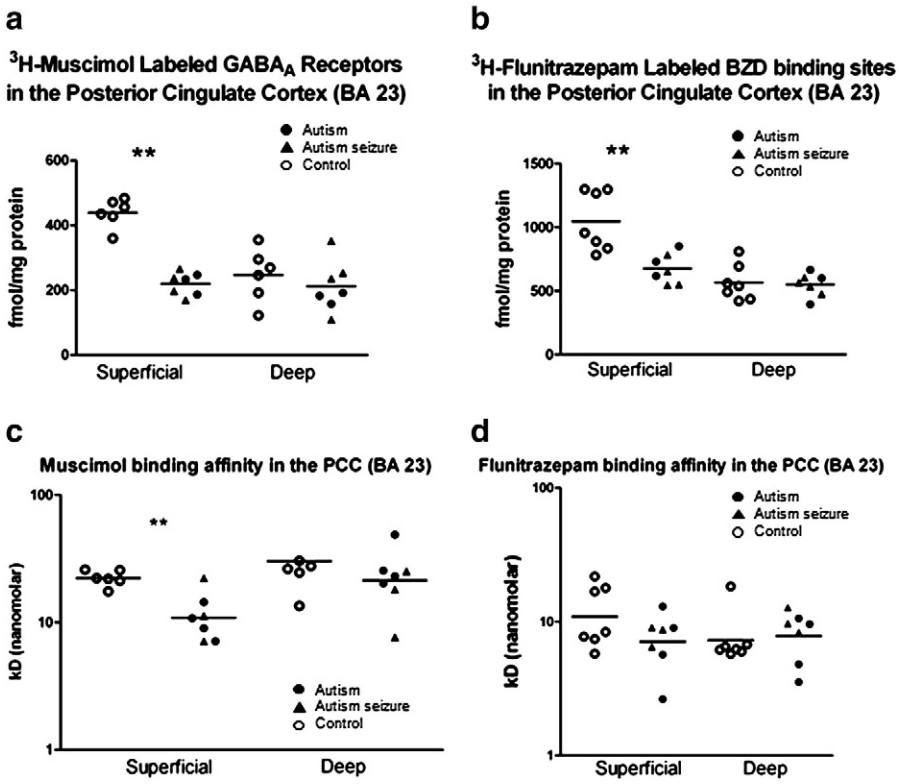

Fig. 3 – Scatter plots demonstrating the density of GABA$_A$ receptors (a) and benzodiazepine binding sites (b) in the posterior cingulate cortex. Each symbol represent an individual case. Significant reductions were found in the superficial layers of both GABA$_A$ receptors (p = 4.00 e-6) and BZD binding sites (p = 0.0044) in autism. No change was found in the deep layers. Binding affinity was calculated for muscimol (c) and flunitrazepam (d). A significant decrease in the K_d (p = 0.0024) was observed in the superficial layers of the autism group in the muscimol study only. Asterisk (**) denotes where significance was found.

the development of the central nervous system (CNS) resulting in deficits in one or more of the cortical regions involved in socio-emotional processing.

3.3. *Effects of seizures and anti-epileptic medication on B_{max} and K_d values of GABA$_A$ and BZD binding*

A significant reduction in the BZD binding site number was found in the superficial layers of the PCC and throughout the FFG in the autism cases paralleled by a change in the binding affinity. Alterations in GABA$_A$ receptor subunit expression are documented in human epilepsy (Loup et al., 2000, 2006) and in animal models of epilepsy (Gilby et al., 2005; Li et al., 2006). The evidence from these studies suggests that a shift in mRNA subunit expression can occur such that the α4 subunit is increased, but α1 is decreased (Brooks-Kayal et al., 2009). If this were the case in autism, it is likely that the number of [^{3}H]-muscimol and [^{3}H]-flunitrazepam binding sites would not decrease in parallel, as alpha-4 containing GABAA receptors bind [^{3}H]muscimol, but do not contain a [^{3}H]flunitrazepam binding site.

In the superficial layers of the PCC and FFG, the number of [^{3}H]-musicmol binding sites was also decreased, arguing that the decrease in [^{3}H]-flunitrazepam binding was due to a loss of receptors rather than subunit switching. However, in the deep layers of the FFG, the number of [^{3}H]-flunitrazepam binding sites was not paralleled by a reduced number of [^{3}H]-muscimol receptor binding. This would argue that it is possible for subunit switching to occur here, in an area prone to seizure, the temporal lobe, however; the binding affinity was not altered in the deep layers. Furthermore, the binding affinity of autism cases with a history of seizure did not differ, indicating that the change in affinity is most likely due to the autism diagnosis. However, this conclusion is tentative due to the small number of cases that had a positive seizure history. These results do not rule out the possibility that changes occur in other regions of the brain that might be more susceptible to epileptiform activity. Therefore, it will be important for future studies to control for seizure and antiepileptics, and increase the number of subjects.

In summary, there are a number of possible explanations for the reduction in GABA$_A$ receptors and BZD binding sites in the brains of individuals with autism. First, the reduction in GABA$_A$ receptors and BZD binding is a consistent deficit in autism, with similar findings in the hippocampus and anterior cingulate cortex (Blatt et al., 2001; Guptill et al., 2007; Oblak et al., 2009) suggesting widespread GABA receptor abnormalities in the autistic cases, irrespective of seizure and medication history. Second, it is possible that a defect in one or more of the GABA$_A$ receptor subunits exists. Fatemi et al. (2009) found reduced proteins in three of the GABA$_A$ receptor subunits in autism in multiple cortical regions and genetic studies found significant association

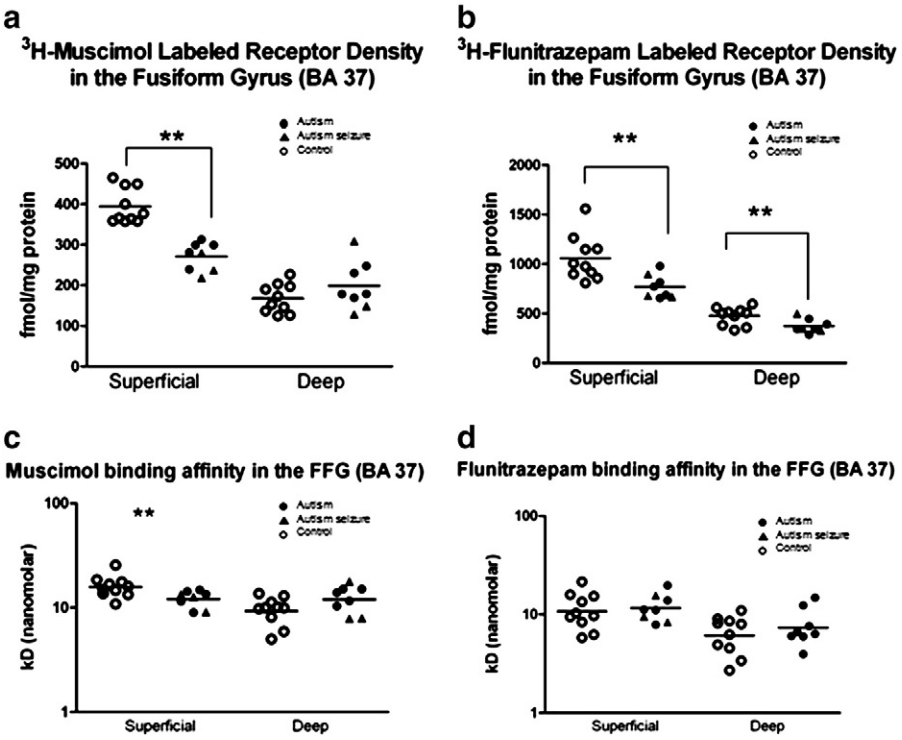

Fig. 4 – Scatter plots demonstrating the density of $GABA_A$ receptors (a) and benzodiazepine binding sites (b) in the fusiform gyrus. Each symbol represents an individual case. Significant reductions were found in the superficial layers of both $GABA_A$ receptors ($p = 5.2$ e-6) and BZD binding sites ($p = 0.0052$) in autism. Further, significant reductions in BZD binding were found in the deep layers (0.017). Binding affinity was calculated for muscimol (c) and flunitrazepam (d). A significant decrease in the K_d was observed in the superficial layers of the autism group ($p = 0.016$) in the muscimol study only. Asterisk (**) denotes where significance was found.

and gene–gene interactions of specific GABA receptor subunit genes in autism (Ma et al., 2005). Molecular and genetic/epigenetic studies are needed to determine whether deficits in subunit composition are in part contributing to the reduced $GABA_A$ receptor binding in autism.

4. Experimental procedures

4.1. *Case data*

The following is a list of cases used in the study (Tables 1 and 2). There were a total of 15 post-mortem cases from patients with autism (7 for the PCC study, 8 for the FFG study) and 17 controls (7 for the PCC study, 10 for the FFG study). The patients with autism ranged in age from 14–37 years and the controls ranged from 16–43 years. The post-mortem interval (PMI) was less than 30 h for all cases. There was no difference between individuals with autism and control group in age or PMI (Student's t-test). Note that seven of fifteen cases (PCC and FFG) from the individuals with autism had a history of at least one seizure (1484, 2825, 3845, 3924, 5173, 6337, 6677). Also, four of the cases (PCC and FFG) from the patients with autism were treated with anticonvulsants (2825, 3845, 5173, 6677). There were seven individuals with autism and six control cases for PCC study; and nine cases from patients with autism and ten control cases from the FFG. All cases used in the study had an autism diagnosis of moderate to severe.

4.2. *Regions of interest*

4.2.1. *Posterior cingulate cortex (PCC; Brodmann Area 23)*

The PCC (BA 23) has a prominent layer IV and a less prominent layer V (Vogt et al., 1995) when compared to the adjacent anterior cingulate cortex. The PCC consists of Areas 23a, 23b, 23d, which reside at the surface of the posterior cingulate gyrus. Area 23c lies in the ventral bank of the caudal cingulate sulcus (Vogt and Pandya, 1987; Vogt et al., 2005). Areas 23a and 23b are divided into ventral and dorsal. These regions are differentiated from each other on the basis of cytoarchitecture and connectivity and are functionally distinct (Goldman-Rakic et al., 1984; Morris et al., 1999; Vogt et al., 2005). It is suggested that both the ventral and dorsal areas of 23a/b contribute to spatial memory (Maguire, 2001). However, ventral Area 23a/b may contribute to verbal and auditory memory (Rudge and Warrington, 1991). For the purposes these studies, only Area 23 is considered, and not the subregions or the dorsal/ventral dichotomy. Blocks from the posterior cingulate were removed at the brain bank, and Nissl stained sections were used to differentiate BA 23 from the surrounding areas (Vogt et al., 2006). In order to reduce heterogeneity within the region, every effort was made to match the levels of the blocks removed from the Brain Bank.

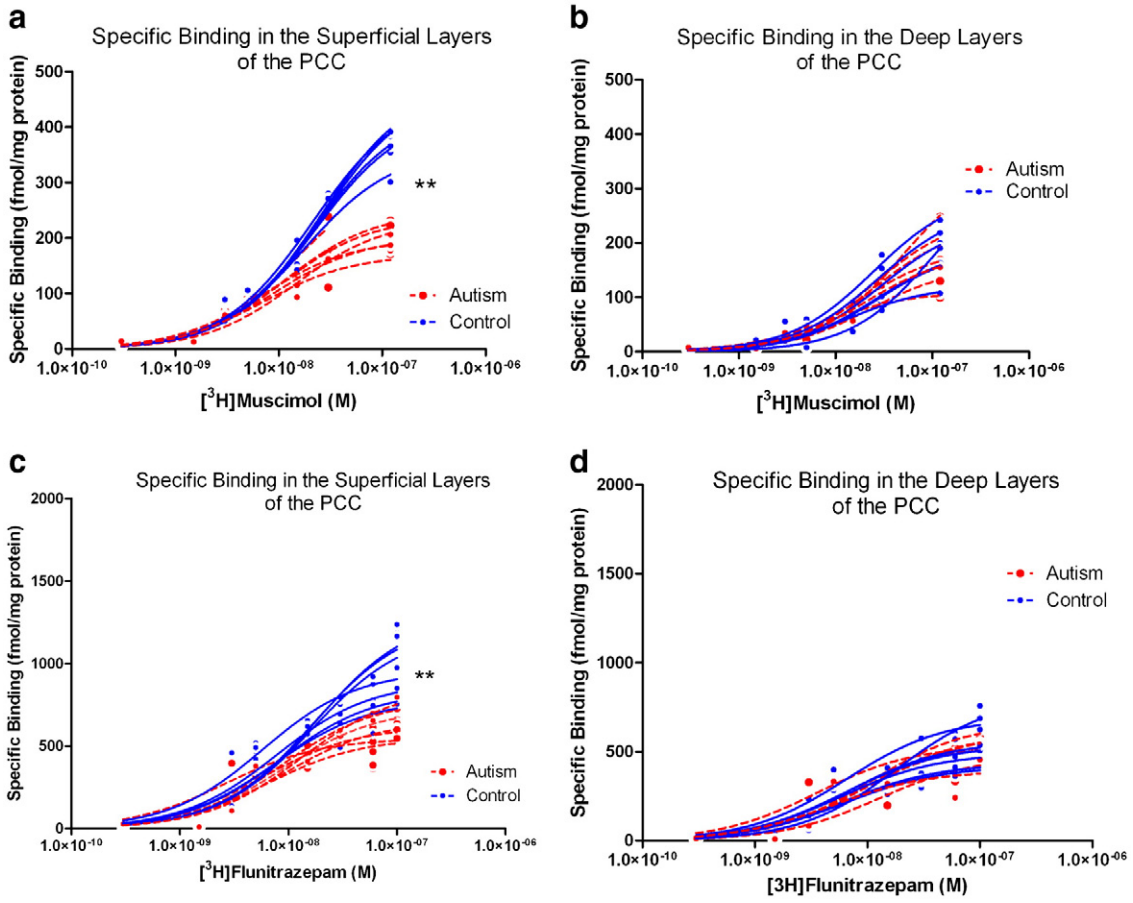

Fig. 5 – Examples of individual binding curves for the ^{3}H-muscimol and ^{3}H-flunitrazepam study is illustrated. Specific binding of ^{3}H-muscimol and ^{3}H-flunitrazepam in the superficial (a,c) and deep (b,d) layers of the PCC is illustrated. Each line represents an individual case. Smooth curves indicate fit to the hyperbolic binding equation. Autism in red, control in blue. Asterisk (**) denotes where significance was found.

4.2.2. *Fusiform gyrus (FFG; Brodmann Area 37)*

The fusiform gyrus (occipitotemporal gyrus) extends the length of the inferior occipitotemporal surface, bound medially by the parahippocampal gyrus and laterally by the occipitotemporal gyrus in humans. BA 37 is a subdivision of the cytoarchitectually defined temporal region of cerebral cortex, located primarily in the caudal portions of the fusiform gyrus and inferior temporal gyrus. The fusiform gyrus was identified on tissue at the brain bank as follows: the medial margin was defined by the collateral and rhinal sulci and the lateral boundary was identified as the sulcus medial to the inferior temporal gyrus (McDonald et al., 2000). Von Economo and Koskinas (1927) has characterized the fusiform gyrus with the following feature: (1) layer I is slightly thicker on average than other cortical regions; (2) layer II is not densely packed, containing mostly granule cells and also some pyramidal cells; (3) layer III is relatively thick and richer in cells; (4) layer IV is compact with large granule cells; (5) layer V is divided into a thin superficial sublayer Va and is composed of densely packed, small, triangular cells, and a deeper sublayer Vb with large cells; (6) layer VI is relatively thin and has a compact layer VIa with large triangular and spindle shaped neurons and a deeper layer VIb which is thinner than other areas but contains spindle cells. Although the FFG has been attributed to several functions including processing of color information, word and number recognition as well as face and body recognition (Allison et al., 1994; Kanwisher et al., 1997), there have not been reports of regions subserving these functions having different cytoarchitectonic patterning. Therefore, the blocks of tissue were taken from similar levels of the FFG, the lateral side of the mid-fusiform gyrus, which has been shown in imaging literature to be responsible for face-processing (Kanwisher et al., 1997) and the entire FFG region was sampled from each block.

4.3. *Multiple-concentration binding assays*

Tissue blocks were coronally sectioned at 20 μm using a Hacker/Brights motorized cryostat at −20 °C. The sections were thaw mounted on 2×3 in. gelatin coated glass slides. Two sections from each case were used to determine total

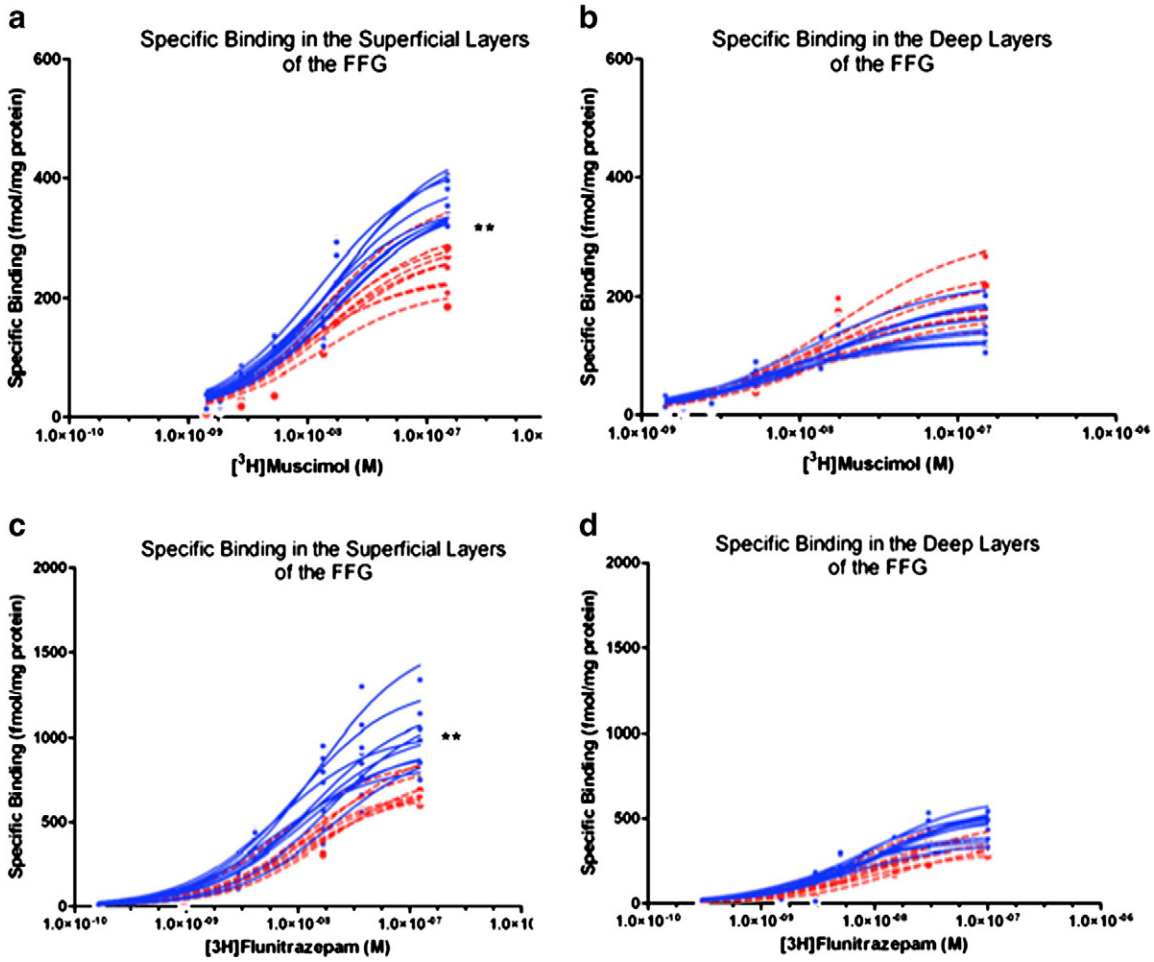

Fig. 6 – Examples of individual binding curves for the ^{3}H-muscimol and ^{3}H-flunitrazepam study. Each line represent an individual case. Specific binding of ^{3}H-muscimol and ^{3}H-flunitrazepam in the superficial (a,c) and deep (b,d) layers of the FFG. Smooth curves indicate fit to the hyperbolic binding equation. Autism in red, control in blue. Asterisk (**) denotes where significance was found.

binding and one section from each case was used to determine non-specific binding for each of seven concentrations. The ligand used for $GABA_A$ receptors was [^{3}H]-muscimol (specific activity 36.6 Curies/millimole; Ci/mmol; New England Nuclear) and the ligand for BZD binding sites was [^{3}H]-flunitrazepam (specific activity 87.2 Ci/mmol; New England Nuclear). Non-specific binding was measured by adding a competitive displacer (see Table 3) to the tritiated ligand and buffer solution at a high concentration. The selected sections from all cases were assayed in parallel in order to eliminate variability in binding conditions. Slides were dried overnight under a cool stream of air, loaded into X-ray cassettes with a tritium standard (Amersham) and apposed to tritium-sensitive film (^{3}H-Hyperfilm, Kodak). Once the exposure time elapsed, the films were developed for 4 min with Kodak D19 developer, fixed with Kodak Rapidfix (3 min) at room temperature, and allowed to air dry. Slides were stained with thionin to determine cytoarchitecture and laminar distribution of the posterior cingulate cortex and fusiform gyrus. Superficial layers corresponded to cortical layers I–IV and deep layers to layers V–VI.

4.4. *Data analysis*

Using an Inquiry densitometry system (Loats Associates), the film autoradiograms were digitized to gather quantitative measurements of optical density. The superficial and deep layers were sampled from each case. The tritiated standards that were exposed with the sections were used to calibrate the autoradiograms to quantify the amount of ligand bound per milligram of tissue in the tissue sections. Optical density for the standards, as a function of specific activity (corrected for radioactive decay), was fitted to the equation optical density $= B1 * (1 - 10^{k1(\text{specific activity})}) + B3$, by nonlinear least squares regression, adjusting the values of the parameters k1, B1, and B3 using the Solver tool of Excel (Microsoft Office XP Professional) to construct a standard curve and to convert the measured optical densities into nCi/mg. Binding in

femtomoles per milligram of tissue (fmol/mg) was calculated based on the specific activity of each ligand.

4.5. *Statistical analyses*

Specific binding of the tritiated ligands in the superficial and deep layers from each case was fitted independently with a hyperbolic binding equation to estimate binding affinity (K_d) and number of receptors (B_{max}) for each region. Examples of binding curves are shown in Figs. 5 and 6. Least-squares fitting was carried out via the Microsoft Excel Solver tool. B_{max} data is reported as mean ± standard error of the mean (SEM). K_d is reported as the geometric average $(K_d)av = 10^x$, where x = mean(log(K_d)), ± SEMav, where SEMav = $((10^{(x+s)} - 10^{(x-s)})/2$, where x = mean(log$K_d$) and s = the SEM of the log (K_d) values. Student's t-tests with unequal variances were performed to determine if there was a significant difference between patients with autism and control cases in the superficial and deep layers of the PCC and FFG. Mann–Whitney U non-parametric tests were carried out to determine if the number of $GABA_A$ receptors and BZD binding sites in cases from patients with autism with a history of seizure differed from those patients with autism with no history of seizure using $p < 0.05$ as criterion. Note that the number of cases that received anticonvulsant therapy did not meet criterion for statistical testing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.brainres.2010.09.021.

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