Autism is a pervasive developmental disorder (PDD) that shares many clinical characteristics with other PDDs such as Asperger syndrome and PDD-not otherwise specified (Volkmar et al. 1996). The shared phenotypes of these disorders suggest some common neurobiological and genetic mechanisms. The core features of the disorder include restricted and repetitive behaviors, delayed language, and abnormal socio-emotional behaviors (APA 1994). Although the etiology of autism is not known, there is growing consensus that the disorder, which ranges from mild to severe, results from a combination of genetic and environmental components (Fombonne 1999). An important consideration when thinking about the neurobiology of the disorder is whether the multitude of symptoms is the result of a number of developmental ‘insults’ to multiple regions of the brain, or if one ‘insult’ results in a multitude of symptoms.

Neuropathology has been reported in the cerebellum, limbic system, and fusiform gyrus. Post-mortem neuropathological studies have found reduced numbers of Purkinje cells (Bauman and Kemper 1985; Ritvo et al. 1986; Bailey et al. 1998; Fatemi et al. 2002; Whitney et al. 2008), abnormal levels of glutamic acid decarboxylase 65 and 67 (Fatemi et al. 2002) and glutamic acid decarboxylase 65/67 mRNA levels (Yip et al. 2007, 2008, 2009), GABA receptors in the cerebellum (Fatemi et al. 2002), decreased neuron size and increased cell-packing density (Bauman and Kemper 1985) in the hippocampus and anterior cingulate cortex (Simms et al. 2009), increased relative density of GABAergic interneurons in the hippocampus (Lawrence et al. 2010), and a reduced number of neurons in the lateral amygdala (Schumann and Amaral 2006) and fusiform gyrus (van Kooten et al. 2008). Bailey et al. (1998) and Simms et al. (2009) have also reported atypical laminar patterns in the frontal cortex and anterior cingulate gyrus, respectively. The abnormal cytoarchitecture observed in the autistic brain is indicative of an early developmental insult within these brain regions.

Functionally, the ACC has been associated with the pain system (Craig et al. 1996), regulation of attention (Botvinick et al. 2004), and face processing, and the fusiform gyrus, important for identification of faces and facial expressions. Significant reductions in GABA<sub>B</sub> receptor density were demonstrated in all three regions examined suggesting that alterations in this key inhibitory receptor subtype may contribute to the functional deficits in individuals with autism. Interestingly, the presence of seizure in a subset of autism cases did not have a significant effect on the density of GABA<sub>B</sub> receptors in any of the three regions.
et al. 1999), emotion (Davidson et al. 1999), vocalization (Jurgens and Ploog 1970), cognition (MacDonald et al. 2000), and reward expectancy (Shidara and Richmond 2002). 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 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, direction of eye gaze, lack of eye contact and impaired 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).

Functional brain imaging studies have described an extensive neural network implicated in face processing in humans. This network includes the fusiform gyrus, the superior temporal sulcus, anterior temporal pole, amygdala, orbitofrontal cortex, retrosplenial cortex, and the anterior and posterior cingulate cortices (Kanwisher et al. 1997; Shah et al. 2001). Several neuroimaging studies have found that individuals with autism display hypoactivation of the fusiform gyrus when compared with controls during a face recognition task (Critchley et al. 2000; Schultz et al. 2000; Pierce et al. 2001) but there are also reports of normal activation of the fusiform gyrus during face processing tasks in autism (Hadjikhanli et al. 2004; Pierce et al. 2004; Dalton et al. 2005).

Schultz et al. (2000) hypothesized that pathology in the fusiform gyrus may account for the hypoactivation during face processing. However, more recently Schultz’s group and others have suggested the hypoactivation of the FFG is a consequence of abnormalities of regions within the face-processing circuit (Grelotti et al. 2002, 2005). Therefore, it is important to determine if neuropathology exists in the FFG and/or if areas conveying information about emotional salience (ACC, PCC) may contribute to the deficits observed in face-processing in autism. Evidence is mounting that the GABAergic system is affected in multiple brain regions in adults with autism (Blatt et al. 2001; Fatemi et al. 2002, 2009a, b; Guptill et al. 2007; Yip et al. 2007, 2008, 2009; Oblak et al. 2009a, b; Lawrence et al. 2010).

GABA is the main inhibitory neurotransmitter in the brain and is important for proper cortical and synapse formation during development. GABAA and GABAC receptors are ligand-gated ion channels and GABAB receptors are metabotropic. Activation of pre-synaptic GABAB receptors, inhibits the release of neurotransmitters and neuropeptides via inhibition of Ca2+ channels. Post-synaptic GABAB receptors activate inwardly rectifying potassium channels and induce the slow, long-lasting component of inhibitory post-synaptic potentials, the fast component of which is mediated through GABAA receptors.

GABA dysregulation has been suggested to play a key role in the increased rate of seizures in autism and others have suggested an imbalance of GABA and glutamate in autism (Rubenstein and Merzenich 2003; Hussman 2001). All reports of decreased GABA receptors in autism have targeted GABAA receptors and benzodiazepine-binding sites (Blatt et al. 2001; Guptill et al. 2007; Oblak et al. 2009a, b). Fatemi et al. (2009a,b) has provided further molecular evidence by showing decreased protein levels of both GABAB1 and GABAB2 subunits in the cerebellum and cortex of individuals with autism. These results raise the question as to whether there are consistent and common alterations in the GABA system throughout affected areas in autism. The current study utilized on-the-slide ligand-binding autoradiographic techniques to examine and quantify the density of the GABAB receptors in three areas involved in socio-emotional and face-processing behaviors, the anterior and posterior cingulate cortex and fusiform gyrus.

Materials and methods

Brain tissue

Fresh frozen brain tissue from the anterior and posterior cingulate cortices and fusiform gyrus was obtained from The Autism Research Foundation, the Autism Tissue Program, Harvard Brain Tissue Resource Center, and the NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, MD. A total of thirty-five blocks were obtained (15 autistic and 19 controls) and stored at ~80°C. A summary of the case details is seen in Table 1. There was no significant difference in age or post-mortem interval between autism and control groups (Student’s t-test).

Case data

The following is a list of cases used in the study (Table 1). Note that seven of 16 cases from the autism group had a history of at least one seizure (1078, 1484, 2825, 3845, 5173, 6337, 6677). As indicated in Table 1, based on availability of tissue blocks, there were seven autistic cases and nine control cases in the ACC study; six autistic and nine control cases for PCC study; and nine autistic and 10 control cases from the FFG. The cases used in the study are designated in the far right column of Table 1 with an ‘X’.

Single-concentration binding assay

Tissue blocks were sectioned coronally at 20 μm using a Hacker/Brights motorized cryostat at ~20°C. Sections were then washed on 2 × 3 inch gelatin coated glass slides. ‘Total binding’ was measured using two sections per case and non-specific binding was determined using one section from each case. The tritiated antagonist ([3H]CGP54626 (1.5 nM; specific activity 50.0 Ci/mmol; Perkin Elmer, Boston, MA, USA; Scheperjans et al. 2005) was used to label GABAB receptors. This antagonist selectively binds to the GABAB receptor subunits 1a and 1b.
Non-specific binding was measured by adding a high concentration of a competitive displacer (CGP55845; 100 μM; Scheperjans et al. 2005) to the tritiated ligand and buffer solution (50 mM Tris–HCl, 2.5 mM CaCl₂, pH 7.2). The following steps were completed at 4°C: pre-incubation with buffer (15 min), incubation with ligand (plus blocker for non-specific binding; 1 h), three washes in buffer (5 min each), and a dip in double-deionized water. Variability in binding conditions was minimized by running all cases in parallel. Slides were dried overnight and loaded into X-ray cassettes with a set of 3H polymer autoradiographic brain tissue standards (Autoradiographic 3H Microscales; GE Healthcare, Piscataway, NJ, USA) and opposed to tritium-sensitive film (3H-Hyperfilm; Kodak, Rochester, NY, USA) for 10 weeks. The exposed films were processed as follows at 23°C: developed with Kodak D19 (4 min), fixed with Kodak Rapidfix (3 min), rinsed in water (10 min) and air dried. Slides were stained with thionin to determine cytoarchitecture and laminar distribution of the ACC, PCC, and FFG. In the ACC, superficial layers corresponded to layers I–III, and deep layers to cortical layers V–VI (Vogt et al. 1995). In the PCC and FFG, superficial layers correspond to layers I–IV and deep layers correspond to layers V–VI.

<table>
<thead>
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<th>Case</th>
<th>Diagnosis</th>
<th>Age</th>
<th>PMI (h)</th>
<th>Cause of death</th>
<th>Gender</th>
<th>ACC</th>
<th>PCC</th>
<th>FFG</th>
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</table>

*aCases had a history of at least one seizure.
The following symbols indicate medication history:
bDilantin, Tegretol, Theodur, Phenobarbital.
cKlonopin, Mysoline, Phenobarbital, Thorazine.
dDilantin, Mellaril, Phenobarbital.
eCisapride, Clorazepate, Depakote, Dilantin, Mysoline, Phenobarbital.

(Kaupmann et al. 1998a, b). Non-specific binding was measured by adding a high concentration of a competitive displacer (CGP55845; 100 μM; Scheperjans et al. 2005) to the tritiated ligand and buffer solution (50 mM Tris–HCl, 2.5 mM CaCl₂, pH 7.2). The following steps were completed at 4°C: pre-incubation with buffer (15 min), incubation with ligand (plus blocker for non-specific binding; 1 h), three washes in buffer (5 min each), and a dip in double-deionized water. Variability in binding conditions was minimized by running all cases in parallel. Slides were dried overnight and loaded into X-ray cassettes with a set of 3H polymer autoradiographic brain tissue standards (Autoradiographic 3H Microscales; GE Healthcare, Piscataway, NJ, USA) and opposed to tritium-sensitive film (3H-Hyperfilm; Kodak, Rochester, NY, USA) for 10 weeks. The exposed films were processed as follows at 23°C: developed with Kodak D19 (4 min), fixed with Kodak Rapidfix (3 min), rinsed in water (10 min) and air dried. Slides were stained with thionin to determine cytoarchitecture and laminar distribution of the ACC, PCC, and FFG. In the ACC, superficial layers corresponded to layers I–III, and deep layers to cortical layers V–VI (Vogt et al. 1995). In the PCC and FFG, superficial layers correspond to layers I–IV and deep layers correspond to layers V–VI.
Data analysis
The film autoradiograms were digitized using an Inquiry densitometry system (Loats Associates, Westminster, MD, USA) to gather quantitative measurements of optical density. Samples were obtained from the superficial and deep layers of the anterior cingulate cortex, posterior cingulate cortex, and fusiform gyrus. For interpolation of measured optical densities, a standard curve was constructed by fitting the optical densities for the $^3$H tissue standards to the single-hit sensistometric equation (Zhu et al. 2003): optical density = $B_1 \times (1 - 10^{fl \cdot \text{specific activity}})$ + $B_3$, to generate a standard curve relating optical density to nCi per estimated tissue equivalent wet weight (Calon et al. 2001). Optimization of the adjustable parameters $k_1$, $B_1$, and $B_3$ by non-linear least squares regression using the Solver tool of Microsoft Excel (Microsoft Office 2007, Redmond, WA, USA) yielded an excellent fit to the measured optical densities of the standards. The standard curve was then used to interpolate the measured optical densities for the tissue sections into nCi/mg. Binding in femtomoles per milligram (fmol/mg) was calculated based on the specific activity of the ligand.

Statistical analyses
Student’s $t$-tests with unequal variances were performed to determine if there were significant differences in the binding density between autistic and control cases in the superficial and deep layers of the ACC, PCC, and FFG. Mann–Whitney $U$ non-parametric tests were also performed to determine if there were differences between the autism subgroup with a history of seizure and the autism subgroup with no seizure history. Although the number of cases with a history of seizure is small in each region (ranging from 3 to 4), this meets the minimal requirements for a non-parametric test of statistical significance. Mann–Whitney $U$ tests were also performed on the autism subgroups receiving anticonvulsant therapy and those not receiving anticonvulsants in the ACC study because the number of cases ($n = 3$) met the criteria to perform this statistical test; however, the PCC and FFG did not meet the requirements and therefore statistical tests were not performed.

Results
Overall, significant decreases were found in GABA$_B$ receptor-binding density in autistic cases throughout the cingulate cortex and fusiform gyrus. Specific-binding densities can be found in the supporting information (Tables S1 and S2). Figure 1(a) and (b) are pseudocolored-digitized images from hyperfilm to demonstrate the division of the anterior cingulate cortex into superficial and deep layers.

GABA$_B$ receptor binding in the anterior cingulate cortex
In Fig. 1(a–c), in the anterior cingulate cortex, there were significant decreases in the binding density of GABA$_B$ receptors in the superficial layers of the autistic cases when compared to controls ($p = 0.018, \downarrow 36.0\%$). In contrast, there was no change in GABA$_B$ receptor density in the deep layers. Of the seven autistic cases, four had a history of seizure. There was also no difference between autistic seizure and autistic non-seizure subgroups or autistic antiepileptic therapy and autistic non-therapy, using a Mann–Whitney $U$ non-parametric test ($p > 0.05$).

GABA$_B$ receptor binding in the posterior cingulate cortex
In Fig. 2(a–c), GABA$_B$ receptor-binding studies in the posterior cingulate cortex showed a significant decrease in $^3$H-CGP54626 binding in the superficial layers ($p = 0.0076; \downarrow 36.3\%$), but unlike the ACC, there was also a significant difference in GABA$_B$ receptor-binding density in the deep layers of the PCC ($p = 0.050; \downarrow 22.8\%$). Three of the six autistic cases had a history of seizure and when these cases were compared to the autistic cases without seizure (Mann–Whitney $U$-test), there was no significant difference between the groups in either the superficial or deep layers of the PCC.

GABA$_B$ receptor-binding in the fusiform gyrus
Similar to the PCC results, Fig. 3(a–c) demonstrates that the binding of $^3$H-CGP54626 to GABA$_B$ receptors was significantly decreased in the superficial layers ($p = 0.019; \downarrow 34.4\%$).
and in the deep layers \( (p = 0.00095; \downarrow 29.8\%) \). Four of the nine autistic cases had a history of seizure, but the comorbid seizure disorder did not have a significant effect on receptor binding in any layer. Note that there appears to be one outlier case with low binding in the autism group (1664) and one in the control group (1026).

**Discussion**

The anterior and posterior cingulate cortex and fusiform gyrus are among many cortical structures implicated in autism because of their correlative functions with core behaviors including social–emotional and face recognition/
expression deficits. Abnormal cortical circuitry is a common theory in autism (e.g. Kleinhans et al. 2008; Weng et al. 2010) and mounting evidence directly implicates cellular and molecular components of the GABAergic system (e.g. Fatemi et al. 2009a; Lawrence et al. 2010), including the GABA$_B$ receptor and its subunits (Fatemi et al. 2009b).

**GABA$_B$ receptor**

The GABA$_B$ receptor is composed of GABA$_{B1}$ and GABA$_{B2}$ subunits that must both be present for functional GABA$_B$ receptors to be expressed on the cell surface. Cell culture studies have shown that the GABA$_{B1}$ subunit binds GABA; however, binding of GABA or any other endogenous ligand to the GABA$_{B2}$ subunit has not been demonstrated (Kniazeff et al. 2002). The GABA$_{B2}$ subunit, which exists as two isoforms, GABA$_{B_{1a}}$ and GABA$_{B_{1b}}$, is necessary for trafficking of GABA$_{B1}$ to the cell surface, and mediates G protein activation in response to binding of agonists to a binding site located on the GABA$_{B1}$ subunit (G$_i$/G$_s$; Kniazeff et al. 2002). Activation of GABA$_B$ receptors can influence long-term changes in synaptic strength and modulation of cortical circuits, and has been reported to restrict long-term potentiation via hyperpolarization, whereas GABA$_B$ autoreceptors have been shown to promote the induction of long-term potentiation by disinhibiting the post-synaptic neuron in rat hippocampal slices (Davies et al. 1991; Olpe et al. 1993). Further evidence suggests that GABA$_B$ receptors have a critical role in developmental processes.

**GABA$_B$ receptors during development**

During pre- and postnatal brain development in the rat, GABA$_B$$_1$ receptors have a similar pre- and post-synaptic distribution as in adulthood in the neocortex, hippocampus, and dorsal cochlear nucleus (Lopez-Bendito et al. 2002; Lopez-Bendito et al. 2004; Lujan et al. 2004). Studies of the development of GABA$_B$ receptors in the cerebellum and olfactory cortex of rats suggest that GABA$_B$$_1$ receptors have a role in synapse and circuitry formation, and blockade of GABA$_B$$_1$ receptor signaling had a significant impact on the distribution of migratory neurons during corticogenesis (Lopez-Bendito et al. 2002; Panzanelli et al. 2004). Neuro-pathologic studies in the autistic brain have demonstrated abnormal neuronal migration and cytoarchitecture in the frontal cortex (Bailey et al. 1998) and anterior cingulate cortex (Simms et al. 2009). Casanova et al. (2002, 2003, 2006, 2010) reported an increase in the number of minicolumns and a decrease in neuropil in a number of cortical regions in autism. Furthermore, van Kooten et al. (2008) found reduced volume and neuron number in the fusiform gyrus in autism cases; however, to our knowledge, neuropathology in the PCC has not yet been reported.

Insulin-like growth factor 1 (IGF-1) is important for normal development of the brain and promotes neuronal survival by rescuing neurons from apoptosis (D’Mello et al. 1993; Cheng et al. 2000). Reduced IGF-1 has been found in the CSF of children with autism (Vanhaela et al. 2001; Riikonen et al. 2006). Tu et al. (2010) have recently found that GABA$_B$ receptors can protect neurons from apoptosis through a mechanism that involves transactivation of the IGF-1 receptor. IGF-1 triggers autophosphorylation of the IGF-1 receptor and activates the PI3 kinase/Akt-signaling cascade which mediates the neuroprotective action of IGF-1 (Delcourt et al. 2007). Akt functions downstream of PI3 kinase and is critical for neuron survival (Bondy and Cheng 2004). Therefore, reductions in GABA$_B$ receptors and/or IGF-1 in autism may result in the death of neurons, possibly contributing to reduced neuron numbers observed in the fusiform gyrus, amygdala and anterior cingulate cortex (Schumann and Amaral 2006; van Kooten et al. 2008; Simms et al. 2009).

The current study demonstrates significant reductions in GABA$_B$ receptor density throughout the cingulate cortex and fusiform gyrus, and although the developmental origin of the GABA$_B$ receptor deficit is unknown, if this system is disturbed during the prenatal or early postnatal period, it could have profound implications towards the maturation of specific behaviors.

**GABA$_B$ receptors and socio-emotional and face processing**

Individuals with autism have severe deficits in processing faces (Klin et al. 1999; Grelotti et al. 2002; Joseph and Tanaka 2003) that may be due to alterations in cortical network signaling because of deficits in the GABA system. The superficial layers of the cortex (layers I–IV) receive information from the thalamus and other cortical regions and project to inter- and intracortical areas while the deep layers also receive thalamic input and project to other cortical and subcortical regions. Based on the present findings, cortical information may be potentially disrupted because of reduced inhibitory receptors that could result in a failure to recruit cortical regions needed for processing emotional responses and facial recognition.

At the synaptic level, neuroligins are a family of post-synaptic cell adhesion molecules in the brain that interact with neurexins and are localized to the post-synaptic specialization of excitatory and inhibitory (including GABAergic) synapses (Ichchenko et al. 1995, 1996). In vitro transfusion studies have suggested a role for neuroligins in synapse formation (Dean et al. 2003); however, cell culture studies suggest that neuroligins are not required for synapse formation but for synapse specification and modulation (Varoqueaux et al. 2006). In autism, mutations in both neuroligins and neurexins have been reported (Chih et al. 2004; Feng et al. 2006; Yan et al. 2008). Therefore, reductions in inhibitory receptors in the present study may result from an alteration in neuroligins and/or neurexins resulting in reduced GABAergic-synaptic protein recruitment...
and stabilization within the cingulate cortex and fusiform gyrus.

**Seizures and GABA_B receptors**
A subgroup of approximately 25–33% of individuals with autism have a relatively high frequency of seizures (Olsson et al. 1988; Volkmar and Nelson 1990). Knock-out mice lacking the GABA_B1 or GABA_B2 subunit exhibit epileptiform activity, enhanced pre-pulse inhibition, altered locomotor activity and impaired memory processing (Prosser et al. 2001; Schuler et al. 2001; Vacher et al. 2006). These knockout mice suggest that differences in the number of GABA_B receptor subunits may lead to the development of seizures due to changes in neurotransmitter release or inhibition of local circuits. Similar deficiencies in GABA_B receptors in autism may result in seizures and decreased effectiveness in controlling cortical circuits.

In the current study, the density of GABA_B receptors in ACC, PCC or FFG did not differ significantly between the autism subgroup with a history of seizure and the subgroup with no history of seizure. However, this result is met with caution because the number of cases (n = 3) was small for seizures. Although several of the cases used in this study were receiving or had received anticonvulsant therapy during their life, none of the drugs used are known to target GABA_B receptors (Table 1). We were unable to determine if the anticonvulsants had an effect on GABA_B-binding density in all three regions because of the small sample size (n = 2 in PCC, n = 1 in FFG). However, in the ACC, there was no effect of pharmacotherapy on binding density, but again the number of cases was small and the results should be met with caution.

We are unable to determine if the significant decrease in the density of binding is caused by a loss of GABA_B receptors on GABAergic or glutamatergic neurons or if there is a reduction in one of the GABA_B receptor subunits. Fatemi et al. (2009b) found significant reductions in the protein level of GABA_B1 subunit in the parietal lobe, frontal lobe, and cerebellum of autistic individuals as well as reduced protein levels of the GABA_B2 subunit in the cerebellum. Based on these results, it is possible that the reductions in the GABA_B-binding density in the present study are caused by decreased availability of either of the subunits required for proper GABA_B receptor surface expression and functioning. A detailed molecular analysis of the subunits across brain areas is thus needed to determine whether the autistic group has fewer GABA_B receptor subunit(s) than controls.

**Closing comments**
The cingulate cortex, an area that modulates emotion, attention, and gaze fixation and activated by socio-emotional events, may be responsible for the proper function of the fusiform gyrus through mechanisms that modulate activity in this region. The alterations in GABA_B receptor densities in the three regions further support the theory that deficits in the GABA system are widespread in autism (Blatt et al. 2010) and that this alteration is not restricted to GABA_A receptors previously reported in these regions (Oblak et al. 2009a,b). The changes in GABA_B receptors thus suggest possible pharmacotherapies since GABA_B receptors are not responsive to antiepileptic drugs that typically target GABA_A receptors. GABA_B agonists such as baclofen might be considered as possible agents for clinical trials.

Finally, although the full syndrome as expressed in later childhood and adolescence appears to involve insults to multiple brain regions, it is not clear if these multiple systemic brain disturbances reflect deficits in multiple-independent control processes, or whether the initial insult might have been more restricted. It is possible that deficits initially confined to one system might negatively impact the development of other neural systems, such that a more pervasive set of impairments evolves. This would suggest that multiple brain areas within specific networks become involved and more widespread behavioral effects emerge.

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**Supporting Information**
Additional Supporting information may be found in the online version of this article:

**Table S1.** Summary of GABAB receptor-binding density values in autistic and control cases from the anterior and posterior cingulate cortices.

**Table S2.** Specific receptor-binding density in the fusiform gyrus in autism and control cases.

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