Published in final edited form as:

Autism Res. 2009 August ; 2(4): 205–219. doi:10.1002/aur.88.

Decreased GABA_A receptors and benzodiazepine binding sites in the anterior cingulate cortex in autism

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Abstract

The anterior cingulate cortex (ACC; BA 24) via its extensive limbic and high order association cortical connectivity to prefrontal cortex is a key part of an important circuitry participating in executive function, affect, and socio-emotional behavior. Multiple lines of evidence, including genetic and imaging studies, suggest that the ACC and GABA system may be affected in autism. The benzodiazepine binding site on the GABA_A receptor complex is an important target for pharmacotherapy and has important clinical implications. The present multiple-concentration ligand-binding study utilized ³H-muscimol and ³H-flunitrazepam to determine the number (B_{max}), binding affinity (K_d), and distribution of GABA_A receptors and benzodiazepine binding sites, respectively, in the ACC in adult autistic and control cases. Compared to controls, the autistic group had significant decreases in the mean density of GABA_A receptors in the supragranular (46.8%) and infragranular (20.2%) layers of the ACC and in the density of benzodiazepine binding sites in the supragranular (28.9%) and infragranular (16.4 %) lamina. In addition, a trend for a decrease in for the density of benzodiazepine sites was found in the infragranular layers (17.1%) in the autism group. These findings suggest that in the autistic group this downregulation of both benzodiazepine sites and GABA_A receptors in the ACC may be the result of increased GABA innervation and/or release disturbing the delicate excitation/inhibition balance of principal neurons as well as their output to key limbic cortical targets. Such disturbances likely underlie the core alterations in socio-emotional behaviors in autism.

Keywords

Autistic; Anterior Cingulate Cortex; GABA; Post-mortem; Ligand binding

Introduction

The anterior cingulate cortex (BA 24; ACC), part of the original Papez circuit of emotion (Papez, 1937), is considered a part of the brain's limbic lobe and is involved in attention and the regulation of cognitive and emotional processing (Allman et al. 2001; Bush et al. 2000; Heimer and Van Hoesen 2006). Anterior cingulate lesions are known to cause blunted affect, impulsivity, disinhibition, aggressive behavior, disabling obsessions and compulsions, and impaired social judgment including the inability to interpret social clues (Devinsky, 1995).

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Neuroanatomical studies have shown that the ACC encompasses a number of specialized subdivisions that subserve a vast array of cognitive, emotional, motor, nociceptive, and visuospatial functions (Bush et al. 2000; Heimer and Van Hoesen 2006; Isomura and Takada 2004; Posner et al., 2007; Zhuo, 2006). The ACC is intimately connected to prefrontal cortex as well as key limbic structures including the hippocampal formation and the amygdala, positioning it as a key structure for roles in executive function, learning and memory and socio-emotional behavior (Pandya et al., 1981; Vogt and Pandya, 1987; Petrides and Pandya, 2007).

In the neurodevelopmental disorder autism, characterized by impairments in communication including language, as well as narrowly focused interests and poor sociability, there is demonstrated neuropathology in the ACC in some reported cases. These were first described by Bauman and Kemper (1985) and recently quantitatively confirmed by Simms et al. (2006), who reported an increased cell packing density and decreased cell size in several subregions within the ACC. Furthermore, positron emission tomography (PET) studies have demonstrated abnormalities in the activation of the ACC in autism during a verbal learning task (Haznedar et al. 1997).

Recent neurochemical research has focused on the gamma-amino-butyric acid (GABA) system and its possible involvement in a number of limbic and cerebellar regions in autistic cases (Blatt et al., 2001; Fatemi et al., 2002; 2009a, b; Guptill et al., 2007; Yip et al., 2007, 2008, 2009). GABA plays a crucial role in normal cortical functioning, information processing, and the formation of brain cytoarchitecture during development (Conti et al., 2004; Di Cristo, 2007; Fritschy et al., 1997; Mohler et al., 1995a). One of the proposed causes of impaired information processing and social behavior in autism is an altered balance between inhibition and excitation in the brain (Rubenstein and Merzenich, 2003). The distribution, electrophysiology, and molecular characteristics of GABA receptors change markedly during development, leaving the formation of the cortex vulnerable to aberrations in neurotransmission at key developmental periods. Seizures are fairly common in individuals with autism, occurring in approximately 25–33% of individuals (Olsson et al., 1988; Volkmar and Nelson, 1990) and may result from abnormal inhibitory control in key cortical areas. Genetic studies have also implicated the GABA system in autism. For example, Schroer et al. (1998) found abnormalities on chromosome 15q11-q13, which includes a cluster of three GABA_A receptor genes (GABR α 5, GABR β 3, GABR γ 3). Several genetic studies have proposed the involvement of multiple GABA_A receptor subunits and suggest that, through complex interactions, these subunits are involved in autism (Ashley-Koch et al., 2006; Ma et al., 2005).

The GABA_A receptor has binding sites for multiple modulators, including benzodiazepines, making it a target for pharmacological intervention. Benzodiazepines (BZDs) enhance GABA_A receptor mediated neurotransmission, and classically act as sedative/hypnotics to reduce anxiety and suppress seizure activity and panic attacks (Mohler et al., 2000). In clinical studies, BZDs have been reported to elicit paradoxical behavioral responses in some autistic individuals, producing increased anxiety and aggressive behavior (Marrosou et al., 1987). This variable response could be due to an alteration in BZD-sensitive GABA_A receptors in the specific subset of cases investigated.

Taken together, there is compelling evidence that the GABA system is impacted in autism and alterations in the GABA_A receptor system likely play a role in the etiology of the disorder during development and may contribute to the abnormal phenotype and the variable response to pharmacotherapy. Changes in these key neural substrates in the ACC could disrupt critical circuits involved in socio-emotional behavior as well as other high-order associative functions especially via its abundant prefrontal cortical connectivity. The aim of

this study was to determine if the number, density, and distribution of GABA_A receptors and BZD binding sites in the anterior cingulate cortex are altered in postmortem adult autistic cases, and to explore how such alterations might impact individuals with the disorder.

Methods and Materials

Brain Tissue and Case Data

Fresh frozen brain tissue from the anterior cingulate cortex was obtained from the Harvard Brain Tissue Resource Center (HBTRC) in Belmont, Massachusetts. All clinical cases were diagnosed as autistic and had Autism Diagnostic Interviews (ADI) completed either pre- or post-mortem. All tissue was provided by The Autism Research Foundation, Autism Tissue Program, and Harvard Brain Tissue Resource Center. A total of seventeen blocks were obtained (seven autism and ten controls) from the rostrum of the anterior cingulate cortex and stored at -80°C . One autism case, #3845, was used for the muscimol study but not for the benzodiazepine study due to limited availability of tissue sections as this case has been used for many of our previous studies. 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 t-test). Four of the seven autism cases had a history of at least one seizure (1078, 1484, 2825 and 3845). Of those four cases, three had received treatment with anticonvulsant therapy (1078, 2825 and 3845). All cases used in the study had an autism diagnosis of moderate to severe.

Multiple-Concentration Binding Assay

All tissue blocks were sectioned coronally at $20\mu\text{m}$ using a Hacker/Brights motorized cryostat at -20°C . Sections were then thaw mounted on 2×3 inch gelatin coated glass slides. For each of the seven concentrations, two sections from each case were used to determine total binding and one section from each case was used to determine non-specific binding. Non-specific binding was measured by adding a high concentration of a competitive displacer (see Table 2) to the tritiated ligand and buffer solution. The ligand used for GABA_A receptors was [^3H]-Muscimol (specific activity 36.6 Ci/mmol; New England Nuclear) and the ligand for BZD binding sites was [^3H]-flunitrazepam (specific activity 85.2 Ci/mmol; New England Nuclear). To eliminate variability in binding conditions, the selected sections from all cases were assayed in parallel. Slides were then dried under a stream of cool air overnight and loaded into X-ray cassettes with a tritium standard (Amersham), apposed to tritium-sensitive film (^3H -Hyperfilm, Kodak) and incubated for a period of time (see table 2). The exposed films were developed for 4 minutes with Kodak D19 developer, fixed with Kodak Rapidfix (3 minutes) at room temperature, and allowed to air dry. Slides were stained with thionin to determine cytoarchitecture and laminar distribution of the anterior cingulate cortex (Figure 1). Supragranular layers corresponded to layers I–III and infragranular layers to layers V–VI as layer IV is absent in the ACC (Vogt et al., 1995).

Data Analysis

The film autoradiograms were digitized using an Inquiry densitometry system (Loats Associates) to gather quantitative measurements of optical density. The supragranular and infragranular layers were sampled from each case. The ^3H standards that were exposed with the sections were used to calibrate the autoradiograms to quantify the amount of ligand bound per milligram of protein in the tissue sections. Optical density for the standards as a function of specific activity (corrected for radioactive decay) was fitted by nonlinear least squares regression to the equation, $\text{optical density} = B1 \times (1 - 10^{k1(\text{specific activity})}) + B3$, by 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, which was used to convert

the measured optical densities into nCi/mg. Binding in femtomoles per milligram of protein (fmol/mg) was calculated based on specific activity of the ligand.

Statistical Analyses

Specific binding of the tritiated ligands in the supragranular and infragranular layers from each subject 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 Figure 2. Least-squares nonlinear regression was carried out using the Microsoft Excel Solver tool. B_{max} data is reported as mean $\pm$ standard error of the mean (SEM). K_d is reported as the geometric average $10^{\text{mean}(\log(K_d))} \pm \text{SEM}_{\text{av}}$, calculated as $\text{SEM}_{\text{av}} = (((10^{(\times + s)} - 10^{\times}) + (10^{\times} - 10^{(\times - s)}))/2)$, where $\times = \text{mean}(\log K_d)$ and s is the SEM of the $\log(K_d)$ values. Significance testing for differences in K_d was carried out using $\log(K_d)$ values. Two-Way Analysis of Variance (ANOVA) was used to determine if there was a significant difference in receptor or binding site number between autistic and control cases, between superficial and deep layers, or if an interaction existed. Non-parametric Mann-Whitney tests were used to determine if seizures or anticonvulsant therapy in the autism cases had an effect on receptor binding.

Results

Table 3 reports specific binding of [^3H]-muscimol and [^3H]-flunitrazepam in the anterior cingulate cortex from autistic and control cases. Overall, the supragranular layers of the anterior cingulate cortex of both autistic and control cases contained a higher density of GABA_A receptors and benzodiazepine binding sites than the infragranular layers, as measured by the B_{max} values ($p=0.0004$, $p=0.0003$, respectively). We found decreases in the density of both GABA_A receptors and BZD binding sites in the supra- and infragranular layers of autistic cases. Binding affinity (K_d) did not differ significantly between autistic and control cases for either ligand (Figure 3; Table 4). Binding data for both autistic and control cases were well-fitted by a single-component hyperbolic binding equation, consistent with a single, homogeneous class of binding sites (Figure 3). Figures 5 and 7 show the specific binding curves for each of the cases. Figures 4 and 6 show typical examples of binding of a single concentration (15 nM) of [^3H]-muscimol and [^3H]-flunitrazepam, respectively, in typical sections from an autistic and a control case. The autoradiograms have been pseudocolored using the Inquiry program to better visualize binding density, with red corresponding to high binding (optical density) and purple to low binding. The Nissl stained section in Figure 1 was used to identify the region of interest as well as the division of the region into supragranular (layers I–III) and infragranular (layers V–VI) layers. The distribution of B_{max} values across layers for GABA_A receptors and benzodiazepine binding sites is given in Table III. Sample binding curves demonstrating specific binding in the supragranular layers from each of the seven concentrations of tritiated ligand can be found in Figure 3.

GABA_A Receptor Binding

Multiple concentration binding experiments revealed that the mean B_{max} value for all autistic cases fell below that for control cases. The results from the seven-concentration binding study indicate a significant reduction of GABA_A receptors (B_{max}) in both the supragranular ($p=5.2 \times 10^{-6}$) and the infragranular ($p=0.04$) layers of the anterior cingulate cortex in the autistic group ($n=7$) when compared to age- and post-mortem interval (PMI) matched controls ($n=9$). The B_{max} values demonstrate that there was a 46.8% reduction in GABA_A receptors in the supragranular layers of the anterior cingulate cortex and a 20.2% decrease in the number of GABA_A receptors in the infragranular layers in autism. The three cases with the lowest B_{max} values in the supragranular layers did have a history of seizure.

However, the case demonstrating the highest binding also had a history of seizure. In the infragranular layers, two of the cases with a history of seizure fell below the mean $B_{\max}$ values of the autistic group. In contrast, there was no difference in binding affinity comparing autistic and control cases (Figure 2).

Benzodiazepine Binding Sites

With respect to the benzodiazepine binding sites, all but one autistic case (in the infragranular layers) fell below the average $B_{\max}$ value of the control group. The results from the seven concentration binding study indicated a significant decrease in the $B_{\max}$ values in the supragranular layers ($p=0.003$) and infragranular layers ($p=0.04$) of the anterior cingulate cortex in autism. The multiple concentration binding study revealed a 28.7% and 16.4% reduction in benzodiazepine binding sites in the supragranular layers and infragranular layers, respectively, in autistic cases. In the supragranular layers, one of the two autistic cases that fell below the mean $B_{\max}$ value for autistic cases had a history of seizure. In the infragranular layers, two of the cases that fell below the mean $B_{\max}$ value had a history of seizure. There was no significant difference observed in binding affinity (Figure 2).

Effect of seizure on binding

Four of the cases in this study had a history of having at least one seizure. In the muscimol binding study, there was no significant difference in binding between autistic cases with seizure ($n=4$) and autistic cases without seizure ($n=3$). Furthermore, in the flunitrazepam binding study no significant difference in binding was observed between autistic cases with seizure ($n=3$) and autistic cases with no seizure history ($n=4$).

Effect of pharmacotherapy on binding

The cases were divided into two groups (autism with a history of anticonvulsant therapy and autism with no history of anticonvulsant therapy) to determine if the medication had a significant effect on binding. In the muscimol binding experiments, three of the seven cases were taking anticonvulsants at the time of death. Using a Mann-Whitney U non-parametric test, a trend ($p=0.06$) was observed in the supragranular layers, such that those receiving anticonvulsant therapy had a trend towards decreased GABA_A receptors. There was no effect of anticonvulsant therapy on the decrease in receptor binding in the infragranular layers ($p = 0.99$ and $p = .113$, respectively). In the flunitrazepam binding study, half ($n=3$) of the cases were receiving anticonvulsant therapy at the time of death. There was no significant difference in the number of benzodiazepine binding sites in the supra- or infragranular layers in the autistic group receiving anticonvulsants compared to the autistic group not receiving anticonvulsants.

Discussion

Normal Functions of the Anterior Cingulate Cortex

The anterior cingulate cortex (ACC; Brodmann area 24; BA 24) has been shown to participate in a variety of functions including executive, evaluative, and cognitive functions and emotion (Vogt et al., 1992; Devinsky et al., 1996; Lane et al., 1998). Processing of high order sensory and multimodal information occurs via connections with the prefrontal cortex and parietal cortex as well as the motor system and the frontal eye fields (Allman et al., 2001; Calzavara et al., 2007; Vogt et al., 1987; Kaitz and Robertson, 1981; Robertson and Kaitz, 1981). When effort is needed to carry out a task such as in early learning and problem solving (Allman et al., 2001), the ACC is recruited, and many studies attribute functions such as error detection and anticipation of tasks, motivation, and modulation of emotional

responses to this region (Bush et al., 2000; Nieuwenhuis et al., 2003; Posner et al., 1999; Posner et al., 2007). The ACC is an integral part of many higher order functions. Disruptions of this region, at any level, cellular or chemical, could lead to social, communication, and other behavioral deficits.

The role of GABA in the anterior cingulate cortex: implications for connectivity

GABAergic neurons are essential to information processing in virtually every brain region, and thus an alteration in GABAergic function, of any nature, in a region such as the anterior cingulate cortex, with widespread cortical connections, may result in impairments of information processing, a feature considered to be central to autism (Belmonte et al. 2004). Information processing in the brain occurs via feed-forward and feedback projections. Long-range cortico-cortical input is provided by “feedback” projections from higher order regions that primarily target the distal dendritic tufts of pyramidal cells from all layers that terminate in layer I (Rockland and Drash 1996). Layer II pyramidal axons make local connections within layers II and III, and layer III pyramidal axons ramify most extensively in layers II/III and V (Thomson et al. 2002). Layer V pyramidal cells project to all other layers of the cortex and represent a major route of excitatory feedback projections to the superficial laminae (Burkhalter, 1989). Abnormalities in the integrity of the layers through cell loss, abnormal cytoarchitecture or receptor changes, as observed in this study, may interfere with cortical processing.

The present observations demonstrate the highest binding to GABA_A receptors and benzodiazepine binding sites in the supragranular layers of the anterior cingulate cortices in agreement with previous autoradiographic and immunohistochemical data (Fritschy et al., 1997; Mohler et al., 1995a; Mohler et al., 1995b). Previous receptor binding experiments have reported that [³H]-muscimol labeled GABA_A receptors and [³H]-flunitrazepam-labeled benzodiazepine binding sites were significantly reduced in the hippocampus of adult autistic individuals (Blatt, 2001; Guptill et al., 2007). The current study found significant reductions in GABA_A receptors and benzodiazepine binding sites in the supragranular and infragranular layers of the ACC. These results suggest abnormal connectivity between the ACC and the regions of the cortex to which the ACC projects (e.g. prefrontal cortex) and also abnormal reciprocal connections between the higher order association cortices and the ACC. This study further suggests that the limbic system is abnormal in autism, which may contribute to the social and emotional aspects of the disorder. In autism, decreases in inhibitory receptors on pyramidal neurons or interneurons may restrict normal connectivity within the anterior cingulate cortex and between cortical regions. Laminar and receptor-specific decreases in the infragranular layers may support a role of these layers in a loss of normal signal transmission and a decrease in the information relaying to and from the thalamus (Kaitz and Robertson 1981; Yakovlev and Locke 1961).

Inadequate inhibitory damping of excitatory projections to other excitatory cells further downstream could result in undesirable positive feedback loops, and could disrupt signaling from the thalamus as it passes through cortical columns. Excitatory projections from pyramidal cells to interneurons would help control the excitatory flow through the cortex by inhibiting cells responsible for their inputs as soon as appropriate outputs to the next stage of processing have been attained (Burkhalter, 1989). However, abnormal receptors in this region would decrease the likelihood of controlling the excitatory flow. This abnormal connectivity may result in changes in cognition and in the proper understanding of socio-emotional interactions that are common in autism.

GABA_A receptors during development and related neuropathology of the autistic brain

Aberrant development of GABAergic circuits is not unique to autism and has been implicated in other neurodevelopmental disorders such as schizophrenia (Benes et al., 1992) and Tourette's syndrome (Kalanithi et al., 2005). Compelling evidence indicates that GABA and GABA receptors play a role in several developmental processes. During development, GABA_A receptors participate in proliferation, migration, and differentiation of precursor cells that orchestrate the development of the embryonic brain (Akerman and Cline 2007; Barker et al. 1998; Conti et al., 2004; Di Cristo, 2007; Mohler et al., 2004). Despite its known effects in adults as a strong inhibitory neurotransmitter, during development GABA_A mediated neurotransmission plays an excitatory role in the developing nervous system, shifting to an inhibitory function in the mature nervous system as a consequence of changes in Cl⁻ gradients (Ben-Ari et al., 2006). Eliminating excitatory GABA action between postnatal days 4 and 6 in rats resulted in severe impairment of the morphological maturation of cortical neurons (Cancedda et al., 2007; Heck et al., 2007). Furthermore, modulation of GABA_A receptor-mediated mechanisms in the immature cerebral cortex of the rat has also been shown to impair neuronal migration *in vitro* and *in vivo* (Conti et al. 2004).

In autism, pathological alterations in the cortical cytoarchitecture in autism resemble neuronal migration disorders previously described in the human cerebral cortex (Meng, et al., 2005; Corfas et al., 2004). Simms et al. (2006) has shown irregular lamination and increased density of neurons in the subcortical white matter of the anterior cingulate cortex in five of nine cases examined, potentially occurring from a disrupted GABAergic receptor system. Furthermore, Bailey et al. (1998) demonstrated abnormal cytoarchitecture in the frontal cortex, demonstrating changes in migrational behavior of the neurons to the cortical layers, and Casanova et al. (2002, 2003, 2006) has shown a reduced intercolumnar width of minicolumns in dorsal lateral prefrontal cortex (PFC) as well as an abnormal number of minicolumns. Again, these results are suggestive of an abnormality in the GABAergic receptor system during development resulting in abnormal cytoarchitecture in the adult autistic brain. The changes observed in a limbic system area (ACC) and a region that it is highly connected with (PFC) suggests further a circuitry/connectivity problem. This could potentially lead to abnormalities in cognitive processing and higher order functioning, a theory central to autism.

Bauman and Kemper (1985) first observed qualitatively a decrease in cell volume in limbic structures of autistic brain. In the anterior cingulate cortex, a significant decrease in cell area was observed in the supragranular and infragranular layers of area 24b (a subregion of the ACC) as well as a significant decrease in cell volume in the supragranular and infragranular layers of the same region (Simms et al., 2006). More recently, Schumann and Amaral (2006) found a quantitative decrease in the volume of neurons in the lateral nucleus of the amygdala, a region with connections to the anterior cingulate (Porrino et al., 1981). An abnormal GABA receptor system during development, such as one that is delayed in the switch from excitatory to inhibitory or one that retains the fetal circuitry (Bauman and Kemper, 1985), could form the basis for abnormal processing and decreased neuron volume in the adult anterior cingulate in autism. These changes could interfere with socio-emotional and cognitive processing similar to what has been found in imaging studies (Calzavara et al., 2007; Haznedar et al., 2000; Kana et al., 2007; Kennedy et al., 2006; Shafritz et al., 2007; Silk et al., 2006; Vollm et al., 2006). A developmental deficiency in any of these roles would adversely effect the temporal ordering of neurogenesis and synaptogenesis, thereby affecting maturation of circuits that are later involved in complex behaviors associated with the anterior cingulate cortex.

Effects of seizure/anti-convulsant treatment on $B_{\max}$ and K_d of GABA_A and benzodiazepine binding

Approximately, 80% of all GABA_A receptors contain the classical benzodiazepine binding site and are characterized largely by the subunit combinations $\alpha 1\beta 2\gamma 2$, $\alpha 2\beta 3\gamma 2$ and $\alpha 3\beta 3\gamma 2$ (Speth et al., 1980). The benzodiazepine site, located at the interface between α and γ subunits, is structurally homologous to the GABA binding site located in the α - β subunit interface. Epileptogenic seizure activity is likely a result of a deficit in GABA-mediated inhibition, and seizures are common in approximately one-fourth to one-third of autistic individuals (Bailey et al., 1998; Gillberg et al., 1987; Olsson et al., 1988; Rutter, 1970; Volkmar and Nelson, 1990). In a PET imaging study, the binding of [¹¹C]-flumazenil to benzodiazepine binding sites in epileptogenic foci was found to be reduced with no change in binding affinity (Savic et al., 1988). In a more recent imaging study using [¹²⁵I]-iomazenil, reductions in central benzodiazepine binding sites were found in 48% of individuals studied in medial temporal lobe regions (Matheja et al., 2001), although this may be a consequence of localized neuronal damage or neuronal loss in the regions with temporal lobe epileptogenic activity. It should be noted that these changes were occurring in the hippocampus and may not be reflective of the changes occurring in the cortex.

Four of the autistic cases studied had a history of seizure, raising the question of whether the changes in [³H]flunitrazepam and [³H]-muscimol binding could be a consequence of seizures. Alterations in GABA_A receptor subunit expression and composition in epilepsy are well documented in human (Loup et al., 2000, 2006) and in animal models (Gilby et al., 2005; Li et al., 2006; Nishimura et al., 2005; Peng et al., 2004; Roberts et al., 2005). In particular, there is evidence that seizures can induce a shift of GABA_A receptor subunit mRNA expression such that $\alpha 4$ subunit expression is increased, whereas $\alpha 1$ subunit expression is decreased (Brooks-Kayal et al., 2009). An increase in $\alpha 4$ -containing GABA_A receptors at the expense of $\alpha 1$ -containing receptors could result in a decreased number of [³H]-flunitrazepam binding sites, as $\alpha 4$ -containing receptors do not contain a [³H]-flunitrazepam binding site, but because [³H]-muscimol binding was also decreased argues that the results reflect an overall deficit in GABA_A receptors.

In addition to the potential effect of seizures on GABA_A receptor expression, three subjects had a history of treatment with anticonvulsants, including the barbiturate phenobarbital. Phenobarbital is a GABA_A receptor positive modulator that has been reported to reduce GABA_A receptor binding in mice (Weizman et al., 1989). Phenobarbital withdrawal is sometimes associated with seizures, suggesting that chronic phenobarbital use alters GABA_A receptor systems. There was no statistically significant difference between the cases with and without seizures or those with and without a history of antiseizure medication, arguing that decreases in binding of GABA_A receptor ligands is not a consequence of antiseizure drug treatment, although this conclusion is tentative given the limited number of cases in both groups. In particular, there was a trend toward lower [³H]-muscimol binding in the supragranular layer of patients receiving anticonvulsant therapy. It is difficult to conclude whether autism, seizures, drug therapy, a small group, or a combination of any of these could be responsible for the reduced binding in one particular layer. Therefore it will be important in future studies to continue to control for seizures, anticonvulsant therapy, and to obtain a larger sample size if possible.

Taken together with previous studies (Guptill et al., 2007; Yip et al., 2007, 2008, 2009; Blatt et al., 2001), the present results support the hypothesis that deficits in GABA_A receptor systems are widespread in autistic brain. Polymorphisms in the genes for the $\alpha 4$ and $\beta 1$ GABA_A receptor subunits have been found to be significantly associated with autism, raising the possibility that decreases in binding of GABA_A receptor ligands in autistic brain may reflect a primary defect in GABA_A receptor subunit expression. Alternatively, it is

possible that down-regulation of GABA_A receptors occurs as a compensatory response to increased GABA release associated with increased numbers, activity, or dendritic branching of GABAergic interneurons. In the hippocampus, increased density of interneurons was found in subfields of the hippocampus (Lawrence et al., 2009) in which reductions in GABA_A and BZD receptors were found (Blatt et al., 2001; Guptill et al., 2007). Alterations in GAD67 and GAD65 mRNA levels have been detected in specific neuronal subpopulations in autistic cerebellum (Yip et al., 2007, 2008, 2009), suggesting enhanced GABA utilization by these cells. If the decrease in GABA_A receptors in ACC is a consequence of excessive GABAergic release by interneurons, then it is likely that interneuron numbers and/or GAD expression will similarly be up-regulated in ACC of autistic cases. Such studies may help to clarify the role of GABA_A receptors with respect to disturbances of executive function that have been described in ASD. ACC hypoactivity has been reported in functional imaging studies of ASD subjects during some executive function dependent tasks (Shafritz et al., 2008; Kana et al., 2006), which would be consistent with excessive release of GABA by ACC interneurons, but abnormally increased ACC activation has also been reported for some tasks (Schmitz et al., 2008; Thakker et al., 2008). A more sophisticated understanding of the role of GABA_A receptor mediated inhibition with respect to executive function deficits will likely require identification of the specific GABA_A receptors affected and their localization with respect to neuronal circuits.

The role of GABA_A receptors in anxiety disorders

Pharmacological evidence suggests that GABA_A receptors play a role in anxiety disorders. Classical benzodiazepines and other GABA_A receptor positive modulators reduce anxiety, whereas competitive and non-competitive GABA_A receptor antagonists and benzodiazepine inverse agonists are anxiogenic (Belzung et al., 1987; Dalvi and Rodgers, 1996), and altered GABA_A receptor binding in the limbic system has been correlated with increased anxiety in both humans (Sundstrom et al., 1997) and animals (Rainnie et al., 1992). Patients with panic disorder have reduced benzodiazepine binding (Cameron et al., 2007; Hasler et al., 2008) and a reduced sensitivity to benzodiazepine drugs (Roy-Byrne et al., 1996). Individuals with Autism Spectrum Disorders (ASDs) have high levels of anxiety disorders (Gillott and Standen, 2007; Juranek et al., 2006; Kuusikko et al., 2008; Sukhodolsky et al., 2008; Towbin et al., 2005) and Gillott (2001) suggests that adults with autism are almost three-times more anxious than their non-autistic peers.

Despite the high frequency of anxiety disorders in patients with ASD, use of benzodiazepines in this group of patients is limited in practice; in 2002, only 4.2% of autistic patients received at least one prescription for a benzodiazepine (Oswald and Sonenklar, 2007), suggesting that the effectiveness of this class of drugs for treatment of anxiety associated with ASD is not high. Marrosu et al. (1987) found that some individuals in a small group of children with autism displayed a paradoxical reaction to benzodiazepines, in which half of the autistic individuals receiving this treatment showed improvement in behavior and the other half exhibited anxiogenic and aggressive response. We hypothesize that the reduction in GABA_A receptors leads to increased anxiety in autism. GABA_A receptors containing the $\alpha 2$ and $\alpha 3$ subunits have been shown to mediate anxiolytic effects of benzodiazepine site ligands in mice, suggesting that these receptors play a role in regulating anxiety (Morris et al., 2006). Moreover, these subunits have been found to be reduced in regions of the brains of individuals with autism (Fatemi et al., 2009b), which could result in both increased susceptibility to anxiety and reduced anxiolytic efficacy of benzodiazepines. Paradoxical reactions to benzodiazepines could reflect different alterations of GABA_A receptor subtype or neuronal distribution in subgroups of individuals with autism. Following up with in situ hybridization studies, immunohistochemical studies, or

western blotting experiments would conclusively determine whether $\alpha 2$ and $\alpha 3$ subunits are reduced in these cases.

Concluding Remarks and Future Experiments

Through many recent neurochemical studies in autism, it is becoming clear that multiple transmitter systems are involved in the neuropathology of the disorder. Interestingly, the system with the most consistent findings is the GABAergic system and specifically the GABA_A and benzodiazepine sites in limbic and cerebellar structures. The present study further elucidated that decreases in these receptor subtypes in the ACC potentially disrupts information processing to high order association cortices in the frontal cortex likely contributing to the core socio-emotional behavior deficits in autistic individuals. As noted in Table 1, all cases used in the study had a history of mental retardation. A subnormal IQ has been observed in most cases of individuals with “classic autism,” similar to all cases within this study. It is unlikely that the presence of mental retardation is the cause of a decreased density of GABA_A receptors and BZD binding sites and specific to autism. However, it would be important to examine Asperger’s cases as well to determine conclusively that the decreased density of GABA_A receptors and BZD binding sites is specific to “classic autism” and not to mental retardation or to high-functioning autism. However, these findings do lend further evidence that the GABA_A receptors should be considered to be a core neural substrate in autism and its alterations in different brain areas in autism are in support of Rubenstein and Merzenich’s (2003) hypothesis of an altered inhibitory:excitatory imbalance directly affecting the autism phenotype.

Acknowledgments

Supported by NIH STAART U54 MH66398.

This work was supported by a “Studies to Advance Autism Research and Treatment” grants from the National Institutes of Health (NIH U54 MH66398) and NIH NINDS NS38975. Brain tissue was provided by the Harvard Brain Tissue Resource Center (HBTRC), the Autism Tissue Program, and the Autism Research Foundation.. We thank Sandy Thevarkunnel for her help in receptor binding autoradiography methodology.

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Figure 1.

A Nissl stained section from a control case (4103) demonstrates the cytoarchitecture of the human anterior cingulate cortex (Area 24a–c). The box illustrates our sampling scheme dividing the cortex into supragranular (I–III) and infragranular (V–VI) layers.

Figure 2.

Summary of mean K_d values for GABA_A (2a) and BZD (2b) receptor binding in autistic and control cases. There was no significant difference between autistic and control cases in either ligand or layers. Geometric means for K_d are reported here.

3a.

Muscimol binding affinity in the ACC (BA 24)

3b.

Flunitrazepam binding affinity in the ACC (BA 24) **Figure 3.**

Sample binding curves from two cases demonstrating specific binding in the supragranular layers from each concentration of tritiated ligand. Data from [^3H]muscimol is in the upper panel and data from [^3H]flunitrazepam is in the bottom panel.

Figure 4.

Examples of pseudocolored images of [^3H]-muscimol binding (15 nM) in autistic (4a) and control (4b) cases. Solid black arrows demonstrate the location of sampling in the supragranular layers; arrows with dashed lines indicate sampling in the infragranular layers. In the [^3H]muscimol binding experiments, the number of GABA_A receptors in the supragranular ($p=0.02$) and infragranular layers ($p=0.04$) was significantly (**) decreased (4c).

5a.

5b.

Figure 5.

Examples of individual [³H]muscimol binding curves. Specific binding of [³H]-muscimol to the supragranular (a) and infragranular (b) layers of the anterior cingulate cortex in seven autistic and nine control subjects. Smooth curves indicate fits to the hyperbolic binding equation.

Figure 6. Examples of autistic (6a) and control (6b) pseudocolored images of [^3H]-Flunitrazepam (15nM) binding in the anterior cingulate cortex. Solid black arrows demonstrate the location of sampling in the supragranular layers; arrows with dashed lines indicate sampling in the infragranular layers. There was a significant decrease (**) in benzodiazepine binding sites in the supragranular layers ($p=0.002$) and a trend (*) in the infragranular layers ($p=0.06$) in autism (6c).

Figure 7. Examples of individual [3 H]-flunitrazepam binding curves. Specific binding of [3 H]-flunitrazepam to the supragranular (7a) and infragranular (7b) layers of the anterior cingulate cortex in six autistic and nine control subjects. Smooth curves indicate fits to the hyperbolic binding equation.

Table 1

Information for cases in the multiple concentration binding studies

CASE	DIAGNOSIS	AGE	PMI (HOURS)	Medication History	CAUSE OF DEATH	GENDER
1078**	Autism	22	14.3	Dilantin, Tegretol, Theodur, Phenobarbital	Drowning	Male
1401	Autism	21	20.6	None	Pneumonia, Sepsis	Female
1484*	Autism	19	15	None	Burns	Male
2825**	Autism	19	9.5	Klonopin, Mysoline, Phenobarbital, Thorazine	Heart attack	Male
3845**	Autism	30	28.4	Dilantin, Mellaril, Phenobarbital	Cancer	Male
4099	Autism	19	3	None	Conj. Heart Failure	Male
5754	Autism	20	30.0	None	Unknown	Male
Mean		21.4	17.3			
4103	Control	43	23	None	Heart attack/disease	Male
4104	Control	24	5	None	Gun shot	Male
4188	Control	16	13	None	Gun Shot	Male
4267	Control	26	20	None	Accidental	Male
4268	Control	30	22	None	Heart attack/disease	Male
4269	Control	28	24	None	Heart disease	Male
4271	Control	19	21	None	Epiglottitis	Male
4275	Control	20	16	None	Accidental	Male
4364	Control	27	27	None	Motor Vehicle Accident	Male
Mean		25.9	19.0			

* Note: Cases with at least one asterisks had a history of at least one seizure.

** Cases with two asterisks were being treated with an anticonvulsant at time of death. All autism cases were considered mentally retarded.

Table 2Multiple Concentration Binding Conditions for GABA_A receptors and Benzodiazepine binding sites

Ligand	Target	Concentration (nM)	Displacer	Exposure Time
³ [H]-Muscimol	GABA _A Receptor	[0.3, 1.5, 3, 8, 30, 60, 110]	100μM GABA	6–360 days
³ [H]-Flunitrazepam	Benzodiazepine binding site	[0.3, 1.5, 3, 5, 15, 30, 120]	100μM Clonazepam	10–180 days

Table 3
Summary of $B_{\max}$ values for GABA_A and BZD receptor binding in autistic and control cases.

Case	Diagnosis	Muscimol Specific Binding (fmol/mg protein)		Flunitrazepam Specific Binding (fmol/mg protein)	
		Supragranular	Infragranular	Supragranular	Infragranular
1078	Autism	361.21	428.58	261.17	229.80
1401	Autism	497.67	473.07	219.45	162.49
1484	Autism	574.10	446.28	321.89	181.76
2825	Autism	371.43	525.64	306.07	198.43
3845	Autism	432.83	345.31		
4099	Autism	558.68	424.41	381.36	217.17
5754	Autism	480.17	387.30	337.30	222.49
Mean ± SEM		468.01 ± 31.80	432.94 ± 21.95	304.54 ± 21.65	202.02 ± 10.66
4103	Control	830.26	493.42	353.76	204.55
4104	Control	1030.31	522.53	335.13	231.79
4188	Control	873.65	328.05	453.82	288.03
4267	Control	712.05	448.30	447.37	214.59
4268	Control	741.45	510.97	391.17	234.53
4269	Control	827.73	742.58	542.08	215.16
4271	Control	1104.62	554.65	509.70	313.10
4275	Control	1003.04	635.73	384.67	192.15
4364	Control	788.97	694.93	420.54	279.98
Mean ± SEM		879.11 ± 47.14	542.35 ± 40.49	427.03 ± 22.90	241.54 ± 14.01
p-value (t-test)		5.16×10⁻⁶	0.035	0.0028	0.043

Table 4

Summary of muscimol and flunitrazepam binding affinity in the anterior cingulate cortex.

Group	Muscimol Binding Affinity (nanomolar)		Flunitrazepam Binding Affinity (nanomolar)	
	Supragranular	Infragranular	Supragranular	Infragranular
Autism	13.5 ± 1.26	9.69 ± 1.70	4.44 ± 0.61	4.79 ± 0.19
Control	13 ± 1.55	9.32 ± 1.60	6.10 ± 1.45	5.76 ± 0.82