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Expression of $GABA_B$ receptors is altered in brains of subjects with autism

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Abstract

Autism is a neurodevelopmental disorder that is often comorbid with seizures. Gamma-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in brain. $GABA_B$ receptors play an important role in maintaining excitatory/inhibitory balance in brain and alterations may lead to seizures. We compared levels of $GABA_B$ receptor subunits GABBR1 and GABBR2 in cerebellum, BA9 and BA40 of subjects with autism and matched controls. Levels of GABBR1 were significantly decreased in BA9, BA40, and cerebellum, while GABBR2 was significantly reduced in the cerebellum. The presence of seizure disorder did not have a significant impact on the observed reductions in $GABA_B$ receptor subunit expression. Decreases in $GABA_B$ receptor subunits may help explain the presence of seizures that are often comorbid with autism, as well as cognitive difficulties prevalent in autism.

Keywords

GABBR1; *GABBR2*; *autism*; *cerebellum*; *BA9*; *BA40*

Introduction

Autism is a neurodevelopmental disorder characterized by impairments in social functioning, stereotypica, including mental retardation [2], and seizure disorders, including epilepsy [3], are often comorbid with autism.

Gamma-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the brain. There are three classes of GABA receptors: $GABA_A$, $GABA_B$, and $GABA_C$. $GABA_B$ receptors are present in the thalamus, cerebellum, hippocampus, cerebral cortex, and interpenduncular nucleus and are coupled via G proteins to membrane K^+ and Ca^{++} channels and to adenylate cyclase in humans [4]. $GABA_B$ receptors are heterodimeric, formed from two subunits: $GABA_B$ receptor 1 (GABBR1) and $GABA_B$ receptor 2 (GABBR2) [5]. $GABA_B$ receptors contribute to synaptic events in the mammalian brain presynaptically by facilitating the release of neurtransmitters, including glutamate and GABA, and postsynaptically by generating inhibitory potentials [4,6]. As a result, $GABA_B$ receptors play an important role in maintaining

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an excitatory/inhibitory balance in the brain, and alterations in $GABA_B$ expression may lead to the development of seizures [7,8].

There is preliminary evidence that polymorphisms of GABBR1 are associated with schizophrenia [9] and obsessive compulsive disorder [10], while the association of GABBR1 polymorphisms with temporal lobe epilepsy is inconclusive [11,12]. Similarly, GABBR2 has also been associated with temporal lobe epilepsy [11], and multiple laboratories have demonstrated altered expression of GABBR1 and GABBR2 in animal models for seizure disorders [13-15].

We investigated whether $GABA_B$ subunits are altered in brains of subjects with autism by measuring expression of GABBR1 and GABBR2 in a well-characterized group of age, sex, and postmortem-interval (PMI) matched brain samples from subjects with autism and matched controls in cerebellum, superior frontal (Brodmann's Area 9 (BA9)) and parietal (BA40) cortices, areas that are involved in the pathology of autism.

Materials and Methods

Tissue preparation

All experimental procedures were approved by the Institutional Review Board of the University of Minnesota School of Medicine. Postmortem blocks of parietal cortex (BA40), superior frontal cortex (BA9), and cerebellum (lobar origin unknown) were obtained from the Autism Research Foundation and several brain banks (the NICHD Brain and Tissue Bank for Developmental Disorders; TARF; the Harvard Brain Tissue Resource Center, which is supported in part by PHS grant number R24 MH068855; the Brain Endowment Bank, which is funded in part by the National Parkinson Foundation, Inc., Miami, Florida; and the Autism Tissue Program). These samples, which have been used by our laboratory previously, are some of the most well-characterized and most-studied brain collections used by multiple groups (for review, [16]). All samples were stored at -80°C until use. Samples were derived from three groups of subjects (cerebellum: N=4-6 from subjects with autism, N=8 from control subjects; BA9: N=4-5 from subjects with autism, N=3 from control subjects; BA40: N=5-8 from subjects with autism, N=6 from control subjects). Consent from next of kin was given to the respective institutions. DSM-IV diagnoses were established prior to death by neurologists and psychiatrists using information from all available medical records and from family interviews. Details regarding the subject selection, diagnostic process, and tissue processing were collected by the Autism Research Foundation. Samples were matched for age, gender, and PMI. All demographic information is listed in Table 1. Seven out of nine subjects with autism had seizure disorder, and all subjects with autism displayed varying degrees of mental retardation (personal communication from Dr. Margaret Bauman). None of the controls had any known history of neuropsychiatric disorders, seizure disorder, or mental retardation.

SDS-PAGE and Western Blotting

Brain tissue (~40 mg per subject) was cut and placed on ice in lysis buffer (3 μl/mg of tissue) [20mM Tris pH 8.0, 0.2 mM EDTA, 150 mM NaCl, 3% Igepal.NP40 (v/v), 1% sodium deoxycholate (w/v), 0.1% SDS (w/v), 50 μl/ml leupeptin, 0.2 mM PMSF, 1 mM sodium orthovanadate, and aprotinin (Sigma, St. Louis, MO; A6279, 30μl/ml buffer)]. Tissue samples were homogenized using a Kontes hand pestle (Kimble-Kontes, Vineland, NJ, USA) while the temperature was maintained at 4°C. Following homogenization, an additional 1 μl of PMSF (0.2 mM) was added to each sample, and the samples were incubated on ice for 30 min. The homogenates were centrifuged for 20 min at 10,000 X *g* at 4°C. Supernatants were collected and assayed for total protein using the Bradford method (BioRad, Richmond, CA). Samples were stored at -86°C until used. Samples were mixed with denaturing SDS sample buffer (20%

glycerol, 100 mM Tris pH 6.8, 0.05% w/v Bromophenol blue, 2.5% SDS (w/v), 5% β-mercaptoethanol) and denatured by heating at 100°C for 5 minutes. SDS polyacrylamide gels were prepared with standard Laemmli solutions (BioRad) (resolving: 6%, stacking: 5%). Sixty μg of protein per lane was loaded onto the gel and electrophoresed for 15 min at 75V followed by 55 min at 150V at room temperature (RT). The proteins were electrotransferred onto nitrocellulose membranes for 2 hr at 300mAmp at 4°C. Protein blots were blocked with 0.2% I-Block (Tropix, Bedford, MA, USA) in PBS with 0.3% Tween 20 for 1hr at RT. The blots were then incubated with anti-GABBR1 (NB300-160, Novus Biologicals (Littleton, CO) 1:1,000), or anti-GABBR2 (56311, QED Bioscience Inc. (San Diego, CA) 1:1,000) for 20 hr at 4°C. Blots were subsequently washed with 0.3% Tween-PBS for 30 minutes, then incubated in secondary antibody for 1 hr at RT (A-9169, Sigma, goat anti-rabbit IgG 1:80,000). Blots were washed twice for 15 minutes with 0.3% Tween-PBS. The immune complexes were visualized using the ECL Plus detection system (Amersham Pharmacia Biotech, Arlington Hts., IL) and exposed to Hyperfilm ECL (Amersham Pharmacia Biotech). Sample densities were analyzed blind to diagnostic nature of the tissue using a BioRad densitometer and the BioRad Multi Analyst software. The molecular weights of approximately 108 kDa (GABBR1) and 105 kDa (GABBR2) immunoreactive bands were quantified with background subtraction. Results obtained are based on at least two independent experiments.

Statistical Analysis

All protein measurements for subjects with autism and control subjects were normalized against β-actin (Table 2). Possible confounding variables were compared between controls and autistics. Neither age nor PMI were found to be statistically different between groups ($t(40) = 0.94$, $p = 0.35$, $t(40) = 0.38$, $p = 0.71$, respectively) with effect sizes for the differences of 0.33 or smaller. We also examined gender and found no significant difference between the two groups (chi-square=0.91, p=0.34). Given this overall lack of difference on potential confounds, we chose to conduct group comparisons (independent group t-tests) without covariates. Significance criteria was set at $p<0.05$ and all tests were two-tailed. There was a significant difference on seizure status between groups. None of the controls had seizures, whereas 75% of the subjects with autism did (chi-square=28.6, $p<0.001$). Therefore, we conducted a second analysis examining subjects with autism comorbid with seizure disorder vs. controls using independent group t-tests. Significance criteria was again set at $p<0.05$ and all tests were two-tailed.

Results

All $GABA_B$ western-blotting experiments were normalized against β-actin and are shown as ratios of the various $GABA_B$ subunits to β-actin. In cerebellum, GABBR1 (108 kDa) was significantly reduced by 67% ($p<0.0049$) in subjects with autism. GABBR2 (105 kDa) was also significantly reduced in subjects with autism (46%, $p<0.026$) when compared with controls (Figure 1, Table 2). GABBR1 was significantly reduced in both BA40 (71%, $p<0.019$) and in BA9 (70%, $p<0.021$) in subjects with autism. In contrast, GABBR2 was not significantly altered in either area (Figure 1, Table 2), despite nonsignificant trends for reduction in both brain areas.

There was a significant difference on seizure status between subjects with autism and controls (chi-square=28.6, $p<0.001$). However, presence of seizure disorder in subjects with autism did not have an impact on the observed significant reductions of GABBR1 and GABBR2 (Table 3).

Discussion

Our results are the first to demonstrate changes in $GABA_B$ subunits in subjects with autism. We found that GABBR1 was significantly decreased in cerebellum, BA9, and BA40, while GABBR2 was significantly altered in cerebellum only. When comparing subjects with autism comorbid with seizure disorder vs. controls, there was no loss of significance, indicating that seizure disorder did not have a significant effect on the observed results.

Multiple laboratories have demonstrated altered expression of GABBR1 and GABBR2 in animal models for seizure disorders [13-15]. Han et al. [15] found that as a result of multiple seizures, there was a long-term decrease in GABBR1 (15 days) and GABBR2 (30 days) expression in rat hippocampus [15]. Following an injection of kainic acid into the dorsal hippocampus in mouse model of temporal lobe epilepsy, Straessle et al. [13] observed a rapid decline in GABBR1 and GABBR2 in CA1, CA3c, and hilus [13]. A study by Princivalle et al. [14] of the corticothalamic circuit, using Genetic Absence Epilepsy Rats from Strasbourg (GARES), found increased GABBR1 and GABBR2 protein expression in somatosensory cortex, ventrobasal, and reticular thalamic nuclei [14]. Moreover, knockout mice lacking GABBR1 exhibit epilepsy, enhanced prepulse inhibition, impaired memory, and die prematurely largely as a result of development of generalized seizures [17,18]. Taken together, these animal studies suggest that the changes in the number $GABA_B$ receptors may lead to epilepsy, due to changes in transmitter release (presynaptic) and inhibition (postsynaptic). There are limited studies of the expression of $GABA_B$ receptors in humans. Princivalle et al. [11] demonstrated altered expression for GABBR1A, GABBR1B, and GABBR2 in the hippocampus of subjects with temporal lobe epilepsy [11]. While we did not include hippocampus, making comparisons to the animal models difficult, we did demonstrate pervasive $GABA_B$ receptor downregulation, which may have a profound affect on the excitatory/inhibitory balance in the brains of subjects with autism and thus, involvement in the development of seizures.

The occurrence of seizure disorders with autism has been estimated anywhere from 4% to 44% [3]. This wide range is thought to be due to the heterogeneity of clinical populations [19]. For example, seizures are more frequent in individuals with autism that display mental retardation, and the type of language disorder is also relevant to the association of autism and seizure disorders [19]. Eplieptiform activity interferes with cognition by causing disturbances of vigilance, shifting attention, and sudden language difficulties [20], and these phenomena may also occur in children with autism and epilepsy. It may be that the regression in language and behavior frequently observed between ages two and three in children with autism may be due to epileptiform activity [19]. Alterations in $GABA_B$ receptors may partially explain the seizure disorders associated with autism. One of our subjects with autism died from seizures (Table 2), and, in total, seven of our subjects with autism were comorbid with seizure disorders. Levels of GABBR1 and GABBR2 proteins that were previously significantly different remained so using this analysis, indicating that presence of seizures did not account for reductions in levels of $GABA_B$ receptors. Future studies should include additional areas including the hippocampus and thalamus, and include data from children with autism with and without seizure disorder. Finally, decreases in levels of glutamic acid decarboxylase 65/67 kDa proteins in subjects with autism may be part of the GABAergic deficits seen in autism [21,22] and be connected mechanistically to the repression in $GABA_B$ receptors observed here.

Conclusion

Our laboratory is the first to show significant decreases in GABBR1 and GABBR2 subunits in subjects with autism when compared to normal controls. These changes may help explain the presence of seizure disorders and cognitive abnormalities in subjects with autism.

Acknowledgements

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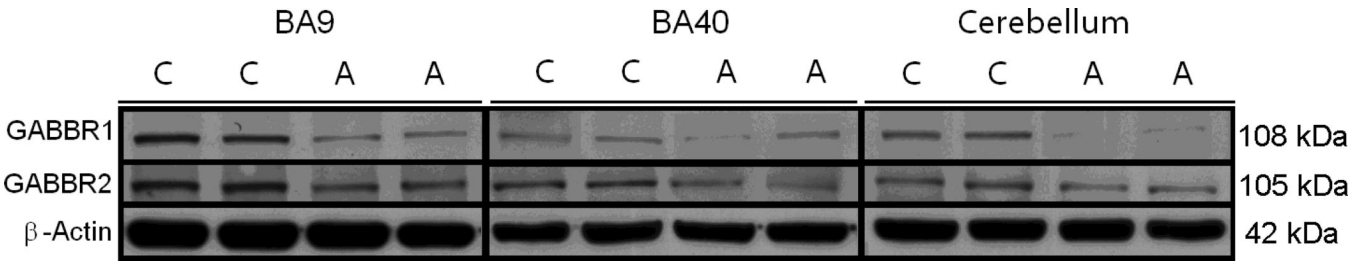

Figure 1.
Representative samples of GABBR1 (108 kDa), GABBR2 (105 kDa), and β-Actin (42 kDa) in BA, BA40, and cerebellum of subjects with autism (A) and matched controls (C).

Table 1

Demographic Data for Subjects with Autism and Controls

Case	Dx	Sex	Age	PMI (Hrs.)	Ethnicity	Medication History	Cause of Death	MR*	Seizure*	Brain Areas
B1078	Autistic	M	22	14.3	Caucasian	Dilantin, Tegretol, Phenobarbital, Theodure Cefobid,	Asphyxia	Yes	Yes	A40
B1045	Autistic	M	28	16.3	Caucasian	Urecholine, Duracef	Cardiac arrest	Yes	Yes	Cer, A40
B5000	Autistic	M	27	8.3	Caucasian	Synthroid	Drowning	Yes	No	Cer
B1401	Autistic	F	21	20.6	Caucasian	Tetracycline	Pneumonia, sepsis	Yes	Yes	Cer, A9, A40
B1664	Autistic	M	20	15	Caucasian	Vitamins B, C	Perforation of ulcer; asphyxia	Yes	Yes	Cer, A9, A40
B2825	Autistic	M	19	9.5	Caucasian	None	Seizure	Yes	Yes	Cer, A9, A40
B3511	Autistic	M	29	15	Caucasian	None	Hit by train	Yes	Yes	Cer, A9, A40
B3845	Autistic	M	30	28.4	Caucasian	Mellaril, Phenobarbital, Dilantin	Shock; acute pancreatitis	Yes	Yes	A9, A40
B1484	Autistic	M	19	15	Caucasian	None	Burns	Yes	No	A9, A40
B3829	Control	M	22	24.3	Caucasian	None	MVA	No	No	Cer
B4267	Control	M	26	20	African-American	None	MVA	No	No	Cer
B4268	Control	M	30	22	African-American	None	Cardio-myopathy	No	No	Cer, A40
B4269	Control	M	28	24	Caucasian	Lidocaine 12.0 mg/L found in blood	Areterio-sclerotic cardiovascular disease	No	No	Cer, A9, A40
B4272	Control	M	19	17	Caucasian	None	Accident; chest injuries	No	No	Cer
B4275	Control	M	20	16	Caucasian	None	Accident	No	No	Cer, A9, A40
B4279	Control	F	20	21	Caucasian	None	MVA	No	No	Cer
B4362	Control	M	30	20	African-American	None	MVA	No	No	Cer, A9
B4101	Control	M	24	5	Unknown	None	Gun shot wound	No	No	Cer, A40
B4271	Control	M	19	21	African-American	EtOH, Advil, Amoxapine	Epiglottitis	No	No	A40
B4363	Control	M	21	9	Caucasian	None	MVA	No	No	Cer, A40

Dx, diagnosis; Hrs, hours; PMI, postmortem interval; M, male; F, female; EtOH, alcohol; MVA, motor vehicle accident; MR, Mental retardation

*Communication from Dr. M. Bauman.

Table 2

Expression of GABBR1 and GABBR2 in Cerebellum, BA40, and BA9 in Subjects with Autism vs. Controls

Cerebellum	**Control**	**Autistic**	**Change**	**P***
GABBR1 / β-Actin	0.051±0.018	0.017±0.006	↓ 67%	**0.0049**
GABBR2 / β-Actin	0.068±0.028	0.037±0.012	↓ 46%	**0.026**
BA40	**Control**	**Autistic**	**Change**	**P**
GABBR1 / β-Actin	0.079±0.049	0.023±0.026	↓ 71%	**0.019**
GABBR2 / β-Actin	0.29±0.27	0.061±0.038	↓ 79%	0.104
BA9	**Control**	**Autistic**	**Change**	**P**
GABBR1 / β-Actin	0.076±0.023	0.023±0.024	↓ 70%	**0.021**
GABBR2 / β-Actin	0.115±0.016	0.053±0.042	↓ 54%	0.064

* two-tailed independent group t-test

Table 3

Impact of Seizure on Expression of GABBR1 and GABBR2 in Cerebellum, BA40, and BA9 in Subjects with Autism vs. Control

Cerebellum	**Control**	**Autistic**	**Change**	**P***
GABBR1 / β-Actin	0.051±0.018	0.015±0.005	↓ 71%	**0.013**
GABBR2 / β-Actin	0.068±0.028	0.037±0.013	↓ 46%	**0.050**
BA40	**Control**	**Autistic**	**Change**	**P**
GABBR1 / β-Actin	0.079±0.049	0.026±0.027	↓ 67%	**0.034**
GABBR2 / β-Actin	0.29±0.27	0.061±0.038	↓ 79%	0.104
BA9	**Control**	**Autistic**	**Change**	**P**
GABBR1 / β-Actin	0.076±0.023	0.028±0.024	↓ 63%	**0.044**
GABBR2 / β-Actin	0.115±0.016	0.067±0.038	↓ 42%	0.115

* two-tailed independent group t-test