Altered GABA\textsubscript{A} Receptor Subunit Expression and Pharmacology in Human Angelman Syndrome Cortex

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ABSTRACT

The neurodevelopmental disorder Angelman syndrome is most frequently caused by deletion of the maternally-derived chromosome 15q11-q13 region, which includes not only the causative UBE3A gene, but also the $\beta_3$-$\alpha_5$-$\gamma_3$ GABA$_A$ receptor subunit gene cluster. GABAergic dysfunction has been hypothesized to contribute to the occurrence of epilepsy and cognitive and behavioral impairments in this condition. In the present study, analysis of GABA$_A$ receptor subunit expression and pharmacology was performed in cerebral cortex from four subjects with Angelman syndrome and compared to that from control tissue. The membrane fraction of frozen postmortem neocortical tissue was isolated and subjected to quantitative Western blot analysis. The ratios of $\beta_3/\beta_2$ and $\alpha_5/\alpha_1$ subunit protein expression in Angelman syndrome cortex were significantly decreased when compared with controls. An additional membrane fraction was injected into Xenopus oocytes, resulting in incorporation of the brain membrane vesicles with their associated receptors into the oocyte cellular membrane. Two-electrode voltage clamp analysis of GABA$_A$ receptor currents was then performed. Studies of GABA$_A$ receptor pharmacology in Angelman syndrome cortex revealed increased current enhancement by the $\alpha_1$-selective benzodiazepine site agonist zolpidem and by the barbiturate phenobarbital, while sensitivity to current inhibition by zinc was decreased. GABA$_A$ receptor affinity and modulation by neurosteroids were unchanged. This shift in GABA$_A$ receptor subunit expression and pharmacology in Angelman syndrome is consistent with impaired extrasynaptic but intact to augmented synaptic cortical GABAergic inhibition, which could contribute to the epileptic, behavioral, and cognitive phenotypes of the disorder.
INTRODUCTION

Angelman syndrome is a severe neurodevelopmental disorder consisting of microcephaly, mental retardation, speech impairment, ataxia, epilepsy, and an apparently happy and excitable demeanor [34]. The cause of the condition is the loss of expression of the maternal copy of the imprinted gene UBE3A (ubiquitin protein ligase E3A), either through deletion of the maternally-inherited chromosome 15q11-13 region (70%), paternal uniparental disomy (2-5%), an imprinting defect (5%), or UBE3A mutation (5-10%) [18]. Epilepsy is extraordinarily common in Angelman syndrome, developing in up to 90% of those with the condition [24, 31].

The GABA\textsubscript{A} receptor is a ligand-gated heteropentameric chloride channel whose activation causes membrane hyperpolarization and decreased neuronal excitability. In addition to the traditional "phasic" form of fast inhibition mediated by synaptic GABA\textsubscript{A} receptors, primarily of the $\alpha_1\beta_2\gamma_2$ composition in cortex, "tonic" GABA\textsubscript{A} currents also serve to regulate neuronal excitability. The tonic GABA\textsubscript{A} current is a continuous inhibitory current mediated by high-affinity, extrasynaptic receptors that are activated by ambient levels of GABA, and in the neocortex is expected to be mediated primarily by receptors of the $\alpha_5\beta_3\gamma_2$ and $\alpha_4\beta_2\delta$ subunit compositions [7].

The potential involvement of GABA\textsubscript{A} receptors in the pathogenesis of epilepsy in Angelman syndrome has been suggested by multiple factors. First, the most common cause of Angelman syndrome, deletion of the maternally-inherited chromosome 15q11-13 region containing the imprinted UBE3A gene, also results in deletion of the non-imprinted GABA\textsubscript{A} $\beta_3$, $\alpha_5$, and $\gamma_3$ subunit genes [18]. Second, multiple studies have demonstrated that individuals with Angelman syndrome due to 15q11-13 deletion have
more severe epilepsy than those with uniparental disomy, imprinting defects, or UBE3A mutations, all of which would be expected to spare the GABA\textsubscript{A} subunit genes [20]. A final line of evidence has come from studies of GABA\textsubscript{A} \(\beta_3\) subunit knockout mice, which demonstrate high voltage EEG slowing and spontaneous myoclonic and clonic seizures [5, 9].

Despite the hypothesized dysfunction of the GABAergic inhibitory system in Angelman syndrome, very few studies have been done in human brain tissue to directly address this question. A postmortem study revealed unchanged GABA levels in the cerebral cortex of an individual with Angelman syndrome as compared with control tissues [15]. Analysis of GABA\textsubscript{A} benzodiazepine receptor binding by positron emission tomography scanning of individuals with Angelman syndrome has produced divergent results, indicating either decreased [11, 22] or increased [1] binding levels. Finally, a decrease in GABA\textsubscript{A} \(\beta_3\) subunit mRNA and protein expression in Angelman cortex has been reported, although expression of other GABA\textsubscript{A} receptor subunits and functional consequences of this reduction were not determined [10, 29]. In the present study, we analyzed protein expression levels of several critical GABA\textsubscript{A} receptor subunits in human Angelman syndrome and control cortex, and then assessed the pharmacologic properties associated with the detected alterations. To do this, we utilized a unique experimental paradigm that allows electrophysiologic analysis of ion channels derived from frozen postmortem tissues.

**MATERIALS AND METHODS**
Western Blot Analysis

Rabbit polyclonal antibodies against GABA_A receptor subunits α_5 and β_3 were obtained from Novus Biologicals, rabbit polyclonal antibodies against the β_2 subunit were from Abcam, and mouse monoclonal α_1 subunit antibody was from Millipore. Other details regarding these antibodies are provided in Supplemental Table 1. Frozen cortical tissue specimens (100-200 mg) were processed to isolate the membrane fraction and subjected to Western blot analysis as detailed in [14]. Infrared fluorescence was used for signal detection and quantitation (Odyssey Infrared Imaging System, LI-COR Biosciences).

Oocyte preparation and injection

Membrane isolation from an additional portion (50-300 mg) of each frozen cortical specimen was performed using the method of [19], with modifications, as detailed in [13]. *Xenopus laevis* oocyte collection and injection procedures were as described previously [13].

Electrophysiology

Injected oocytes (1-3 days after injection) were placed in a recording chamber and bathed in oocyte Ringer’s solution containing (in mM) NaCl 82.5, KCl 2.5, CaCl_2 2.5, MgCl_2 1, and Hepes 5 (pH 7.4). Two-electrode voltage-clamp recordings were made at room temperature using electrodes with a resistance of 0.5 – 2.5 MΩ filled with 3 mM KCl. Oocyte membrane potential was held at ~80 mV. Solutions were applied using a gravity-driven, valve-controlled perfusion system.
Data analysis

Graphing and curve-fitting were carried out using Excel (Microsoft) and OriginPro 8 software (OriginLab Corporation). Statistical analysis was performed using OriginPro 8 and InStat (GraphPad Software).

RESULTS

Cortical specimens

Frozen postmortem human cortical tissue was acquired from the NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland. Although there are many limitations inherent to the use of postmortem tissue, in this and our previous studies [14], GABA<sub>A</sub> receptor subunit protein expression and pharmacologic properties were not correlated with postmortem interval (data not shown). Four samples were obtained from individuals with Angelman syndrome and were analyzed along with four age-matched control specimens obtained from individuals with non-neurologic causes of death. Supplemental Table 2 lists basic clinical details for each specimen. All Angelman syndrome patients were noted to have epilepsy and cognitive impairment. Chromosome 15q11-q13 deletion was identified in Angelman Cases 1, 3, and 4; genetic information was not available for Case 2. Medications at time of death were available for Case 1 (clorazepate) and Case 2 (topiramate and clonazepam) only.

GABA<sub>A</sub> receptor subunit expression in Angelman syndrome
The expression levels of several GABA\textsubscript{A} receptor subunits were analyzed by quantitative Western blotting of cortical membranes isolated from the Angelman and control autopsy specimens. Selected for analysis were the \(\beta_3\) and \(\alpha_5\) subunits, which contribute to cortical extrasynaptic \(\alpha_5\beta_3\gamma_2\) GABA\textsubscript{A} receptors [3, 21] and are deleted from the maternal 15q11-q13 region along with the UBE3A gene in the majority of Angelman syndrome cases. Also analyzed were the \(\beta_2\) and \(\alpha_1\) subunits, which contribute to cortical synaptic GABA\textsubscript{A} receptors, most of which are of the \(\alpha_1\beta_2\gamma_2\) composition [3, 21]. Expression of the \(\gamma_3\) subunit, which is also usually heterozygously deleted in Angelman cases, was not assessed as this subunit is present at extremely low levels in human cortex [23]. As shown in a representative Western blot (Fig. 1A) and graphs summarizing subunit expression levels relative to \(\beta\)-tubulin (Fig. 1B), expression of the \(\beta_3\) subunit was not substantially altered in the cortical specimens obtained from the individuals with Angelman syndrome as compared with the controls. In contrast, expression of the \(\beta_2\) subunit was markedly elevated. Alpha5 subunit expression was reduced in the Angelman syndrome samples, while levels of the \(\alpha_1\) subunit were unchanged. As a result of these alterations, the ratio of \(\beta_3\) to \(\beta_2\) subunit expression, as well as the ratio of \(\alpha_5\) to \(\alpha_1\), was significantly reduced in Angelman syndrome cortex as compared with age-matched control cortex (Fig. 1C). This indicates a relative deficit in expression of protein subunits that make up cortical extrasynaptic \(\alpha_5\beta_3\gamma_2\) GABA\textsubscript{A} receptors in Angelman syndrome as compared with the subunits that compose cortical synaptic \(\alpha_1\beta_2\gamma_2\) GABA\textsubscript{A} receptors.

\textit{GABA\textsubscript{A} receptor pharmacology in Angelman syndrome}

The alterations of cortical GABA\textsubscript{A} receptor subunit expression in Angelman
syndrome detected above predict several pharmacologic changes. To investigate this, we utilized the technique of injection of brain cellular membrane preparations into the *Xenopus* oocyte, which results in the “microtransplantation” of human GABA\textsubscript{A} receptors in their native configuration into the oocyte plasma membrane [19]. With this method, the appearance of receptor currents does not depend on mRNA translation, but instead arises from incorporation into the oocyte membrane of the injected membrane vesicles harboring native receptors. This allows analysis by standard two-electrode voltage clamp electrophysiology. The advantages and limitations of this technique are discussed in detail elsewhere [13]. Of particular note, absolute current amplitudes measured using this method do not accurately reflect those present in the source tissue; rather, this technique is ideal for determining relative receptor responses to pharmacologic manipulations [19].

GABA dose-response curves were generated from oocytes expressing receptors from control and Angelman syndrome cortex (Fig. 2A). Although there was a trend towards higher GABA\textsubscript{A} receptor affinity in the Angelman specimens, the differences in half-maximal effective GABA concentration (EC\textsubscript{50}) did not reach statistical significance (Control = 96.4 \pm 10.4 \textmu M, Angelman = 69.0 \pm 7.8 \textmu M, \textit{P} > 0.05). We next assessed responses to exogenous and endogenous agents known to modulate GABA\textsubscript{A} currents. Zolpidem is a benzodiazepine-site agonist with selectivity for \(\alpha_1\beta_2\gamma_2\) receptors that is used clinically in the treatment of insomnia. In contrast, diazepam is a non-selective benzodiazepine used in the treatment of seizures and anxiety which acts equally on \(\alpha_1\beta_2\gamma_2\) and \(\alpha_5\beta_3\gamma_2\) receptors. GABA\textsubscript{A} current enhancement by zolpidem was significantly increased in Angelman syndrome cortex as compared with controls (Fig. 2B,C), while enhancement by diazepam was equivalent (Fig. 2D). GABA\textsubscript{A} receptor current is directly
inhibited by endogenous zinc, which acts with low affinity on synaptic $\alpha_1\beta_2\gamma_2$ receptors and with higher affinity on extrasynaptic $\alpha_5\beta_3\gamma_2$ receptors [4]. The degree of $\text{GABA}_A$ receptor current inhibition by zinc was significantly less in Angelman syndrome cortex than in control cortex (Fig. 3A,B). These pharmacologic findings are consistent with a relative predominance of synaptic $\alpha_1\beta_2\gamma_2$ receptors over extrasynaptic $\alpha_5\beta_3\gamma_2$ receptors in Angelman syndrome, as suggested by the protein expression studies above.

We next analyzed responses to the barbiturate phenobarbital and the endogenous neurosteroid 5$\alpha$-pregnan-3$\alpha$-ol-20-one (5$\alpha$3$\alpha$), both of which enhance $\text{GABA}_A$ receptor currents and function as anticonvulsant and sedative agents. Based on studies of recombinant receptors, these agents should act with similar efficacy on synaptic $\alpha_1\beta_2\gamma_2$ and extrasynaptic $\alpha_5\beta_3\gamma_2$ combinations [2, 32]. Interestingly, current augmentation by phenobarbital was substantially larger in Angelman syndrome cortex as compared with control cortex (Fig. 3C,D). In contrast, there was no difference in the degree of $\text{GABA}_A$ current potentiation by the neurosteroid 5$\alpha$3$\alpha$ (Supplemental Figure 1). Unfortunately, none of the currently available $\text{GABA}_A$ receptor agonists or antagonists are able to discriminate between $\beta_2$ and $\beta_3$-subunit containing receptors [8], so the relative prevalence of these subtypes could not be directly assessed pharmacologically.

DISCUSSION

The present study represents the first to systematically analyze $\text{GABA}_A$ receptor subunit expression and pharmacology in human Angelman syndrome cortex. This is despite the frequently stated hypothesis that reduced $\text{GABA}_A$ receptor function is a prominent mechanism contributing to the development of epilepsy and cognitive
impairment in the disorder. Our study has demonstrated that GABA$_A$ $\beta_3/\beta_2$ and $\alpha_5/\alpha_1$ subunit expression ratios are decreased in Angelman syndrome cortex as compared with age-matched control cortex. These reductions predict relative impairment of inhibition by extrasynaptic GABA$_A$ $\alpha_5\beta_3\gamma_2$ receptors, but unchanged to increased inhibitory function by synaptic GABA$_A$ $\alpha_1\beta_2\gamma_2$ receptors. These alterations in subunit expression patterns were accompanied by changes in receptor pharmacology, with increased efficacy of the $\alpha_1$-selective benzodiazepine site agonist zolpidem, and decreased efficacy of zinc, which acts preferentially on $\alpha_5\beta_3\gamma_2$ as compared with $\alpha_1\beta_2\gamma_2$ receptors. GABA$_A$ current enhancement by phenobarbital, which acts equally on all receptor subunit combinations, was substantially increased, while that of the neurosteroid 5a3a was unchanged.

Based on the heterozygous deletion of chromosome 15q11-q13, including the non-imprinted GABA$_A$ $\beta_3$-$\alpha_5$-$\gamma_3$ gene cluster, one would predict a 50% reduction in $\beta_3$ expression and no change in $\beta_2$, which is located on chromosome 5q in a cluster with the $\alpha_6$-$\alpha_1$-$\gamma_2$ subunit genes. Instead, what we found was a marked increase in $\beta_2$ expression with no significant change in $\beta_3$ expression. We feel the most likely explanation for this finding is that expression of both $\beta_2$ and $\beta_3$ subunits have been reported to be elevated in temporal lobe epilepsy patients, possibly as a consequence of repeated seizures [25]. In Angelman syndrome patients, this would predominantly be manifested as $\beta_2$ elevation, given underlying deficits in $\beta_3$ expression. Other possible explanations include effects of antiepileptic drugs [27] or compensatory overexpression of $\beta_2$ during development in the setting of relative $\beta_3$ deficiency, similar to that observed in $\alpha_1$ subunit knockout mice [16].
The demonstrated alterations in Angelman syndrome patients would be predicted to have complex clinical effects, due to the divergent functions of \( \beta_3 \)- and \( \beta_2 \)-containing GABA\(_{A}\) receptors in the brain. Receptors containing \( \beta_3 \) subunits seem to be primarily responsible for mediation of the anesthetic effects of GABA\(_{A}\) receptor agonists, while those containing \( \beta_2 \) subunits mediate the sedative effects of these agents [28]. The anticonvulsant effects of non-selective GABA\(_{A}\) agonists are mediated by both \( \beta_3 \)- and \( \beta_2 \)-containing receptors [6]. This would predict that administration of non-selective GABA\(_{A}\) agonists to individuals with Angelman syndrome would have reduced anesthetic effects but unchanged to enhanced sedative and anticonvulsant effects. In keeping with this hypothesis, benzodiazepine medications are among the most commonly prescribed to individuals with Angelman syndrome and epilepsy, and are found to be efficacious and well-tolerated [31]. In addition, our results suggest that those affected by Angelman syndrome may be more sensitive to GABAergic agents used to treat insomnia. It is interesting to consider the clinical implications of our results for phenobarbital, which demonstrated considerably increased current enhancement in Angelman syndrome cortex as compared with control cortex. Phenobarbital is commonly prescribed for the management of epilepsy in Angelman syndrome, although reports have indicated limited effectiveness in monotherapy [31, 33]. In addition, phenobarbital exhibits an adverse side effect profile including lethargy, which individuals with Angelman syndrome may be more sensitive to given our findings.

Comparison of our results to those found in GABA\(_{A}\) \( \beta_3 \) homozygous or heterozygous knockout mice, a purported model of Angelman syndrome, reveals both similarities and areas of divergence. The heterozygous condition would be expected to be
more analogous to the human disorder. Homozygous β3 knockout mice exhibited significantly reduced GABA_A and benzodiazepine receptor binding, markedly decreased current amplitudes, and unchanged to increased GABA affinity, while heterozygotes had unchanged to slightly decreased GABA_A and benzodiazepine receptor binding, unchanged to minor decreases in current amplitudes, and no change in GABA affinity [12, 17, 30]. Our current study on human Angelman cortex also identified no change in GABA affinity or modulation by the benzodiazepine diazepam. Studies in cultured cortical neurons from homozygous β3 knockout mice revealed no change in α1 subunit expression and increased current potentiation by zolpidem [26], similar to what we have found in the present study using human tissue.

In conclusion, our studies of GABA_A receptor subunit expression and pharmacology in Angelman syndrome reveal alterations that predict impairment of cortical extrasynaptic, but unchanged to enhanced synaptic, GABAergic activity. While these changes are unlikely to explain the development of epilepsy and neurodevelopmental impairments in this disorder, they may modify their characteristics and responsiveness to GABAergic medications.
Legends to Figures

**Figure 1.** GABA<sub>A</sub> receptor subunit expression is altered in Angelman syndrome cortex.

A) Representative Western blot demonstrating GABA<sub>A</sub> receptor subunit β<sub>3</sub>, β<sub>2</sub>, α<sub>5</sub>, and α<sub>1</sub> protein expression in control and Angelman syndrome cortical specimens. Antibody staining of β-tubulin is shown as a control for protein loading. B) Fluorescence intensities for the receptor subunits normalized to the intensity of β-tubulin. Each connected pair of squares represents the average normalized band intensities from a single blot, while the asterisks represent the average of all blots performed. C) Graph depicting the ratios of the fluorescence intensities of the β<sub>3</sub>/β<sub>2</sub> subunits and of the α<sub>5</sub>/α<sub>1</sub> subunits in Angelman syndrome cortex normalized to the ratios measured in control specimens on the same blot. Average values determined from five (β<sub>3</sub>/β<sub>2</sub>) or four (α<sub>5</sub>/α<sub>1</sub>) separate blots ± SEM are shown. *P < 0.05 versus corresponding control, one sample t-test.

**Figure 2.** GABA<sub>A</sub> current enhancement by zolpidem is increased in oocytes incorporating GABA<sub>A</sub> receptors from individuals with Angelman syndrome, while GABA affinity and current enhancement by diazepam are unchanged. A) Dose-response curves illustrating current produced by increasing GABA concentrations. Each point represents the average current ± SEM normalized to that produced by 1000 μM GABA. B) Representative traces from an oocyte incorporating receptors from a Control individual and an individual with Angelman syndrome showing current generated by 30 μM GABA in the absence and presence of 100 nM zolpidem. The traces obtained in the presence of GABA alone are equivalently scaled to allow direct comparisons of the degree of modulation by the agents of interest. C) Enhancement of GABA<sub>A</sub> receptor
currents by 10-100 nM zolpidem in control and Angelman syndrome specimens. D) Enhancement of GABA<sub>A</sub> receptor currents by 100-1000 nM diazepam. In this and all subsequent figures results were obtained from four Control and four Angelman syndrome specimens (at least 4 injected oocytes analyzed per specimen). *P < 0.05 versus corresponding Control, two-way repeated measures ANOVA with Tukey post-tests.

**Figure 3.** GABA<sub>A</sub> current inhibition by zinc is reduced in oocytes incorporating GABA<sub>A</sub> receptors from individuals with Angelman syndrome, while enhancement by phenobarbital is increased. A) Representative traces showing current generated by 100 μM GABA in the absence and presence of 100 μM ZnCl<sub>2</sub>. B) Inhibition of GABA<sub>A</sub> receptor currents by 1-100 μM ZnCl<sub>2</sub> in Control and Angelman syndrome specimens. C) Representative traces showing current generated by 30 μM GABA in the absence and presence of 1 mM phenobarbital. D) Enhancement of GABA<sub>A</sub> receptor currents by 30-1000 μM phenobarbital in Control and Angelman syndrome specimens. *P < 0.05 versus corresponding Control.
REFERENCES


Figure 3

(A) Control
100 μM GABA

GABA +
100 μM ZnCl₂

Angelman

50 nA
5 sec

(B) Percent of 100μM GABA alone

Control
Angelman

* * *

(C) Control
30 μM GABA

GABA +
1 mM Phenobarbital

Angelman

20 nA
5 sec

(D) Percent of 30μM GABA alone

Control
Angelman

* * *