

Altered Expression of Genes Involved in GABAergic Transmission and Neuromodulation of Granule Cell Activity in the Cerebellum of Schizophrenia Patients

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Objective: Deficits in gamma-aminobutyric acid (GABA) signaling have been described in the prefrontal cortex, limbic system, and cerebellum in individuals with schizophrenia. The purpose of the present study was to further investigate cerebellar gene expression alterations as they relate to decreases in GABAergic transmission by examining the expression of GABAergic markers, *N*-methyl-D-aspartic-acid (NMDA) receptor subunits, and cerebellum neuromodulators in individuals with schizophrenia.

Method: Subjects were postmortem men with a diagnosis of schizophrenia (N=13) and a postmortem interval-matched non-psychiatric male comparison group (N=13). The authors utilized real-time-quantitative polymerase chain reaction (PCR) to measure mRNA levels of the following GABAergic markers: glutamic acid decarboxylase (GAD) 65 and 67; GABA plasma membrane transporter-1 (GAT-1); GABA type A (GABA_A) receptor subunits α_6 , β_3 , and δ ; and parvalbumin. In addition, real-time-quantitative PCR was utilized to assess mRNA levels of the NMDA receptor (NR) subunits NR1, NR2-A, NR2-B, NR2-C, and NR2-D as well as the cerebellar neuromodulators glutamate

receptor (GluR)-6, kainate-preferring glutamate receptor subunit-2 (KA2), metabotropic glutamate receptor (mGluR)-2 and mGluR3, and neuronal nitric oxide synthase. Measurements for mRNA levels were determined using lateral cerebellar hemisphere tissue from both schizophrenia and comparison subjects.

Results: Schizophrenia subjects showed significant decreases in mRNA levels of GAD₆₇, GAD₆₅, GAT-1, mGluR2, and neuronal nitric oxide synthase. Increases in GABA_A- α_6 and GABA_A- δ as well as GluR6 and KA2 were also observed. Medication effects on the expression of the same genes were examined in rats treated with either haloperidol (Sprague-Dawley rats [N=16]) or clozapine (Long-Evans rats [N=20]). Both haloperidol and clozapine increased the levels of GAD₆₇ in the cerebellum and altered the expression of other cerebellar mRNAs.

Conclusions: These findings suggest that GABA transmission is decreased in the cerebellar cortices in individuals with schizophrenia and additional gene expression changes may reflect an attempt to increase GABA neurotransmission at the cerebellar glomerulus.

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One of the most consistent molecular findings from schizophrenia research is the dysfunction of specific subsets of gamma-aminobutyric acid (GABA) expressing interneurons (1–4). Reductions in mRNA and protein levels of glutamic acid decarboxylase 67 (GAD₆₇) have been found in specific interneurons of the prefrontal cortex (5–7) and limbic system in individuals with schizophrenia (8, 9), and similar findings have been observed in analyses of whole cerebellar tissue (10, 11). The same prefrontal cortex interneurons with GAD₆₇ reductions have also shown decreases in the presynaptic GABA plasma membrane transporter-1 (GAT-1) (12). Compensatory postsynaptic changes, such as increases in GABA type A (GABA_A)- α_2 receptor density and GABA_A receptor radioligand binding, in the prefrontal cortex and anterior cingulate cortex have

also been reported (13–15). In addition, single nucleotide polymorphisms (SNPs) in the promoter region of the GAD₆₇ gene expression have been associated with gray matter reductions in patients with childhood onset schizophrenia (16) and decreased cognitive functioning in adult schizophrenia (17). Given the role of GABAergic interneurons in the modulation of excitatory output, dysfunction in specific interneurons may mediate some of the positive, negative, and cognitive symptoms seen in schizophrenia.

Aside from its role in motor coordination, the cerebellum has been shown to contribute to cognitive functioning (18) through connections of the posterior lateral hemispheres to the prefrontal cortex (19). Thus, physiological deficits in this brain region could contribute to the

This article is featured in this month's AJP **Audio** and is discussed in an editorial by Dr. Akbarian (p. 1507).

pathophysiology of schizophrenia. Supporting this hypothesis, neuroimaging studies have described increased blood flow and glucose consumption in the cerebellum among schizophrenia patients (20–22). Molecular studies have revealed decreased cerebellar expression of the GABAergic protein reelin and the GABA synthesizing enzymes GAD₆₇ and GAD₆₅ (10, 11) as well as increased expression of the axonal chemorepellent Semaphorin 3A (23) and increased expression of granule cell-selective, activity-dependent, brain-derived neurotrophic factor, and growth-associated protein of 43 kDa (GAP-43) genes (24). To further explore the function of cerebellar interneurons in schizophrenia, we investigated changes in transcript levels of GABAergic markers, *N*-methyl-D-aspartate (NMDA) receptor subunits, and local circuit neuromodulators of GABA release using total RNA prepared from the posterior lateral cerebellar hemispheres of post-mortem male schizophrenia subjects and age- and post-mortem interval-matched male comparison subjects. Alterations in specific GABAergic transcripts point to a common pathophysiological mechanism across different brain regions and implicate the cerebellum in the cognitive and motor dysfunction observed in individuals with schizophrenia.

Method

Human Subjects

Subjects were postmortem men (N=13) with a diagnosis of schizophrenia (according to DSM-IV criteria) and a sex-, age-, and postmortem interval-matched comparison group (N=13). Cerebellar tissue from both schizophrenia and comparison subjects was acquired from the Maryland Brain Collection (Table 1). All subjects were between 25 and 65 years of age at the time of death, with a postmortem interval ≤ 24 hours. None of the subjects had a history of alcohol abuse or dependence. No significant differences were found between schizophrenia and comparison subjects for the following factors: 1) mean age (schizophrenia group: 43.5 years [SD=11], range=31–62 years; comparison group: 40.9 years [SD=8], range=27–50 years); 2) mean postmortem interval (schizophrenia group: 12.1 hours [SD=5.1], range=5–21 hours; comparison group: 16.5 hours [SD=5.4], range=6–24 hours); 3) mean brain pH (schizophrenia group: 6.53 [SD=0.2], range=6.21–6.74; comparison group: 6.50 [SD=0.1], range=6.29–6.64); 4) mean RNA integrity number (schizophrenia group: 7.98 [SD=0.12], range=7.30–8.50; comparison group: 7.73 [SD=0.11], range=7.00–8.60); and 5) mean freezer storage time (schizophrenia group: 71 months [SD=24], range=27–157 months; comparison group: 49 months [SD=29], range=10–100 months). At the time of death, six subjects were receiving typical antipsychotics and seven were receiving atypical antipsychotics. Neither schizophrenia nor comparison subjects were receiving medications for mood disorders at the time of death. The subjects' cerebellar tissue originated from the lateral cerebellar hemisphere corresponding to crus I of lobule VIIa, which has projections to the prefrontal cortex (19). Specimens were dissected at -20°C and frozen at -80°C until ready for use.

Haloperidol-Treated Rats

The RNA from the cerebellum of Sprague-Dawley rats (N=16) treated with haloperidol was acquired from a previous study

(25). The rats were injected (intramuscular) with either haloperidol depot (38 mg/kg/month [corresponding to approximately 1 mg/kg/day]) or sesame oil (vehicle [comparison group]). After a 7-day acclimation period, the rats were injected once a month for 6 months. They were anesthetized using isoflurane and decapitated 1 month after the last injection. The cerebellum of each rat was removed intact, frozen on dry ice, and stored at -80°C .

Clozapine-Treated Rats

Adult male Long-Evans rats (N=20) were injected (intraperitoneal) with either clozapine (10 mg/kg/day), dissolved in 0.4% acetic acid in 0.9% saline, or a vehicle (comparison group) for 21 days. This paradigm was modified from methods described by Bai et al. (26), which suggested a maximum D₂ receptor occupancy for clozapine. The cerebellum of each rat in both the clozapine-treated group and vehicle-treated comparison group was removed 18 hours after the last injection, and the lateral hemispheres were dissected and frozen at -80°C .

Quantitative Real-Time Polymerase Chain Reaction (PCR)

All experimental procedures and data analyses were performed blind to the diagnosis. The cerebellar tissue from both human and rat subjects was homogenized using a Polytron homogenizer (Brinkmann Instruments, Westbury, N.Y.). Total RNA was isolated using TriReagent (Sigma, St. Louis) and analyzed with Bioanalyzer 2100 (Agilent Technologies, Santa Clara, Calif.). As detailed in Table 1, all RNA integrity number values were ≥ 7.0 . The cDNA was synthesized from 4 μg of the total RNA using MMLV Reverse Transcriptase (Promega, Madison, Wisc.). Real-time-quantitative PCRs were run via an Applied Biosystems (Foster City, Calif.) 7000, 7300, or 7500 fast-quantitative real-time PCR machine. Gene expression levels for all human tissue samples as well as those for tissue samples of neuroleptic-treated rats were examined using SYBR Green (Applied Biosystems, Foster City, Calif.), with the exception of the GABAergic markers GABA_A α_6 and GABA_A δ , which were analyzed via TaqMan Assays-on-Demand probes (Applied Biosystems, Foster City, Calif.). All gene expression levels were examined according to the manufacturers' protocols. Exon spanning primer pairs (Operon, Huntsville, Ala.) specific to 1) GABAergic markers GABA_A β_3 , GAD₆₇, GAD₆₅, parvalbumin, and GAT-1; 2) NMDA receptor (NR) subunits NR1, NR2-A, NR2-B, NR2-C, and NR2-D; and 3) cerebellar neuromodulators metabotropic glutamate receptor (mGluR)-2 and mGluR3, kainate-preferring glutamate receptors GluR6 and KA2, and neuronal nitric oxide synthase were designed using Primer Express 3.0 (Applied Biosystems, Foster City, Calif. [see the table in the data supplement accompanying the online version of this article]). Expression levels of genes of interest were normalized to those of β -actin, and expression levels were reconfirmed using cyclophilin for selected genes. Validation curves for all primer sets were run with housekeeping genes and shown to have a linear slope of $<|0.1|$. Dissociation curves for all SYBR Green primer sets showed no evidence of dimerization.

Data and Statistical Analysis

Cerebellar tissue samples from the 13 schizophrenia subjects and 13 comparison subjects were run in triplicate in three separate plates and compared with β -actin or cyclophilin on the same plate. The relative levels of expression of each mRNA were corrected by housekeeping genes according to a previously described method (24), and the relative GABAergic transcript levels for schizophrenia subjects versus comparison subjects were calculated using the $2^{-\Delta\Delta\text{Ct}}$ method (27). The averaged values per tissue sample were entered into Prism 4.0 (GraphPad Software, San Diego) and analyzed using the Wilcoxon Signed-Rank Test

TABLE 1. Demographic and Clinical Characteristics of Postmortem Male Schizophrenia Subjects and Age- and Postmortem Interval-Matched Male Comparison Subjects

Matched Pairs ^a	Subject Code ^b	Race	Age (years)	Postmortem Status for Schizophrenia (N=13) and Comparison (N=13) Subjects					
				Postmortem Interval (hours)	Brain pH	RNA Integrity Number	Cerebellar Tissue Freezer Storage Time (months)	Antipsychotic Treatment at the Time of Death	Cause of Death
Schizophrenia subjects									
1	42a	African American	33	12	6.21	7.40	28	Olanzapine	Appendicitis
2	39*	African American	31	14	6.74	8.30	104	Clozapine	Arteriosclerotic cardiovascular disease
3	7s	Caucasian	32	7	6.58	8.00	99	Clozapine	Suicide
4	41	Caucasian	37	14	6.68	7.30	27	Risperidone	Suicide
5	3s	African American	38	6	6.22	7.80	69	Fluphenazine	Asphyxia
6	11s	African American	38	5	6.64	8.50	157	Chlorpromazine	Pneumonia
7	5s	African American	42	21	6.25	7.80	71	Perphenazine	Tuberculosis
8	18	Caucasian	45	6	6.65	8.40	56	Olanzapine	Suicide
9	31	Caucasian	46	20	6.42	8.50	52	Olanzapine	Arteriosclerotic cardiovascular disease
10	5	Caucasian	49	16	6.55	7.50	78	Thioridazine	Intoxication
11	36	Caucasian	59	13	6.67	8.40	64	Haloperidol	Embolism
12	1s	Caucasian	53	11	6.69	7.90	58	Perphenazine	Arteriosclerotic cardiovascular disease
13	1	African American	62	12	6.56	8.00	55	Olanzapine	Arteriosclerotic cardiovascular disease
Comparison subjects									
1	35	African American	33	14	6.50	8.60	81		Morbid obesity
2	8s	African American	27	14	6.56	7.80	10		Asthma
3	12s	Caucasian	31	16	6.61	7.80	12		Polytrauma
4	34*	Caucasian	35	16	6.44	7.70	23		Arteriosclerotic cardiovascular disease
5	4s	Caucasian	38	24	6.55	7.70	46		Asphyxia
6	4*	African American	38	12	6.50	7.50	100		Cardiomyopathy
7	40	Caucasian	43	20	6.29	7.50	69		Arteriosclerotic cardiovascular disease
8	12	Caucasian	47	6	6.46	8.30	80		Arteriosclerotic cardiovascular disease
9	10s	Caucasian	46	24	6.64	7.00	15		Arrhythmia
10	6s	Caucasian	47	17	6.52	7.50	20		Arrhythmia
11	17	Caucasian	48	19	6.51	8.00	79		Aneurysm
12	21*	Caucasian	49	10	6.49	7.30	79		Arteriosclerotic cardiovascular disease
13	2s	Caucasian	50	22	6.38	7.80	23		Arteriosclerotic cardiovascular disease

^a Pairs matched according to age and postmortem interval.

^b Maryland Brain Collection code numbers for schizophrenia and comparison subjects. Asterisk marks are part of the code number.

for pair-matched human subjects and Student's t tests for rats. For further analysis of human subjects, SPSS 15.0 for Windows (SPSS, Chicago) was employed, which included analyses of covariance (ANCOVA) to determine the effect of age, postmortem interval, brain pH, RNA integrity number, and freezer storage time at the levels of each GABAergic transcript. Initial analyses demonstrated no significant effects of brain pH and postmortem interval at the levels of any of the transcripts. Thus, for all subsequent analyses, diagnosis was used as the independent variable, and age, RNA integrity number, and freezer storage time were used as covariates. A bivariate Spearman's r correlation analysis was employed to examine putative gene-gene in-

teractions. Any statistical analysis with a p value <0.05 was considered significant.

Results

GABAergic Marker Expression in the Lateral Cerebellar Hemisphere of Schizophrenia Subjects

Given the evidence of GABA dysfunction among individuals with schizophrenia, we sought to characterize the condition of inhibitory interneurons in the cerebellum of

TABLE 2. Gene Expression Changes in Postmortem Male Schizophrenia Subjects and Neuroleptic-Treated Rats

Gene of Interest	Human Subjects					Animal Subjects			
	Schizophrenia Group (N=13)					Haloperidol-Treated Sprague-Dawley Rats (N=16)	Clozapine-Treated Long-Evans Rats (N=20)		
	Change (%) ^a	F	df	Pairwise p ^b	ANCOVA p	Change (%) ^c	p ^d	Change (%) ^c	p ^d
GABAergic markers									
GABA _A - α_6	+44	5.286	4, 21	0.005	0.004	+29	0.1	+25	<0.0001
GABA _A - β_3	+16	2.138	4, 21	0.12	0.1	+33	0.01	+35	<0.0001
GABA _A - δ	+52	8.124	4, 21	0.0002	<0.001	+4	0.87	+25	<0.0001
GAD ₆₅	-26	2.696	4, 21	0.02	0.06	-19	0.01	+28	0.002
GAD ₆₇	-38	6.551	4, 21	0.01	0.001	+20	0.001	+31	<0.0001
GAT-1	-43	4.348	4, 21	0.002	0.01	+17	0.02	-23	<0.0001
PV mRNA	-12	0.36	4, 21	0.89	0.83	e	e	e	e
NMDA receptor subunits									
NR1	-5	0.476	4, 21	0.5	0.75	+13	0.04	+34	<0.0001
NR2-A	-6	1.037	4, 21	0.27	0.41	+23	0.08	-24	<0.0001
NR2-B	-16	3.942	4, 21	0.07	0.02	+34	0.18	-20	0.004
NR2-C	+7	0.978	4, 21	0.54	0.44	-15	0.28	-19	0.0005
NR2-D	+6	0.78	4, 21	0.27	0.55	-49	<0.0001	-10	0.1009
Cerebellar neuro-modulators									
GluR6	+23	4.353	4, 21	0.03	0.01	+26	0.03	+24	0.0005
KA2	+29	7.722	4, 21	0.0002	0.001	-10	0.13	-23	<0.0001
mGluR2	-27	4.418	4, 21	0.002	0.01	+32	0.0008	+10	0.62
mGluR3	-5	1.871	4, 21	0.54	0.2	e	e	e	e
Neuronal nitric oxidase synthase	-28	2.65	4, 21	0.03	0.06	+11	0.34	-19	0.02

^a Change represents the percentage of gene expression alteration in schizophrenia subjects (versus nonpsychiatric comparison subjects).

^b Values determined using Wilcoxon Signed-Rank Test for matching pairs.

^c Change represents the percentage of gene expression alteration in drug-treated rats (versus vehicle-treated rats).

^d Values determined using Student's t tests.

^e Value not determined.

schizophrenia subjects in order to 1) investigate the mechanisms underlying cerebellar abnormalities and 2) examine the nature and extent of GABA dysfunction in different neuronal populations. Initial studies examined the expression of seven markers of GABA function in the lateral cerebellar hemisphere of 13 pair-matched schizophrenia and comparison subjects using real-time PCR. To normalize the levels of a specific mRNA, β -actin was utilized because the expression of this particular mRNA did not differ between schizophrenia and comparison subjects (see the figure in the data supplement accompanying the online version of this article). Analysis of GABAergic transcript levels in schizophrenia subjects and matched comparison subjects using the Wilcoxon Signed-Rank Test revealed significant decreases in mRNA levels of GAD₆₇, GAD₆₅, and GAT-1 (schizophrenia to comparison group ratio=0.62, 0.74, and 0.57, respectively). However, no decreases in mRNA levels of parvalbumin were found (Table 2, Figure 1). Similar decreases in these transcripts were observed when cyclophilin was used as the housekeeping gene (see the data supplement accompanying the online version of this article). Subjects with schizophrenia also showed significant increases in mRNA levels for the granule-cell extrasynaptic GABA_A receptor subunits α_6 (schizophrenia to comparison group ratio=1.44) and δ (schizophrenia to comparison group ratio=1.53) relative to β -actin mRNA. However, no changes were found in GABA_A- β_3 mRNA, which is expressed in several cell types. In addition

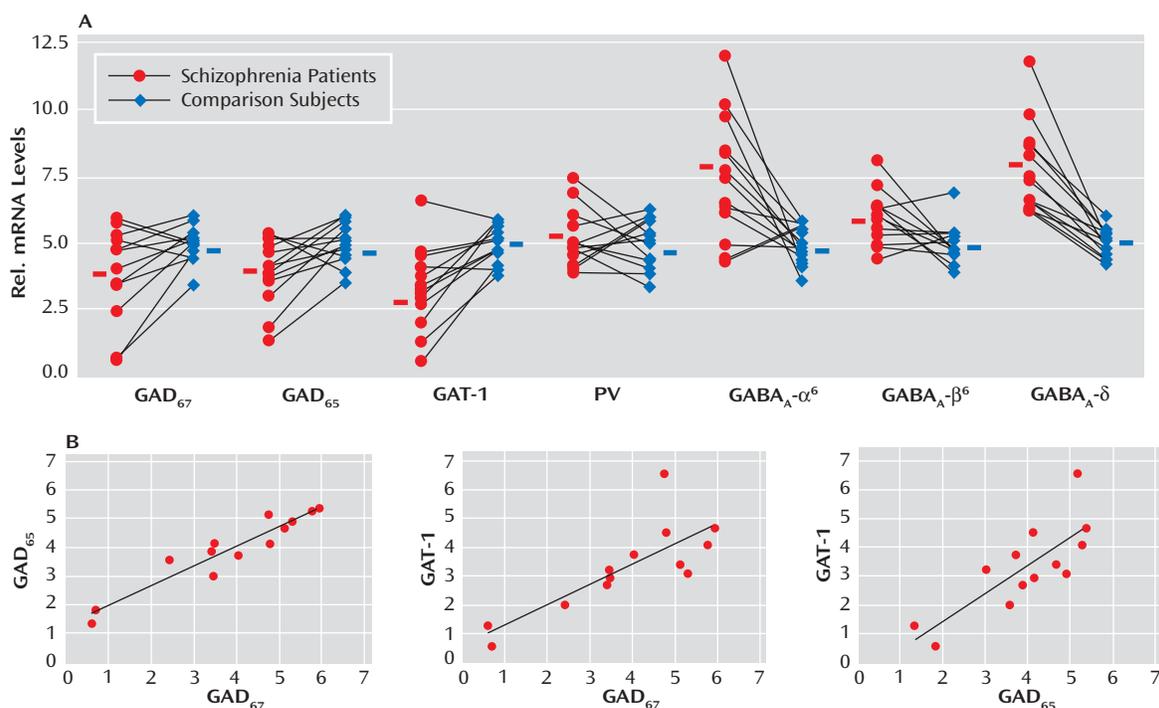
to pairwise analyses, we evaluated the effect of potential confounding variables, such as age, freezer storage time, and RNA integrity number, using ANCOVA. As detailed in Table 2, ANCOVA results confirmed that mRNA levels of GAD₆₇, GAT-1, GABA_A- α_6 , and GABA_A- δ were significantly different for schizophrenia subjects relative to comparison subjects ($p < 0.05$), while mRNA levels of GAD₆₅ were close to significant ($p = 0.06$).

To examine possible gene-gene interactions, mRNA levels were analyzed using Spearman's r correlations. In the schizophrenia group, positive correlations were 1) GAD₆₇ with GAD₆₅ ($r = 0.922$, $p < 0.001$) and 2) GAT-1 ($r = 0.797$, $p = 0.001$) and GAD₆₅ with GAT-1 ($r = 0.770$, $p = 0.002$) (Figure 1). Comparison subjects also showed a positive correlation between GAD₆₇ and GAT-1 ($r = 0.646$, $p = 0.02$).

NMDA Receptor Subunit Expression in the Lateral Cerebellar Hemisphere of Schizophrenia Subjects

In addition to GABA deficits, NMDA dysfunction has been found in schizophrenia patients (28). In the present study, analyses of the levels of NMDA receptor subunits NR1, NR2-A, NR2-B, NR2-C, and NR2-D, using the Wilcoxon Signed-Rank Test, revealed no significant changes except for a tendency toward decrease in the Golgi cell-selective subunit NR2-B (29) (schizophrenia to comparison group ratio=0.84, $p = 0.07$ [Table 2]). Furthermore, when considering the effect of age, freezer storage time, and RNA integrity number using ANCOVA, we found a signifi-

FIGURE 1. GABAergic Marker Transcript Expression and Correlations in the Cerebellum of Postmortem Schizophrenia Subjects and Postmortem Interval-Matched Comparison Subjects^a



^a The top row illustrates significant decreases seen in GABAergic marker transcript expression levels for GAD₆₇ and GAT-1 and significant increases seen in GABA_A-α₆ and GABA_A-δ among schizophrenia subjects (Wilcoxon Signed-Rank Test [see Table 2]). Expression levels are relative to β-actin. Values show mRNA levels in matched pairs and averages for schizophrenia and comparison subjects. The bottom row illustrates GABAergic marker transcript expression levels for GAD₆₇, GAD₆₅, and GAT-1, which are all significantly correlated using Spearman's r correlation coefficient.

cant decrease in the levels of NR2-B among schizophrenia subjects (p=0.004 [Table 2]). In addition, the levels of NR2-B in the schizophrenia group were positively correlated with the levels of NR1 (r=0.553, p=0.05), NR2-A (r=0.553, p=0.05), and NR2-D (r=0.663, p=0.01).

Cerebellar Neuromodulator Expression in the Lateral Cerebellar Hemisphere of Schizophrenia Subjects

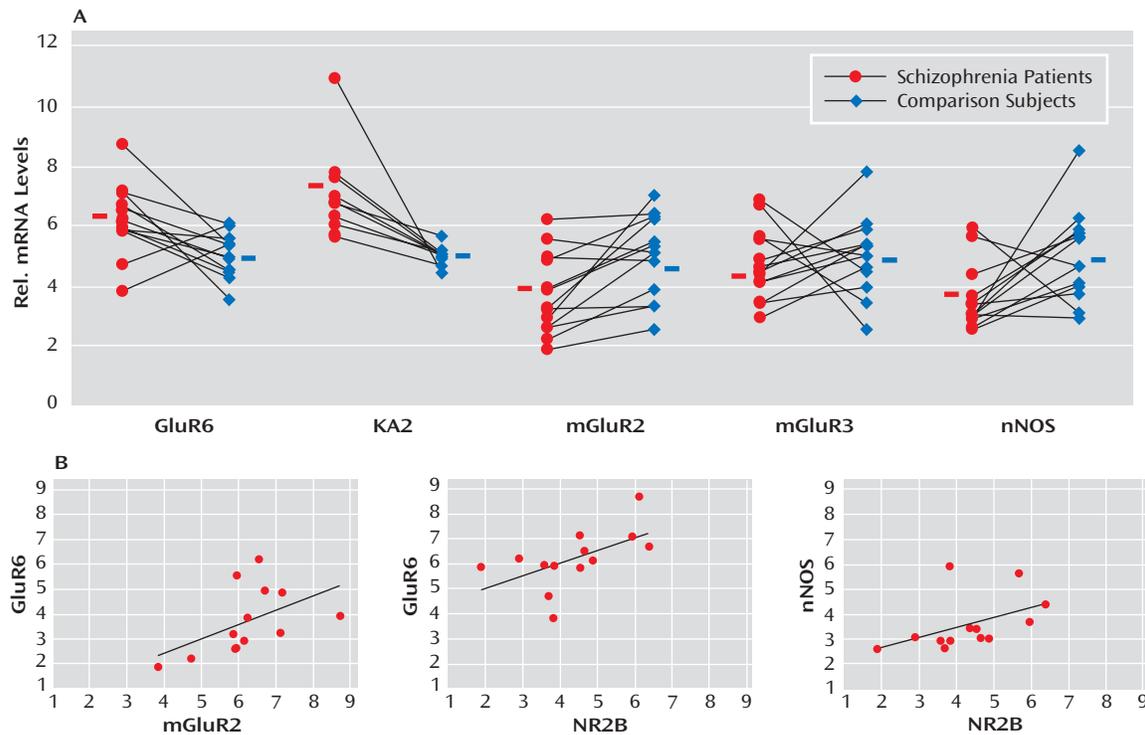
GABA release onto cerebellar granule cells is regulated at the cerebellar glomerulus, where pontine mossy fibers and Golgi cells synapse onto granule-cell dendrites in a close structural and functional unit. Thus, we examined the major modulatory factors of this unit, which could contribute to decreased GABA neurotransmission to granule cells. Of these modulatory factors, we observed significant decreases in GABAergic transcript levels of neuronal nitric oxide synthase (schizophrenia to comparison group ratio=0.72), present in granule cells and molecular layer interneurons (30), and the Golgi cell-specific mGluR2 (31) (schizophrenia to comparison group ratio=0.73) but not mGluR3 (Figure 2). Interestingly, both nitric oxide production by neuronal nitric oxide synthase and presynaptic mGluR2 receptor activation modulate GABA release from Golgi cells (32). Moreover, we found significantly increased levels of the glutamatergic kainate receptor sub-

units GluR6 (schizophrenia to comparison group ratio=1.23) and the granule cell-specific subunit KA2 (schizophrenia to comparison group ratio=1.29 [33]). ANCOVA revealed that all changes in GABAergic transcript levels were significant (p<0.05) except for changes in neuronal nitric oxide synthase, which were near significant (p=0.06 [Table 2]).

Correlations of Transcripts Levels in Golgi Cells, Granule Cells, and Local Cerebellar Circuitry Components

To further investigate the functional integrity of the cerebellar glomerulus in schizophrenia subjects, interactions between specific markers in the Golgi-granule cell circuit were analyzed. We found positive correlations for the Golgi cell-selective NR2-B with GAD₆₇ (r=0.922, p<0.001), GAD₆₅ (r=0.854, p<0.001), and GAT-1 (r=0.671, p=0.01) and for mGluR2 with GAD₆₅ (r=0.575, p=0.04), all of which were decreased in the schizophrenia group. In addition, GABAergic transcripts expressed in granule cells showed correlations for NR2-C with GABA_A-α₆ (r=0.639, p=0.02), GABA_A-β₃ (r=0.722, p=0.005), GABA_A-δ (r=0.652, p=0.02), and KA2 (r=0.726, p=0.005), which were all increased among schizophrenia subjects. Finally, we found that neuronal nitric oxide synthase correlated with GABA_A-β₃ (r=0.757, p=0.003);

FIGURE 2. Neuromodulator Transcript Expression and Golgi-Granule Cell-Circuit Correlations in the Cerebellum of Post-mortem Schizophrenia Subjects and Postmortem Interval-Matched Comparison Subjects^a



^a The top row illustrates significant increases seen in the levels of the kainate receptor subunits GluR6 and KA2 and significant decreases found in levels of mGluR2 and neuronal nitric oxide synthase among schizophrenia subjects (Wilcoxon Signed-Rank Test [see Table 2]). Expression levels are relative to β -actin. Values show mRNA levels in matched pairs and averages for schizophrenia and comparison subjects. The bottom row illustrates the significant correlations between markers expressed in the Golgi-granule cell circuit (using Spearman's r correlation coefficient).

GABA_A- α_6 correlated with GluR6 ($r=0.659$, $p=0.01$); and KA2 correlated with GABA_A- δ ($r=0.600$, $p=0.03$).

Positive correlations for components of Golgi cell-granule cell circuitry were 1) NR2-B with GABA_A- β_3 ($r=0.600$, $p=0.03$), GluR6 ($r=0.594$, $p=0.03$), and neuronal nitric oxide synthase ($r=0.624$, $p=0.02$) and 2) mGluR2 with GluR6 ($r=0.713$, $p=0.006$) and GABA_A- α_6 ($r=0.797$, $p<0.001$). Significant negative correlations were observed for GAT-1 with NR2-C ($r=-0.553$, $p=0.05$) and KA2 ($r=-0.779$, $p=0.002$), presumably reflecting a compensatory mechanism to increase GABA neurotransmission in the synaptic cleft.

Medication Effects on Gene Expression in Haloperidol- and Clozapine-Treated Rats

Both schizophrenia and comparison subjects were receiving antipsychotics at the time of death, with approximately one-half of the schizophrenia group being treated with typical antipsychotics and one-half being treated with atypical antipsychotics (Table 1). Analysis of gene expression levels between schizophrenia subjects who were receiving atypical versus typical antipsychotics showed a significant increase in GAD₆₇ gene expression for the former ($p=0.03$ [Mann-Whitney Rank Sum Test]). This finding suggests that medication type may have an effect on gene expression levels in individuals with schizophre-

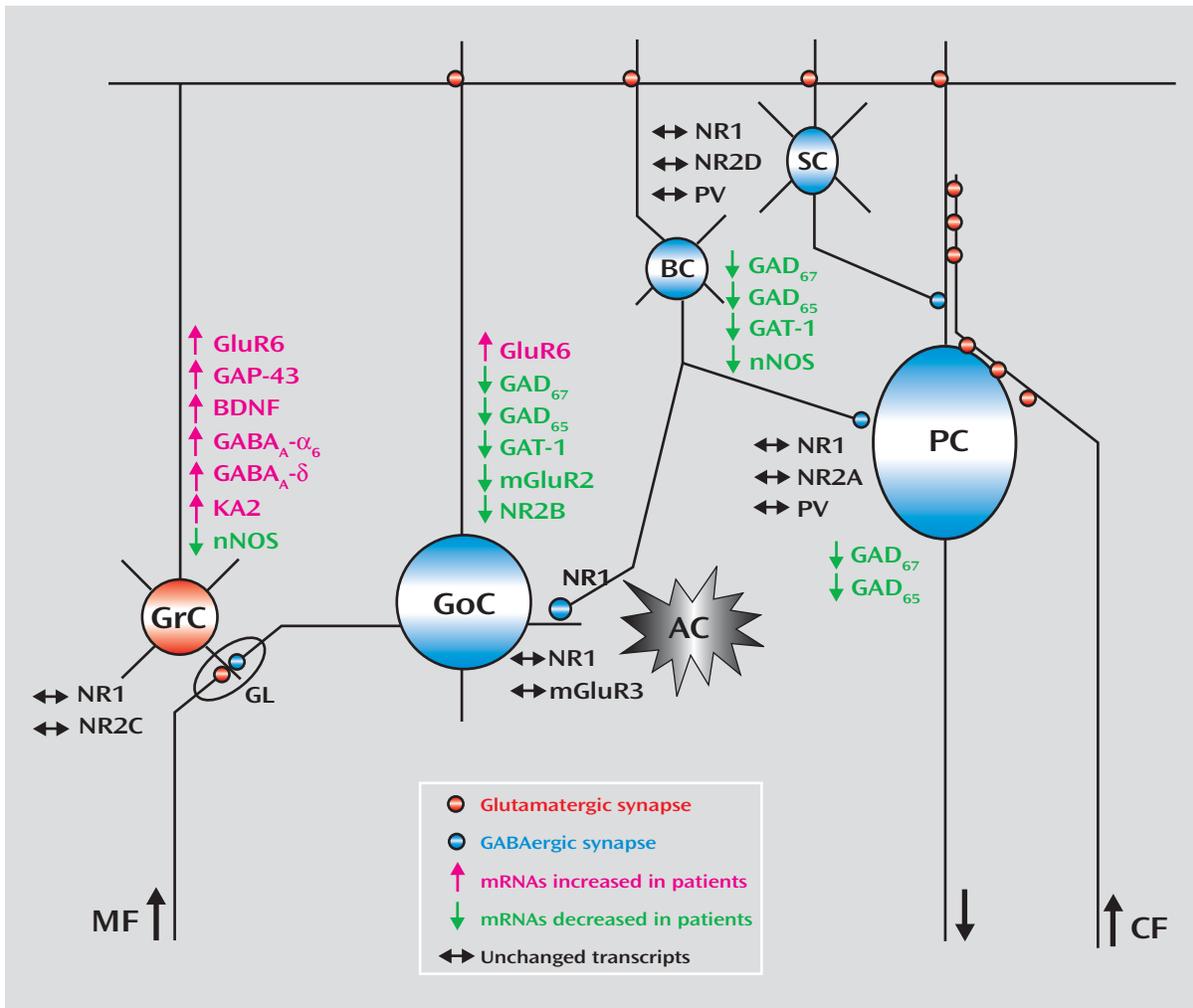
nia. Therefore, we analyzed the levels of the same mRNAs in the cerebellum of rats chronically treated with approximately 1 mg/kg/day of the typical neuroleptic haloperidol. As detailed in Table 2, we found significant increases in GABA_A- β_3 , GAD₆₇, GAT-1, NR1, GluR6, and mGluR2 in these rats. However, a significant decrease was seen in the expression of GAD₆₅ and NR2-D transcripts.

To examine the effects of atypical antipsychotics, rats were treated with clozapine for 21 days (10 mg/kg/day). As detailed in Table 2, these animals expressed increased mRNA levels of GABA_A- β_3 , GAD₆₇, and GAD₆₅, while GAT-1 expression was significantly decreased. The NMDA receptor subunit NR1 was significantly increased, whereas NR2-A, NR2-B, and NR2-C were all decreased. Finally, GluR6 was significantly increased, KA2 and neuronal nitric oxide synthase were decreased by clozapine treatment. Overall, our results indicate that clozapine could compensate, in part, for the decreased level of GABA synthesizing enzymes and other gene expression changes observed in schizophrenia subjects.

Discussion

An increasing number of studies support the hypothesis that GABA neurotransmission is deficient in individuals

FIGURE 3. Gene Alterations in the Cerebellum of Postmortem Schizophrenia Subjects and Postmortem Interval-Matched Comparison Subjects^a



^a The graph illustrates decreased GABA gene expression in Golgi cells and concomitant increases in granule-cell gene expression. Expression patterns are based on the Allen Brain Atlas (www.brain-map.org) and data from the literature. PV=parvalbumin; GrC=granule cells; GoC= Golgi cells; BC=basket cells; SC=stellate cells; PC=Purkinje cells; GL=cerebellar glomerulus; AC=astrocyte; MF=mossy fibers (from pontine nuclei); and CF=climbing fibers (from inferior olive).

with schizophrenia (2–4). Although GABAergic deficits have been well characterized in the prefrontal cortex (5–7) and limbic system (8, 9), few studies have examined these deficits in the cerebellum (10, 11). The present study demonstrated GABA transmission deficits in the lateral cerebellar hemisphere in individuals with schizophrenia, which implicates selective impairment in local granule-cell Golgi-cell communication, along with altered expression of neuromodulators of granule cell activity. Disinhibition of granule cells by deficient tonic inhibition from Golgi cells could disrupt cerebellar output by Purkinje cells, which in turn may affect other brain regions in the cortico-cerebellar-thalamic-cortical circuit, including the prefrontal cortex (34).

Several discrete GABAergic interneurons are located in the cerebellum, most notably Golgi cells in the granule layer and basket and stellate cells in the molecular layer.

Although basket and stellate cells provide feed-forward inhibition from granule cells to Purkinje cells, Golgi cells provide the sole source of feedback inhibition to granule cells (35). All of these interneurons express the GABA synthesizing enzymes GAD₆₅ and GAD₆₇. Our findings indicate that the levels of these GABAergic transcripts are decreased in individuals with schizophrenia relative to comparison subjects, suggesting that GABA synthesis may be impaired in schizophrenia patients. Consistent with our findings, Fatemi et al. (11) reported decreases in protein levels of both GAD₆₅ and GAD₆₇ in the cerebellum of a separate cohort of schizophrenia subjects.

Although GABA synthesis cannot be directly measured in postmortem tissue, other markers can be investigated to infer whether GABAergic transmission is reduced. For example, decreased levels of the presynaptic GAT-1 may reflect a compensatory attempt to increase synaptic

GABA. Mature Golgi and basket/stellate cells express GAT-1, while Purkinje cells and glia do not (36), suggesting that GAT-1 deficits may be restricted to specific GABAergic interneurons. This is consistent with a similar decrease in GAT-1 observed in the prefrontal cortex of schizophrenia patients in association with GAD₆₇ deficits (12). Interestingly, the calcium binding protein parvalbumin, which has been found to be decreased in the prefrontal cortex in individuals with schizophrenia (6), was not decreased in the present study, suggesting that different subtypes of interneurons may be affected in the cerebellum. Parvalbumin is expressed primarily in Purkinje and basket/stellate cells but only in a small subpopulation of Golgi cells (37). Conversely, the significant increases in the expression of the extrasynaptic GABA_A receptor subunits α_6 and δ suggest that a compensatory mechanism may occur in order to increase GABA receptivity by granule cells. As a result of freezing the tissue samples, we were unable to confirm the localization of specific transcripts by *in situ* hybridization. However, our findings point to Golgi cells as the predominantly affected cerebellar interneuron in schizophrenia.

A competing, but not mutually exclusive, hypothesis for schizophrenia proposes that NMDA receptor-mediated neurotransmission may be deficient in individuals with schizophrenia (28) and that GABAergic interneurons are particularly vulnerable to NMDA receptor hypofunction (38). In the present study, GABAergic interneuron function in the cerebellum was further investigated by measuring the expression of cell-selective NMDA receptor subunits. Although each cell type expresses the obligatory NR1 subunit, Purkinje cells express primarily NR2-A; Golgi cells express primarily NR2-B; granule cells express primarily NR2-C; and basket/stellate cells express primarily NR2-D (29). Among these subunits, we found a decrease in the levels of the Golgi cell-selective NR2-B. The strong correlations between NR2-B expression and GAD₆₇, GAD₆₅, and GAT-1 expression in the schizophrenia group further suggests that GABA transmission deficits may be localized to Golgi cells. Alternatively, it is possible that the number of Golgi cells and not their activity may be decreased in individuals with schizophrenia.

GABA release by Golgi cells is regulated by various factors in both Golgi and granule cells, including Golgi cell-specific mGluR2 (31) and the granule cell-located neuronal nitric oxide synthase (32). Activation of both systems normally decreases GABA release by Golgi cells, and both were decreased in our cohort of schizophrenia subjects (Figure 2), presumably compensating for decreased levels of GABA in the synapse. On the other hand, mGluR3, which is expressed in Golgi cells and glia (31), was not altered in the schizophrenia group. In addition, although decreased levels of mRNA in the receptor subunits GluR6 and KA2 have been previously reported in the prefrontal cortex and hippocampus of schizophrenia patients (33, 39), we observed increased cerebellar expression of these kainate receptors. Since both markers are expressed in

granule cells and these cells have shown increased expression of activity dependent-genes in the same group of schizophrenia subjects (24), it is possible that the changes in kainate receptor subunits may reflect a change in basal granule cell activity.

The changes in gene expression and putative cerebellar microcircuit alterations are illustrated in Figure 3. Although postmortem human tissue studies only provide a limited view of the premortem state and the use of tissue homogenates does not allow localization of transcripts to specific cell types, analyses of gene expression patterns using gene-gene correlations are valuable tools to infer the functional relevance of the observed changes across different cells in the network. An alteration in GABA neurotransmission between Golgi and granule cells is further evidenced by the positive correlations between the modulators of GABA release neuronal nitric oxide synthase and mGluR2 and between the Golgi specific marker NR2-B and neuronal nitric oxide synthase. Moreover, the negative correlations for GAT-1 with the granule cell-specific receptor subunits NR2-C and KA2 suggest a defect in modulation of GABA release by Golgi cells and increased granule cell firing. Although these findings are intriguing, animal models will need to be developed in order to confirm the dynamic nature of the interactions between granule cell and Golgi cell gene-expression patterns reported in the present study.

Our gene expression findings in schizophrenia subjects point to dysfunctional regulation of GABA release between Golgi and granule cells but do not take into account any effects of antipsychotic medication. Although antipsychotics have been shown to affect gene expression in several brain regions, these alterations have not been demonstrated in the cerebellum. As detailed in Table 2, haloperidol treatment resulted in increased levels of GAD₆₇, mGluR2, NR2-B, GAT-1, and neuronal nitric oxide synthase in chronically treated rats, whereas clozapine was more effective in increasing the expression of GAD₆₇ and GAD₆₅. Comparison of the levels of GluR6, GABA_A- α_6 , and GABA_A- δ receptor mRNAs in schizophrenia subjects and treated animals suggests that the observed increases of these transcripts in the schizophrenia group could be explained by medication effects. In contrast, the increases in KA2 mRNA seen in the schizophrenia group could not be attributable to antipsychotic treatment, since both haloperidol and clozapine decreased the levels of KA2 mRNA. In addition, medication effects could account for an underrepresentation of the changes in NR2-C and NR2-D gene expression in the schizophrenia group. Based on the results of the chronic neuroleptic exposure in rats, haloperidol seems to have a more beneficial effect on gene expression in the local cerebellar circuit relative to clozapine. Furthermore, although both medications were effective in increasing GAD₆₇ expression in rats, neither treatment was effective in schizophrenia subjects. Our results support the idea that combining different types of antipsychotics with GABA_A

receptor agonists may provide enhanced benefits for treating the underlying GABA-glutamate pathophysiology of schizophrenia.

In summary, we found that the expression of multiple GABAergic markers is decreased in the lateral cerebellar hemisphere among individuals with schizophrenia and this deficit is most likely localized to Golgi cells. Overall, patient mRNA expression data suggest that GABA neurotransmission deficits could be responsible for increased granule cell activity and disinhibition of glutamatergic signaling in the cerebellum, leading to aberrant cerebellar output. These changes may underlie not only the alterations in cerebellar blood flow observed in our schizophrenia cohort, but also, and most importantly, the clinical symptoms associated with abnormal cerebellar function in schizophrenia (40).

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