

# Decrease in Reelin and Glutamic Acid Decarboxylase<sub>67</sub> (GAD<sub>67</sub>) Expression in Schizophrenia and Bipolar Disorder

## A Postmortem Brain Study

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**Background:** Reelin (RELN) is a glycoprotein secreted preferentially by cortical  $\gamma$ -aminobutyric acid-ergic (GABAergic) interneurons (layers I and II) that binds to integrin receptors located on dendritic spines of pyramidal neurons or on GABAergic interneurons of layers III through V expressing the disabled-1 gene product (DAB1), a cytosolic adaptor protein that mediates RELN action. To replicate earlier findings that RELN and glutamic acid decarboxylase (GAD)<sub>67</sub>, but not DAB1 expression, are down-regulated in schizophrenic brains, and to verify whether other psychiatric disorders express similar deficits, we analyzed, blind, an entirely new cohort of 60 postmortem brains, including equal numbers of patients matched for schizophrenia, unipolar depression, and bipolar disorder with nonpsychiatric subjects.

**Methods:** Reelin, GAD<sub>65</sub>, GAD<sub>67</sub>, DAB1, and neuron-specific-enolase messenger RNAs (mRNAs) and respective proteins were measured with quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) or Western blot analyses. Reelin-positive neurons were identified by immunohistochemistry using a monoclonal antibody.

**Results:** Prefrontal cortex and cerebellar expression of RELN mRNA, GAD<sub>67</sub> protein and mRNA, and prefrontal cortex RELN-positive cells was significantly decreased by 30% to 50% in patients with schizophrenia or bipolar disorder with psychosis, but not in those with unipolar depression without psychosis when compared with nonpsychiatric subjects. Group differences were absent for DAB1, GAD<sub>65</sub> and neuron-specific-enolase expression implying that RELN and GAD<sub>67</sub> down-regulations were unrelated to neuronal damage. Reelin and GAD<sub>67</sub> were also unrelated to postmortem intervals, dose, duration, or presence of antipsychotic medication.

**Conclusions:** The selective down-regulation of RELN and GAD<sub>67</sub> in prefrontal cortex of patients with schizophrenia and bipolar disorder who have psychosis is consistent with the hypothesis that these parameters are vulnerability factors in psychosis; this plus the loss of the correlation between these 2 parameters that exists in nonpsychotic subjects support the hypothesis that these changes may be liability factors underlying psychosis.

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**S**CHIZOPHRENIA is presumed to be a disorder of brain cortical function expressing a variable array of devastating psychiatric symptoms. Such florid symptoms contrast with comparatively modest anatomopathological alterations characterized by (1) dilation of cerebral ventricles<sup>1</sup>; (2) reduction of cortical neuropil, resulting in an apparent increase in cell-packing density<sup>2</sup>; (3) decrease in apical dendrite spine density on cortical pyramidal neurons<sup>3,4</sup>; (4) important morphological (axon terminals of chandelier neurons)<sup>5</sup> and neurochemical (decrease of glutamic acid decarboxylase<sub>67</sub> [GAD<sub>67</sub>])<sup>6-8</sup> alterations of inhibitory  $\gamma$ -aminobutyric acid-ergic (GABAergic) interneurons.

The origin of such changes is unknown, but a few interesting models are

emerging. One such model is the heterozygous reeler mice,<sup>9</sup> which express 50% the amount of reelin (RELN) found in the brain of the wild-type mice, and exhibit<sup>9</sup> the following: (1) the postpubertal appearance of sensory motor gating down-regulation reminiscent of that associated with psychosis, (2) a selective decrease of cortical neuropil expression associated with an apparent increase in cortical cell packing, (3) a selective decrease of nicotinamide adenine dinucleotide phosphate-positive GABAergic neurons in superficial white matter in the overlying cortex but an increase in the deeper white matter.

Reelin is a glycoprotein (3461 amino acids) displaying structural analogies with extracellular matrix proteins.<sup>10</sup> During ontogenesis, the mammalian cerebral cortex lamination is regulated by RELN secreted

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## SUBJECTS AND METHODS

### SUBJECTS

Superior PFC gyrus, Brodmann area 9, and cerebellar hemisphere (cerebellum) specimens from 60 subjects obtained blind from the Stanley Foundation Brain Bank Neuropathology Consortium, Bethesda, Md, consist of 4 matched groups: 15 brains with schizophrenia (10 undifferentiated and 5 paranoid), 15 brains with bipolar disorder (11 with psychosis and 4 without psychosis), 15 brains with depression without psychotic features, and 15 control brains, free of psychiatric disorders before or at the time of death, and herein defined as nonpsychiatric subjects.<sup>25</sup> Their psychiatric diagnosis was established by 2 senior psychiatrists, based on clinical and family history, using DSM-IV criteria.<sup>27</sup> Demographic data are summarized in **Table 1**. Most patients with schizophrenia and patients with bipolar disorder with psychosis were receiving antipsychotic medication at the time of death. The total lifetime intake of antipsychotic medication was established in fluphenazine gram equivalent as described.<sup>28</sup> Table 1 also summarizes the history of alcohol and other substance abuse. Brain, blood, and urine toxicology screen for alcohol, cocaine, amphetamine, codeine, methadone, marijuana, and benzodiazepines was performed by the Stanley Foundation Consortium.<sup>27</sup>

### QUANTITATIVE ANALYSES OF RELN-IMMUNOPOSITIVE CELLS

Coronal sections (20  $\mu$ m) of formalin-fixed PFCs were Nissl stained and/or immunostained with RELN-142 (1:500) antibody.<sup>11,12</sup> Cells were counted in a 1000- $\mu$ m-wide column that spans from the pial surface to the underlying white matter. Six columns per section and a total of 3 sections per patient were counted. Since most (>95%) of RELN-immunopositive cells were found in layer I<sup>7</sup> (**Figure 2**), cell counting was reported for layer I, only in **Table 2**. To prevent overestimation, only neurons with an evident nucleus were counted, and only neurons that bordered the right and the top edges of the counting field were included. Nissl-positive neurons were differentiated from glia using the criteria described by Benes et al.<sup>29</sup>

### QUANTITATIVE mRNA AND PROTEIN ANALYSES

Reelin mRNA, GAD<sub>67</sub>, or GAD<sub>65</sub> protein levels were determined in all samples. In 6 PFC samples of each cohort we also compared the abundance of GAD<sub>65</sub> or GAD<sub>67</sub> proteins with the expression level of the respective mRNAs. In the same samples we also measured neuron-specific enolase (NSE) mRNA that was used as a marker to correct for non-specific loss of mRNA owing to neuronal damage. The analyses of GAD<sub>65</sub>, GAD<sub>67</sub>, and NSE mRNAs was carried out in only 6 samples per group because, only in these samples, there was a sufficient amount of mRNA available for the assay. The content of the DAB1 protein was determined only in a small sample chosen randomly from the PFC of all patient cohorts because the supply of specific anti-DAB1 antibody was limited.

The RNA quality of each brain sample was assessed by the Stanley Foundation Consortium by measuring

glyceraldehyde or actin mRNA and then grading the yield: A (excellent), B (good), C (fair), D (poor), and F (very poor); only samples graded A to C were used.<sup>27</sup>

In each extract, the yield of RNA was established by absorbance at 260/280 nm. Samples with a ratio below 1.8 were rejected. In samples with abnormally low levels of RNA, possible major mRNA degradation was assessed using denaturing agarose gel electrophoresis and evaluating the sharpness of the 2 ribosomal RNA bands (28S and 18S). If these 2 bands were smeared, the sample was discarded.<sup>7</sup>

The quantity of RELN mRNA was determined using internal standards as described.<sup>7</sup> Primers for GAD<sub>67</sub> mRNA were forward 1855 to 1878 base pairs (bp); reverse 2246 to 2269 bp (Gene Bank Accession No. M81883); internal standard contained a *Bgl*III restriction endonuclease, which on digestion generated fragments of 199 and 216 bp. Primers for GAD<sub>65</sub> mRNA were forward 8 to 103 bp, and reverse 507 to 532 bp (Gene Bank Accession No. M72422); the internal standard contained an *Xba*I restriction endonuclease, which on digestion generated fragments of 215 and 235 bp. To establish whether neuronal RNA contributes equally to the total RNA pool, NSE mRNA was determined using the following primers forward 328 to 379 bp and reverse to 792 to 815 bp (Gene Bank Accession No. M22349); the internal standard contained a *Bam*HI restriction endonuclease, which on digestion generated fragments of 199 and 220 bp.

For the determination of GAD<sub>65</sub>, GAD<sub>67</sub>, and DAB1 protein, we used Western blot analyses. Two to 3 aliquots of brain extracts (10-40  $\mu$ g of protein) were resolved on acrylamide gel.<sup>7</sup> The blots were developed with GAD<sub>65</sub>/GAD<sub>67</sub> (Chemicon International, Temecula, Calif) or with DAB1-B3<sup>17</sup> polyclonal antibody (1:1000) and subsequently with  $\beta$ -actin (1:5000) antiserum (Sigma, St Louis, Mo).

The levels of GAD<sub>65</sub>, GAD<sub>67</sub>, and DAB1 were calculated as a ratio of the optical density of the antibody of interest to the optical density of the antibody directed against  $\beta$ -actin.<sup>7</sup> The reliability of duplicated blots for GAD<sub>67</sub> and GAD<sub>65</sub> was 0.85 and 0.95 for cortex and 0.92 and 0.95 for cerebellum, respectively ( $P < .001$  for all comparisons).

### STATISTICAL ANALYSES

Differences in the postmortem interval (in hours) between death and sample freezing, or between the right and left hemispheres, or in the clinical variables or in concomitant medications among diagnostic groups were evaluated with 1-way analysis of variance (ANOVA) or  $\chi^2$  analysis. Because background variables did not differ among diagnostic groups, statistical adjustment for these variables was not used. To test statistically whether there were effects of diagnosis on RELN mRNA or on GAD<sub>67</sub>, GAD<sub>65</sub>, DAB1 content, and on the density of RELN-positive cells, we used ANOVA. For GAD<sub>67</sub> and GAD<sub>65</sub>, the values were analyzed using a randomized-block ANOVA with blots as the blocking variable. Multiple comparisons were conducted and we reported unadjusted  $P$  values. We have indicated in each table and text the Bonferroni adjusted  $\alpha$  level and the rationale for its adjustment. Finally, Pearson correlations were determined between RELN mRNA, GAD<sub>67</sub> protein, RELN-immunopositive neurons, and lifetime antipsychotic doses, and between RELN mRNA and GAD<sub>67</sub>.  $P$  values were 2 tailed.

from pioneer Cajal Retzius cells, located in the marginal zone. After birth in rats,<sup>11,12</sup> or during the last months of gestation in primates,<sup>13</sup> when the Cajal Retzius cells disappear, RELN is secreted by a select population of GABAergic interneurons including bitufted and horizontal cells of layers I and II of various cortical areas. Such secretion has been documented throughout life in rats<sup>11,12,14</sup> and primates.<sup>15</sup>

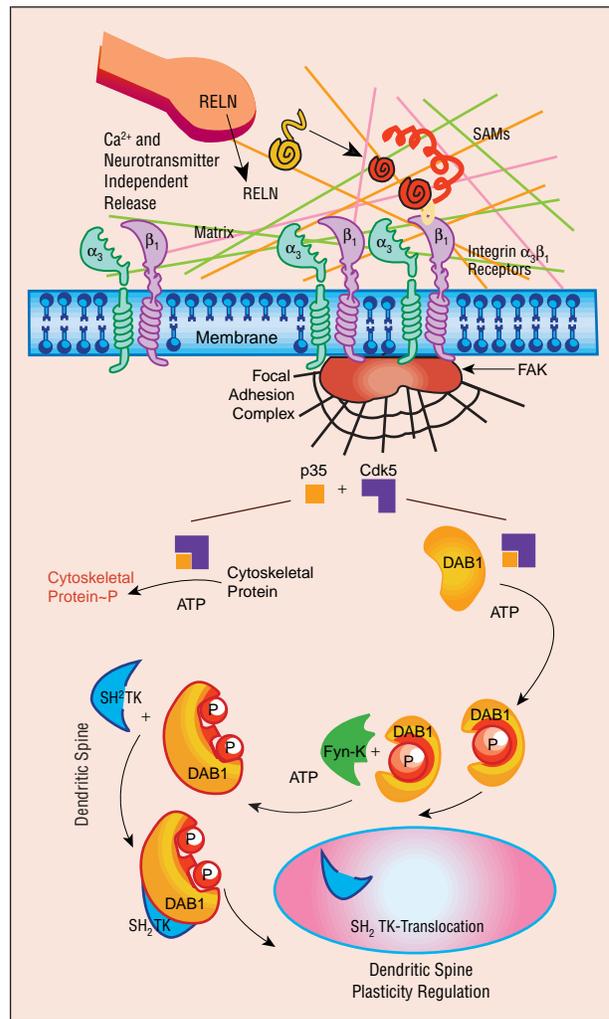
In layers I and II of the cortex, the apical dendrites of glutamatergic pyramidal neurons, and in deeper layers (III-V), the dendrites and somata of basket, and chandelier, GABAergic interneurons may be surrounded by RELN immunoreactive halos.<sup>12,15</sup> Some of these cells may also express RELN messenger RNA (mRNA).<sup>12</sup> These RELN immunoreactive halos probably reflect RELN secreted into the extracellular matrix that binds to domains of integrin receptors including the  $\alpha_3$  subunit,<sup>16</sup> expressed on dendritic spines<sup>15</sup> (Figure 1). The RELN-integrin-receptor interaction triggers an intracellular transduction cascade involving the cytosolic disabled-1 gene product (DAB1) that, in the phosphorylated state, functions as an adaptor protein,<sup>17,18</sup> (Figure 1). In the phosphorylated state DAB1 can bind members of the soluble tyrosine kinase family and presumably can translocate them to various cellular compartments including the nucleus where these kinases may initiate transcription of specific genes operative in synaptic plasticity and in promoting cytoskeletal changes including synaptic spine maturation and their bifurcation associated with learning.<sup>19-22</sup>

Recent evidence places apolipoprotein E<sub>2</sub> and very low-density-lipoprotein receptors in a common signaling pathway operative with integrin and DAB1 in the control of cell positioning during cortical embryonic development.<sup>23,24</sup> However, since the knockout mice for either 1 of these 2 receptors fails to show phenotypic traits proper of the *reeler* mice, further studies are needed before proposing that apolipoprotein E<sub>2</sub> and very low-density lipoprotein receptors are operative in the cascade triggered by RELN in embryonic brain.

In a previous report we showed that RELN expression is down-regulated in GABAergic neurons of the prefrontal (Brodmann areas 10 and 46) temporal and parietal cortices, hippocampus, caudate nucleus, and glutamatergic cerebellar neurons of schizophrenia patients.<sup>7</sup> In neocortex of the same patients, the expression of GAD<sub>67</sub>, the enzyme responsible for keeping cortical GABA levels at steady state,<sup>25,26</sup> is also reduced.

Using 4 entirely new cohorts of postmortem brains comprising patients who were affected by schizophrenia, unipolar depression, or bipolar disorder, or were non-psychiatric subjects,<sup>27</sup> we have addressed the following questions:

1. Is brain down-regulation of RELN and GAD<sub>67</sub> expression a specific feature of schizophrenia or is it found associated with other psychiatric disorders?
2. Is GAD<sub>67</sub> and RELN down-regulation correlated with lifetime antipsychotic dosages or with other demographic parameters?
3. Is RELN and GAD<sub>67</sub> down-regulation associated with psychosis?
4. Are prefrontal cortex (PFC) expression levels of RELN and GAD<sub>67</sub> correlated? Is this correlation altered in psychotic patients?



**Figure 1.** Putative reelin (RELN) signaling pathway in dendritic spines of cortical pyramidal neurons: involvement of the adaptor protein disabled-1 gene product (DAB1) and various protein kinases. Reelin expressed preferentially in bitufted or horizontal GABAergic interneurons<sup>11,12</sup> is secreted by a Ca<sup>2+</sup>- and neurotransmitter-independent constitutive process<sup>33</sup> into the extracellular matrix space. It adheres to dendritic spines of pyramidal neurons or dendrites or somata of basket and chandelier interneurons<sup>15</sup> either via a binding to substrate adhesion molecules (SAM) or directly to the integrin ( $\alpha_3\beta_1$ ) receptors, promoting clustering of these receptors. The RELN-integrin receptor interaction mediates focal adhesion kinase (FAK) activation, to establish a link of extracellular matrix to cytoskeletal proteins and the formation of a cytoplasmic focal adhesion complex. Thus, RELN may activate serine-threonine kinases (P35/Cdk5) and the Src-tyrosine kinase family (ie, Fyn-K) that phosphorylate cytosolic DAB1. Phosphorylated DAB1 may serve as a docking site for Src homology 2 tyrosine kinase (SH<sub>2</sub>-TK) domain of members of the Src-tyrosine kinase family that may be transferred by DAB1 to various neuronal compartments and become operative in synaptic and dendritic spine plasticity. This model is supported by knockout studies of genes encoding for P35/Cdk5 kinase<sup>32,33</sup> and DAB1<sup>17</sup> and by electronmicroscopy evidence of RELN and integrin association in dendritic spine synapses.<sup>15</sup> ATP indicates adenosine triphosphate; GABAergic,  $\gamma$ -aminobutyric acidergic.

## RESULTS

### PREFRONTAL CORTEX

In patients with schizophrenia and those with bipolar disorder, but not in patients with unipolar depression without psychosis, the expression of GAD<sub>67</sub> protein and RELN mRNA was decreased by about 50% when compared with

**Table 1. Demographic Characteristics of the Study Groups<sup>a</sup>**

	Nonpsychiatric Subjects (n = 15)	Patient Cohort			Statistical Analysis
		Unipolar Depressed (n = 15)	Schizophrenia <sup>b</sup> (n = 15)	Bipolar Disorder <sup>c</sup> (n = 15)	
M/F ratio	9/6	9/6	9/6	9/6	$\chi^2_3 = 0.00, P = .99$
Age, y	48 ± 11	46 ± 9.3	44 ± 12	42 ± 13	$F_{3,56} = 0.39, P = .76^d$
Postmortem interval, h	24 ± 9.9	27 ± 4.7	34 ± 16	32 ± 15	$F_{3,56} = 1.7, P = .18^d$
Brain pH	6.3 ± 0.06	6.2 ± 0.09	6.2 ± 0.09	6.2 ± 0.09	$F_{3,55} = 0.6, P = .62^d$
Age of illness onset, y	NA	34 ± 13	23 ± 8.3	21 ± 7.9	$F_{2,42} = 6.6, P = .003^d$
Duration of illness, y	NA	13 ± 11	21 ± 11	23 ± 10	$F_{2,42} = 3.8, P = .03^d$
Cause of death					
Suicide	None	7	4	9	$\chi^2_3 = 20, P = .01^e$
Cardiopulmonary	13	7	8	4	
Accident	2	0	2	1	
Other	0	1	1	1	
Family history of psychosis	1	1	6	7	$\chi^2_3 = 11, P = .01^e$
Severe sociofunctional deficit <sup>f</sup>	None	None	9	5	Fisher exact test, $P < .001$
Antipsychotic drug use <sup>g</sup>					
None	15	15	3	5 <sup>h</sup>	Only bipolar and schizophrenic patients received antipsychotic drugs. Fisher exact test between bipolar and schizophrenic illness was not significant; $P = .15$
Clozapine only	None	None	2	2	
Others <sup>i</sup>	None	None	7	8	
Clozapine/others	None	None	3	None	
Antidepressants	None	9	5	7	$\chi^2_3 = 13, P < .001^e$ Fisher exact test, $P = .004$
Mood stabilizers <sup>j</sup>	None	3	5	10	
Abuse or dependence <sup>k</sup>					
Alcohol	0	3	2	2	Fisher exact test, $P = .49$
Cocaine	0	1	0	1	Fisher exact test, $P = .99$
Polysubstance	0	0	1	1	Fisher exact test, $P = .99$

<sup>a</sup>Values are expressed as mean ± SD, unless otherwise indicated. NA indicates not applicable.

<sup>b</sup>This cohort includes 10 undifferentiated and 5 paranoid schizophrenic patients.

<sup>c</sup>This cohort includes 11 patients with psychosis and 4 patients without psychosis.

<sup>d</sup>One-way analysis of variance among patient cohorts.

<sup>e</sup> $\chi^2$  Analysis among patient cohorts.

<sup>f</sup>Lack of independent functioning in living, maintaining a job, or social activity.

<sup>g</sup>Antipsychotic drug present at the time of death.

<sup>h</sup>Of the 5 patients with bipolar disorder who were not receiving antipsychotic medication, there were 3 who did not have psychosis and 2 who had psychosis.

<sup>i</sup>Includes the following: haloperidol, chlorpromazine, risperidone, and thioridazine.

<sup>j</sup>Includes the following: valproate, carbamazepine, and lithium.

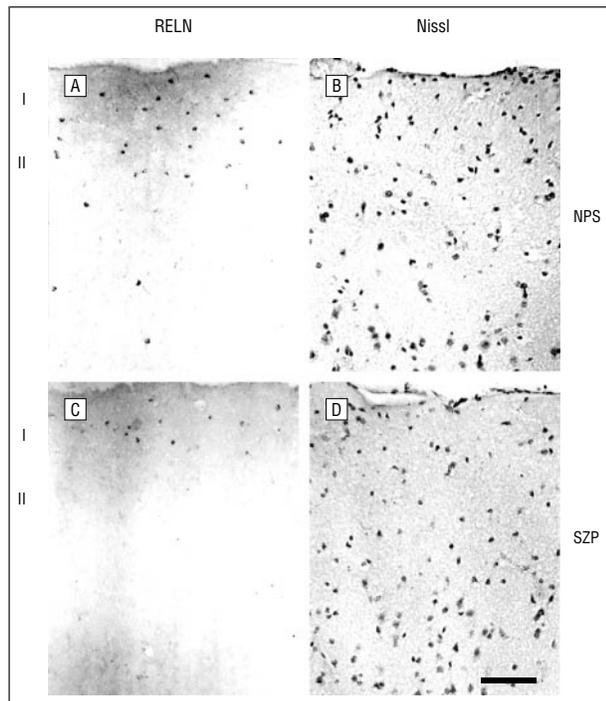
<sup>k</sup>Subjects with a history of recent alcohol or other drug abuse or dependence.

nonpsychiatric subjects (Table 2). In psychiatric patients, GAD<sub>65</sub> protein expression was virtually identical to nonpsychiatric subjects (Table 2). In Table 2 and Figure 2 we also show that in layer I of PFCs, the density of RELN-immunopositive neurons in schizophrenic patients or patients with bipolar disorder is decreased by 25% to 30% when compared with nonpsychiatric subjects, and this decrease occurs in the absence of a Nissl-positive neuron loss.

The cohort of patients with bipolar disorder can be subdivided into 2 subgroups: (1) those with with psychosis (11 patients) and (2) those without psychosis (4 patients) (Table 1). Two pair-wise comparisons were of interest here: (1) patients with bipolar disorder with psychosis vs nonpsychiatric subjects and (2) patients with bipolar disorder without psychosis vs nonpsychiatric subjects; therefore, a Bonferroni-adjusted  $\alpha$  of 0.05/2 = 0.025 was used. Only the group with psychosis showed

statistically significant down-regulations of PFC RELN and GAD<sub>67</sub> expression (ANOVA: RELN mRNA,  $F_{2,27} = 5.4, P = .01$ ; RELN-positive neurons,  $F_{2,19} = 21, P < .001$ ; and GAD<sub>67</sub>,  $F_{2,27} = 6.8, P = .004$ ). Pair-wise comparisons of bipolar patients with psychosis vs nonpsychiatric subjects yielded  $P$  values of .010 for RELN mRNA,  $< .001$  for RELN-positive cells, and .001 for GAD<sub>67</sub> protein. Comparing these  $P$  values to the adjusted significance  $\alpha$  level of .025 indicates that patients with bipolar disorder with psychosis are significantly different from nonpsychiatric subjects for RELN mRNA and GAD<sub>67</sub> protein expression levels.

In the PFC of 34 nonpsychotic subjects combined (15 nonpsychiatric subjects, 15 patients with unipolar depression, and 4 patients with bipolar disorder without psychosis), there was a significant correlation between expression levels of RELN mRNA and of GAD<sub>67</sub> protein (Figure 3, left). These subjects also had a higher



**Figure 2.** Photomicrographs of 20- $\mu$ m serial sections of prefrontal cortex (superior gyrus, Brodmann area 9) of a typical nonpsychiatric subject (NPS) (A and B) and of a schizophrenic patient (SZP) (C and D) immunolabeled for reelin (RELN) (left panels) or Nissl-stained (right panels) neurons. Reelin-positive neurons are mostly localized in layer I. Other layers combined contain only 2% to 3% of the total number of RELN-positive cells<sup>†</sup> and are excluded from the Figure. Note that the nonpsychiatric subject has a higher density of RELN-positive cells<sup>†</sup> and also has an extracellular diffuse RELN-immunostaining halo that is stronger than that observed in the schizophrenic patient. Bar indicates 150  $\mu$ m.

density of RELN-positive cells and exhibited a strong extracellular matrix halo of RELN immunostaining (Figure 2). In contrast, in the pool of 26 psychotic patients (15 with schizophrenia and 11 with bipolar disorders) (Figure 3, right), the expression levels of RELN mRNAs and GAD<sub>67</sub> protein were significantly decreased (RELN,  $t_{55}=3.8$  [unequal variance],  $P<.001$ ; GAD<sub>67</sub>,  $t_{57}=4.4$ ,  $P<.001$ ), but the changes in these 2 variables were not correlated (Figure 3, right).

## CEREBELLUM

In the cerebellum of the same patients with schizophrenia or bipolar disorder, we also found a decrease of GAD<sub>67</sub> protein expression (approximately 50%) without changes in GAD<sub>65</sub> when compared with nonpsychiatric subjects (Table 2). Reelin mRNA was also significantly decreased by about 38% in schizophrenic patients, but in patients with bipolar disorder a similar decrease (about 35%) failed to reach significance (Table 2). In contrast in patients with depression but without psychosis, neither RELN nor GAD<sub>67</sub> expression were down-regulated (Table 2).

## DAB1 PROTEIN, GAD<sub>65</sub> AND GAD<sub>67</sub> mRNAs AND PROTEINS, AND NSE mRNA EXPRESSION

The expression of DAB1 was virtually identical in the PFC of all 4 groups (Table 2). We also compared the expression of GAD<sub>67</sub> or GAD<sub>65</sub> proteins with that of their respective mRNAs, in 6 PFC samples. **Table 3** shows that GAD<sub>67</sub> mRNA expression is down-regulated in schizo-

**Table 2.** RELN- and Nissl-Positive Neurons, RELN mRNA, GAD<sub>67</sub>, GAD<sub>65</sub>, and DAB1 Protein Content in Prefrontal Cortex (PFC) Brodmann Area 9 and Cerebellum of Nonpsychiatric and Psychiatric Patients\*

Variable	Patient Cohort				Overall ANOVA			Multiple Comparison†			
	1	2	3	4	F	df	P	Schizophrenia vs		Bipolar Disorder vs	
	Nonpsychiatric Subjects (n = 15)	Unipolar Depressed (n = 15)	Schizophrenia (n = 15)	Bipolar Disorder (n = 15)				1	2	1	2
<b>PFC</b>											
RELN-positive cell‡	72 ± 7.2 (11)	76 ± 9.4 (10)	56 ± 11 (12)	52 ± 8.0 (11)	27	3,40	<.001	<.001	<.001	<.001	<.001
Nissl-stained cell‡	134 ± 20 (11)	134 ± 20 (10)	132 ± 28 (12)	128 ± 19 (11)	0.3	3,40	NS	...	...	...	...
RELN mRNA§	78 ± 34 (15)	74 ± 41 (13)	31 ± 20 (14)	41 ± 27 (15)	7.1	3,54	<.001	<.001	.003	.002	.003
GAD <sub>67</sub> protein	1.1 ± 0.24 (15)	1.1 ± 0.70 (15)	0.51 ± 0.35 (15)	0.63 ± 0.45 (15)	6.4	3,56	<.001	.001	<.001	.01	.009
GAD <sub>65</sub> protein	1.3 ± 0.40 (15)	1.5 ± 0.38 (15)	1.4 ± 0.54 (15)	1.3 ± 0.58 (15)	0.5	3,56	NS	...	...	...	...
DAB1 protein¶	1.0 ± 0.38 (6)	0.7 ± 0.47 (6)	1.1 ± 0.47 (6)	0.87 ± 0.39 (6)	0.8	3,20	NS	...	...	...	...
<b>Cerebellum</b>											
RELN mRNA§	1200 ± 390 (14)	1100 ± 500 (14)	750 ± 430 (14)	780 ± 440 (13)	3.8	3,51	.02	.008	.04	.01	.06
GAD <sub>67</sub> protein	1.2 ± 0.43 (15)	1.2 ± 0.32 (15)	0.54 ± 0.32 (15)	0.63 ± 0.29 (15)	15	3,56	<.001	<.001	<.001	<.001	<.001
GAD <sub>65</sub> protein	1.4 ± 0.42 (15)	1.4 ± 0.52 (15)	1.4 ± 0.60 (15)	1.2 ± 0.43 (15)	0.2	3,56	NS	...	...	...	...

\*RELN indicates reelin; mRNA, messenger RNA; GAD, glutamic acid decarboxylase; DAB1, disabled-1 gene; ANOVA, analysis of variance; NS, not significant; and ellipses, not applicable. All values are expressed as mean ± SD (number of subjects per group).

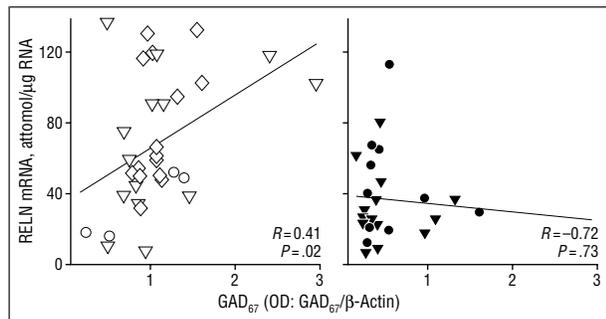
†For multiple comparison, the P values are compared with a Bonferroni-adjusted  $\alpha = 0.05/6 = 0.0083$ . Multiple comparison tests with  $P \leq .008$  was considered statistically significant.

‡The number (measured in square millimeters) of RELN-positive and Nissl-stained neurons in the PFC are referred to layer I because this layer contains most (approximately 95%) of the RELN-positive neurons (Figure 2). Reelin- and Nissl-positive cell studies were not carried out when cytoarchitecture was not preserved.

§Reelin mRNA equals attomoles per microgram of total RNA. Reelin mRNA determinations were not performed when RNA was degraded (See "Subjects and Methods" section).

||GAD<sub>67</sub>, GAD<sub>65</sub>, and DAB1 protein values are expressed as the optical density ratio with  $\beta$ -actin.

¶DAB1 was determined in 6 randomly chosen patients from each group (see "Subjects and Methods" section).



**Figure 3.** Pearson correlations between reelin messenger RNA (RELN mRNA) and glutamic acid decarboxylase<sub>67</sub> (GAD<sub>67</sub>) protein content in the prefrontal cortex of patients without psychosis (left) and with psychosis (right). Left, Open squares indicate nonpsychiatric subject; open triangles, patients with unipolar depression without psychosis; and open circles, patients with bipolar disorder without psychosis. Right, Solid triangles indicate schizophrenic patients; solid circles, patients with bipolar disorder and psychosis.

phrenic patients and in patients with bipolar disorder with psychosis, when compared with nonpsychiatric patients; the extent of this down-regulation is similar or even greater than that of the GAD<sub>67</sub> protein (Table 3). In contrast, the GAD<sub>65</sub> mRNA and protein expression and that of the mRNA encoding for NSE fail to change. Table 3 also shows that the expression of GAD<sub>67</sub> or RELN mRNAs is still down-regulated when expressed as a function of the respective NSE mRNA content to correct for nonspecific loss of mRNA owing to a putative neuronal damage.

#### DEMOGRAPHIC VARIABLES AND ANTIPSYCHOTIC MEDICATION

The average values of postmortem interval and other demographic variables (sex, age of death, cause of death, or pH of brain) were similar in all 4 subject groups (Table 1). Pearson correlations of postmortem interval to RELN, GAD<sub>67</sub>, and GAD<sub>65</sub> in PFC and cerebellum in all 4 groups ( $r=0.10, 0.06,$  and  $0.18$  for PFC and  $0.06, 0.22,$  and  $0.02$  for cerebellum) or for each diagnostic group, all nonsignificant, and the correlations between the above parameters and age at death, or age at disease onset or duration, or cause of death, or brain pH were virtually 0. Moreover, there were no significant sex differences in RELN mRNA, in GAD<sub>67</sub> or GAD<sub>65</sub> protein levels, or in the number of RELN-positive cells in the PFC.

In view of the known anatomical and physiological differences between the 2 brain hemispheres, we also evaluated whether there were any differences in the levels of RELN mRNA and GAD<sub>67</sub> protein between the right (8 samples in each cohort) and the left (7 samples in each cohort) hemispheres. No overall left vs right difference was observed, and the decreases in these 2 variables were similar in schizophrenic patients and in patients with bipolar disorder when compared with nonpsychiatric patients or patients with depression.

In our cohort only a few patients had a current history of alcohol or other substance abuse (Table 2).<sup>27</sup> No statistically significant interaction exists between alcohol abuse or dependence and PFC or cerebellar RELN or GAD<sub>67</sub> expression (PFC RELN,  $F_{2,51}=0.1, P=.84$ ; PFC

GAD<sub>67</sub>,  $F_{2,53}=0.1, P=.91$ ; cerebellum RELN,  $F_{2,48}=1.8, P=.18$ ; cerebellum GAD<sub>67</sub>,  $F_{2,53}=0.2, P=.79$ ).

The levels of RELN mRNA and GAD<sub>67</sub> protein in the PFC or cerebellum were virtually identical whether subjects with a history of alcohol abuse were included (Table 2) or excluded (ie, PFC RELN mRNA, patients with unipolar depression [ $n=10$ ],  $77 \pm 43$ ; schizophrenic patients [ $n=12$ ],  $33 \pm 20$ ; and patients with bipolar disorder [ $n=13$ ],  $0.65 \pm 0.46$ ). Only a few patients (Table 1) had a history of psychoactive drug abuse; nevertheless, their levels of GAD<sub>67</sub> protein and RELN mRNA were close to the average levels detected in drug-free patients with unipolar or bipolar disorder or schizophrenic patients.

Brain, blood, and urine were screened for alcohol and other substance abuse (see "Subjects and Methods" section). In the patients with depression, 3 patients with a current history of alcohol abuse had detectable blood alcohol levels (0.2-0.6 g/dL), and 1 patient with a history of cocaine abuse had a high level of cocaine and a benzodiazepine. In the group with bipolar disorder, 1 patient with a history of methadone abuse and 1 patient with a history of cocaine abuse were positive for methadone and cocaine, respectively. In the schizophrenic group, 1 patient without a history of drug abuse was positive for morphine and codeine, and 1 patient also without a history of drug abuse was positive for ketamine. In all groups, the other patients were free of other substance abuse or alcohol. All nonpsychiatric subjects were free of substances of abuse, except for 1 subject with a blood alcohol level of 0.06 g/dL. Importantly, the toxicology parameters were not correlated with changes of RELN or GAD<sub>67</sub> expression in any group.

Most schizophrenic patients or patients with bipolar disorder exhibiting severe psychotic symptoms had received antipsychotic medications (Table 1). However, in a combined group of patients, including both those with schizophrenia and those with bipolar disorder and psychosis, there was no significant or consistent correlation between total lifetime intake of antipsychotics, normalized as fluphenazine gram equivalent, and expression levels of RELN mRNA or GAD<sub>67</sub> protein content measured in PFC (RELN,  $r=0.05, P=.82$ ; GAD<sub>67</sub>,  $r=0.11, P=.59$ ) or in cerebellum (RELN,  $r=0.05, P=.81$ ; GAD<sub>67</sub>,  $r=0.02, P=.92$ ). We also evaluated the possible effect of antipsychotic medication at time of death classifying the groups as: no medication, clozapine therapy only, other antipsychotic drugs, or these combined (Table 1). We found no effect of medication on RELN mRNA or GAD<sub>67</sub> protein. In patients never treated with antipsychotic drugs or who had received relatively small amounts of antipsychotic medication and were antipsychotic drug free for several years before death (Table 1), RELN mRNA and GAD<sub>67</sub> protein expressions were virtually identical to the average levels detected in treated patients with bipolar disorder or patients with schizophrenia.

#### COMMENT

The expression of GAD<sub>67</sub> and RELN proteins and mRNAs is down-regulated in PFC and to a lesser extent in the cerebellum of patients with schizophrenia or patients with bipolar disorder with psychosis. In contrast, in patients

**Table 3. GAD<sub>67</sub>, GAD<sub>65</sub>, Proteins and Messenger RNAs (mRNAs), RELN, and Neuron-Specific Enolase (NSE) mRNAs in Prefrontal Cortex of 6 Selected Patients From Each Cohort\***

Patient Cohort	NSE		GAD <sub>67</sub>		GAD <sub>65</sub>			RELN	
	mRNA/ Total RNA	Protein†	mRNA/ Total RNA‡	mRNA/ NSE mRNA§	Protein	mRNA/ Total RNA	mRNA/ NSE mRNA	mRNA/ Total RNA	mRNA/ NSE mRNA¶
Nonpsychiatric	356 ± 56	1.1 ± 0.29	342 ± 127	9.9 ± 4.3	1.2 ± 0.1	20 ± 5.6	0.50 ± 0.22	84 ± 35	2.4 ± 1.0
Depressed	338 ± 159	0.87 ± 0.23	327 ± 89	11 ± 4.7	1.4 ± 0.41	22 ± 2.2	0.65 ± 0.20	80 ± 36	2.7 ± 1.6
Schizophrenic	294 ± 42	0.49 ± 0.31#	86 ± 29#	3.1 ± 1.7#	1.5 ± 0.50	28 ± 7.4	0.55 ± 0.21	13 ± 8.0#	0.90 ± 0.64#
Bipolar disorder and psychosis	387 ± 100	0.48 ± 0.28#	110 ± 39#	3.1 ± 1.4#	1.6 ± 0.55	20 ± 4.4	0.70 ± 0.35	32 ± 17#	0.89 ± 0.61#

\*All values are the mean ± SD. GAD<sub>67</sub> indicates glutamic acid decarboxylase 67; GAD<sub>65</sub>, glutamic acid decarboxylase 65; and RELN, reelin. Only 2 pair-wise comparisons were of interest (1) schizophrenic vs nonpsychiatric subjects and (2) patients with bipolar disorder and psychosis vs nonpsychiatric subjects. A Bonferroni-adjusted  $\alpha$  of 0.05/2 = 0.025 was used. Protein is expressed as an optical density ratio with  $\beta$ -actin; mRNA, attomoles per total RNA or attomoles per 10 attomoles NSE mRNA.

†Subject group difference is  $F_{3,19} = 6.5$ ,  $P = .003$ .

‡Subject group difference is  $F_{3,20} = 8.8$ ,  $P < .001$ .

§Subject group difference is  $F_{3,20} = 9.5$ ,  $P < .001$ .

||Subject group difference is  $F_{3,20} = 10.3$ ,  $P < .001$ .

¶Subject group is  $F_{3,20} = 5.1$ ,  $P = .009$ .

# $P < .03$  was considered statistically significant.

with unipolar disorder and depression, but without psychosis, the expression of these 2 variables is virtually identical to that of nonpsychiatric subjects.

Patients with bipolar disorder and psychosis share severe sociofunctional deficits with schizophrenic patients (Table 1), including the auditory-gating deficit,<sup>30</sup> and like schizophrenic patients, they often receive antipsychotic medication. However, in a combined group of patients including both those with schizophrenia and patients with bipolar disorder and psychosis, no sign of correlation was noted between the levels of GAD<sub>67</sub> protein and RELN mRNA expression with the lifetime dosages of antipsychotic medication. Even psychotic patients who had never received antipsychotic treatment evidenced GAD<sub>67</sub> protein and RELN mRNA down-regulation.

A protracted haloperidol treatment of rats failed to change RELN mRNA content in cortex and cerebellum<sup>7</sup> and, in a recent study, it was shown that protracted haloperidol treatment failed to change the expression of GAD<sub>67</sub> mRNA in the PFC of nonhuman primates.<sup>8</sup> We have reported previously that in rats the turnover rate of GABA fails to change with haloperidol, but it increases with clozapine treatment.<sup>31</sup> Collectively, these data suggest that the down-regulation of RELN and GAD<sub>67</sub> is independent from haloperidol treatment, although a more extensive study including other typical or atypical neuroleptics would be desirable.

Statistical analyses of these data exclude a relevance of differences in postmortem interval, age, sex, cause of death, or any other demographic variable. Moreover, the amnesic and toxicology data indicate that there is not a statistically significant interaction between the abuse of alcohol or other illicit substances and PFC or cerebellar RELN or GAD<sub>67</sub> expression.

The RELN and GAD<sub>67</sub> down-regulation observed in schizophrenic patients or patients with bipolar disorder and psychosis seems to be selective and not dependent on neuronal loss because in the same brains other proteins or mRNAs, including GAD<sub>65</sub> and DAB1, are equal to that of nonpsychiatric subjects.

Moreover, a decrease of GAD<sub>67</sub> and RELN, but not GAD<sub>65</sub> mRNA expression, can be observed also when the measurements are calculated with reference to mRNA encoding for NSE, a specific neuronal marker. In fact, if the down-regulation of GAD<sub>67</sub> protein and RELN expression were due to a decrease in neurons, these changes should not occur if measured against NSE mRNA.

The RELN-immunopositive neurons in PFC (layers I and II) appear small and ovoid.<sup>7</sup> Experiments in rats<sup>11,12</sup> and nonhuman primates<sup>15</sup> have identified these neurons to be horizontal and bitufted GABAergic interneurons.

The density of RELN-immunopositive neurons in schizophrenic patients or patients with bipolar disorder and psychosis is lower (25%-30%) than in patients with unipolar depression or in nonpsychiatric subjects. This decrease appears to be smaller than that of RELN mRNA expression (40%-50%) (Table 2) and RELN protein.<sup>7</sup> This apparent discrepancy may be related to the presence of extracellular RELN bound with high affinity to specific receptors (ie, integrins), which is reflected by RELN mRNA and Western blot measurements, but not by the cell count. Hence, such a discrepancy was expected because RELN immunoreactive extracellular halos were considerably reduced in histological preparations of schizophrenic patients and patients with bipolar disorder and psychosis.

Cerebellar RELN mRNA is almost exclusively expressed in glutamatergic granule neurons.<sup>7,11</sup> If the decrease of cerebellar RELN mRNA found in schizophrenia were to be a consequence of a substantial decrease in the number of these neurons, the size of the cerebellum in schizophrenic patients should be proportionally decreased, because the cerebellar mass to a large extent is influenced by the number of granule neurons. However, no gross alterations of the cerebellar size or shape were observed in the patient cohorts (E. Fuller-Torrey, MD, oral communication, 2000).

Based on the selective down-regulation of RELN and GAD<sub>67</sub> expression in the brain of both schizophrenic pa-

tients with bipolar disorder and patients with psychosis, we hypothesize that RELN and GAD<sub>67</sub> down-regulations may be liability factors that play a role in psychosis vulnerability. Since such a hypothesis may cut across classic boundaries of psychiatric diagnosis,<sup>32</sup> before accepting this conclusion, we need to study a substantial number of patients with unipolar depression and psychosis and a larger group of patients with bipolar disorder without psychosis, to test statistically this hypothesis. Also, in these 2 groups, we need to have a substantial group of psychotic patients who were never treated. Finally, we need to extend these findings to a greater number of cortical, hippocampal, and corpus striatum structures.

Our findings on GAD<sub>67</sub> expression are in keeping with the study of Akbarian et al,<sup>6</sup> and Volk et al<sup>8</sup> who also showed by *in situ* hybridization in the superior frontal gyrus (Brodmann area 9) of schizophrenic patients, a decrease of GAD<sub>67</sub> mRNA expression but no differences in the total number of neurons.

To interpret the nature of GAD<sub>67</sub> expression down-regulation and the functional link between GAD<sub>67</sub> and RELN, we hypothesize that the GABAergic neurons of layers I and II of the PFC, by a constitutive process,<sup>33</sup> secrete RELN into the extracellular matrix, which, in turn, may bind to integrin receptors expressed by basket and chandelier cells. Most of these interneurons whose cell bodies reside in layers III through V do not express RELN,<sup>11,12</sup> but presumably express DAB1,<sup>7,11</sup> which in response to a RELN-initiated signaling cascade (Figure 1), selectively regulates GAD<sub>67</sub>, but not GAD<sub>65</sub> mRNA expression. When RELN is down-regulated (as in the brain of schizophrenic patients and patients with bipolar disorder and psychosis), the DAB1-dependent regulation of protein synthesis declines perhaps leading to the down-regulation of GAD<sub>67</sub> gene expression found in basket or chandelier cells.<sup>8</sup> This hypothesis can be addressed by studying whether RELN-integrin interaction can be demonstrated with electron microscopy in chandelier or basket GABAergic neurons.

The generalized decrease of RELN mRNA and protein in several structures—PFC, temporal and limbic cortices, striatum, and cerebellum—of psychotic patients in the absence of a detectable loss of neurons found in this and in previous studies<sup>6-8</sup> suggests that a genetic RELN mRNA down-regulation (owing to haploinsufficiency or to a dysfunction of the RELN promoter) or an epigenetic (gestational or postgestational viral, hypoxic, or excitotoxic) event may, in part, be responsible for the GAD<sub>67</sub> down-regulation and perhaps for the neuropil reduction reported by Selemon and Goldman-Rakic<sup>2</sup> in PFC of schizophrenic patients.

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## Welcome, Joe!

I AM delighted to welcome Joseph T. Coyle, MD, as the fourth editor of *Archives of General Psychiatry*. Dr Coyle is the Eben S. Draper Professor of Psychiatry and Neuroscience at Harvard Medical School. He is an internationally renowned and respected scientist, educator, and clinician who will add substantially to our team of *JAMA* and ARCHIVES editors. We look to Joe to carry on the great tradition of Daniel X. Freedman, MD, a mentor of Dr Coyle's, and the other former editors of the journal.

I also wish to publicly express my gratitude to Richard M. Glass, MD, who has served as interim editor for the past 3 months. We are happy to have him back full-time as deputy editor of *JAMA*.

Watch for great things to happen with *Archives of General Psychiatry* under Joe's leadership.

Catherine D. DeAngelis, MD, MPH  
Editor-in-Chief, Scientific Publications &  
Multimedia Applications  
Editor, *JAMA*

### Correction

**Misspelling of an Author's Surname.** In the byline of the article titled "Decrease in Reelin and Glutamic Acid Decarboxylase<sub>67</sub> (GAD<sub>67</sub>) Expression in Schizophrenia and Bipolar Disorder," published in the November 2000 issue of the ARCHIVES (2000;57:1061-1069), the fourth author's full name should have read Valeria Di-Giorgi-Gerevini, PhD. The journal regrets the error.