Decreased Glutamic Acid Decarboxylase\textsubscript{67} Messenger RNA Expression in a Subset of Prefrontal Cortical \(\gamma\)-Aminobutyric Acid Neurons in Subjects With Schizophrenia

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Background: Markers of \(\gamma\)-aminobutyric acid (GABA) neurotransmission seem to be altered in the prefrontal cortex (PFC) of subjects with schizophrenia. We sought to determine whether the expression of the messenger RNA (mRNA) for the synthesizing enzyme of GABA, glutamic acid decarboxylase\textsubscript{67} (GAD\textsubscript{67}), is decreased in the PFC of subjects with schizophrenia, whether this change is present in all or only some GABA neurons, and whether long-term treatment with haloperidol decanoate contributes to altered GAD\textsubscript{67} mRNA expression.

Methods: Tissue sections from 10 pairs of subjects with schizophrenia and control subjects and 4 pairs of haloperidol-treated and control monkeys were processed for in situ hybridization histochemical analysis with sulfur-35–labeled oligonucleotide probes for GAD\textsubscript{67} mRNA and exposed to nuclear emulsion. Within each layer of PFC area 9, neurons expressing a detectable level of GAD\textsubscript{67} mRNA were quantified for cell density and the relative level of mRNA expression per cell (grain density per neuron).

Results: In subjects with schizophrenia, the density of labeled neurons was significantly (\(P<.05\)) decreased by 25% to 35% in cortical layers 3 to 5. In contrast, the mean grain density per labeled neuron did not differ across subject groups. Similar analyses in monkeys revealed no effect of long-term haloperidol treatment on either the density of the labeled neurons or the grain density per labeled neuron.

Conclusions: These findings indicate that in subjects with schizophrenia, GAD\textsubscript{67} mRNA expression is relatively unaltered in most PFC GABA neurons but is reduced below a detectable level in a subset of GABA neurons. Altered GABA neurotransmission in this subset may contribute to PFC dysfunction in subjects with schizophrenia.

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CORTICAL markers of \(\gamma\)-aminobutyric acid (GABA) neurotransmission seem to be altered in subjects with schizophrenia. The amount and activity of the synthesizing enzyme for GABA, glutamate decarboxylase (GAD), and the release and uptake of GABA are decreased in subjects with schizophrenia.1-4 Other studies have found alterations in GABA\(_A\) receptors, including increased hydrogen-3–muscimol binding,5,6 an increased density of structures immunoreactive for the \(\alpha\) and \(\beta_{2/3}\) subunits of the GABA\(_A\) receptor,\(^6,7\) and a shift in the ratio of the messenger RNAs (mRNAs) encoding the splice variants of the \(\gamma_2\) subunit in the prefrontal cortex (PFC) of subjects with schizophrenia.9

Akbarian et al\textsuperscript{10} found that the number of neurons expressing a detectable level of mRNA for the 67-kd isoform of GAD (glutamic acid decarboxylase\textsubscript{67} [GAD\textsubscript{67}]) was decreased in PFC area 9, located in the superior frontal gyrus, of subjects with schizophrenia. However, the potential confounding factors associated with postmortem studies require that this finding be independently replicated. Furthermore, since the relative level of GAD\textsubscript{67} mRNA expression per neuron was not determined by Akbarian and colleagues, it is unclear whether GAD\textsubscript{67} mRNA expression was decreased in all GABA neurons such that some were no longer detectable or whether the decrease was primarily limited to a subset of GABA neurons.11 Finally, the long-term pharmacological treatment of schizophrenia necessitates determining if long-term treatment with antipsychotic medications contributes to alterations in GAD\textsubscript{67} mRNA expression by examining, for example, monkeys treated with haloperidol in a manner that reflects clinical practice. Consequently, in our study we sought to answer the following questions: (1) Is the density of neurons expressing a detectable level of GAD\textsubscript{67} mRNA decreased in PFC area 9 in a new cohort of subjects with schizophrenia? (2) If so, is the relative level of GAD\textsubscript{67} mRNA expression decreased in all or only a subset of PFC GABA neurons?
SUBJECTS, MATERIALS, AND METHODS

SUBJECTS

Brain specimens were obtained during autopsies conducted at the Allegheny County Coroner’s Office, Pittsburgh, Pa., after obtaining consent from the next of kin. Ten subjects with schizophrenia, each matched to one control subject for sex, age, and postmortem interval, were used in our study (Table). Pairs were completely matched for sex, and the mean (SD) differences within pairs was 5.0 (4.0) years for age and 4.8 (2.8) hours for postmortem interval. Additionally, subject groups did not differ in mean (SD) freezer storage time or postmortem brain pH (Table). An independent panel of experienced clinicians arrived at consensus DSM-III-R diagnoses for each subject after reviewing medical records and the results of structured interviews conducted with family members of the deceased.13 These interviews also revealed a history of depressive disorder (not otherwise specified) in 1 control subject (case 635), and the presence of alcohol abuse, current at the time of death, in another control subject (case 558); no psychiatric disorders were present in the other control subjects. Four subjects with schizophrenia also had a history of an alcohol and/or other substance abuse disorder (Table). Findings from toxicology studies conducted on all subjects were positive for alcohol (46-276 mmol/L) in 3 control subjects; no other drugs of abuse were detected in any subject. Two subjects with schizophrenia (cases 537 and 622) had not been receiving antipsychotic medications for 9.6 and 1.2 months prior to death, respectively (Table). The mean (SD) age of subjects with schizophrenia at the onset of illness was 26.4 (10.4) years, and the average duration of illness was 18.8 (8.0) years. The brain specimens used in our study were obtained from a community-based population; consequently, most subjects (7 with schizophrenia and 9 controls) died suddenly outside of a hospital setting.

Findings from neuropathological examination of each brain revealed abnormalities only in 1 subject (case 622) in whom an infarction limited to the distribution of the inferior branch of the right middle cerebral artery was discovered. However, PFC area 9 appeared to be unaffected. Additionally, thioflavine S staining revealed a few senile plaques in 1 subject (case 685), but clinical and neuropathological criteria for Alzheimer disease were not met.14 All procedures in our study were approved by the University of Pittsburgh’s Institutional Review Board for Biomedical Research.

TISSUE PREPARATION AND IN SITU HYBRIDIZATION PROCEDURE

The PFC of the right hemisphere was blocked coronally and immediately frozen and stored at -80°C. Coronal tissue sections (20 µm) containing the superior frontal gyrus were cut and thaw mounted onto slides and stored at -80°C until processed. Every 10th section was stained for Nissl substance, and these sections were used to identify the location of area 9 using cytoarchitectonic criteria.15

A cocktail of 3 oligonucleotide probes (Oligos Etc., Wilsonville, Ore) complementary to bases 808 through 849, 1059 through 1106, and 2657 through 2704 of human GAD67 complementary DNA were used to detect GAD67 mRNA.16 The probe specificity for GAD67 mRNA was previously demonstrated by experiments in which (1) an excess of unlabeled probes eliminated specific hybridization signal, (2) the use of each probe alone revealed similar patterns of cellular localization, and (3) the combination of 2 probes produced an increase in hybridization signal.17 The probes were labeled with 35S-DATP (NE, Boston, Mass) using terminal deoxynucleotidyl transferase (Bethesda Research Laboratories, Gaithersburg, Md).

All tissue sections from each subject pair were processed together. Eight sections from each subject were processed for in situ hybridization as previously described.18 Briefly, sections were incubated overnight in hybridization buffer containing a cocktail of sulfur 35–labeled oligonucleotide probe (1.5 × 106 disintegrations per minute per section). After a series of stringent washes, sections were coated with photographic emulsion (NTB2; Eastman Kodak, Rochester, NY) and developed after 4 weeks of exposure. Tissue sections were then counterstained with cresyl violet.

QUANTIFICATION OF GAD67 mRNA EXPRESSION

Slides were coded to conceal subject number and diagnosis from the investigator (D.W.V.). Four tissue sections from each subject were randomly chosen for analysis. Using a Microcomputer Imaging Device (MCID; Imaging Research Inc, London, Ontario), three 140 × 140-µm sampling frames were randomly placed in each cortical layer (Figure 1). The bright-field image (objective ×40) of the sampling frame was digitized, and the outline of each Nissl-stained soma with at least 2 overlying silver grains was traced on the computer monitor at a final magnification of ×1510.19 In a dark-field image of the same sampling frame, the number of grains over each encircled cell was then counted by the Microcomputer Imaging Device system. More than 10 000 cells were sampled in our study, ranging from 310 to 649 cells per subject. The mean (SD) coefficient of error for cell counts in each subject, averaged across layers, was identical in the

(3) Does long-term treatment with haloperidol alter GAD67 mRNA expression in the PFC of monkeys?

RESULTS

SPECIFICITY OF OLIGONUCLEOTIDE PROBES FOR GAD67 mRNA

The specificity of the oligonucleotide probes for GAD67 mRNA was confirmed by the morphological character-
schizophrenic and control groups (0.14 [0.05]) and did not differ in any layer between the groups (paired t test, \( t < 2.4, P > .24 \)). For background measurements, the number of grains in 3 sampling frames randomly placed in the subjacent white matter was determined. The thickness of the gray matter in area 9 was determined by measuring the distance from the pial surface to the layer 6–white matter border at 3 locations on 2 Nissl-stained sections located immediately rostral and caudal to the tissue sections processed for in situ hybridization.

HALOPERIDOL-TREATED MONKEYS

To mimic the clinical treatment of subjects with schizophrenia, 4 male cynomolgus (Macaca fascicularis) monkeys were treated with haloperidol and benztpine mesylate for 9 to 12 months as previously described. Mean (SD) trough serum haloperidol levels (11 [3] nmol/L) were within the reported therapeutic range for the treatment of schizophrenia. Each haloperidol-treated animal was matched to one control animal for sex, age (using bone dating and/or an assessment of developmental stage), and weight (haloperidol-treated animals, 3.8 [1.1] kg; control animals, 4.0 [1.0] kg). Both animals in each matched pair were euthanized at the same time, and following a 45-minute postmortem interval, coronal tissue blocks were frozen and stored at \(-80^\circ C\).

Tissue processing procedures were identical to those described previously in this article with a few minor modifications in section thickness and exposure time. The mean (SD) coefficient of error for neuron counts in each subject averaged across layers was 0.07 (0.01) in both subject groups and did not differ in any layer between the groups (paired t test, \( t < 1.4, P > .26 \)).

DATA ANALYSIS

To measure the relative level of GAD67 mRNA expression per neuron, grain density per neuron (grains per 100 \( \mu m^2 \) somal area) was determined for each sampled cell. Grain density per neuron was then corrected for nonspecific background labeling by subtracting the average background level of grains in the subjacent white matter of the same tissue section. To identify a threshold of grain density per neuron that would exclude nonspecifically labeled cells from the neuron population, histograms of grain density per neuron for all sampled neurons per layer from the controls and subjects with schizophrenia were constructed. These histograms revealed a distribution that appeared bimodal in each layer, representing the modes of nonspecifically and specifically labeled neuron populations. Similar histograms including only neurons with a grain density greater than the background \( \times 5 \) showed a distribution that appeared normal and unimodal in both the schizophrenic and control groups. Therefore, a threshold of the background \( \times 5 \) provided a cutoff at the point of rarity in the distribution of all cells that permitted the identification of specifically labeled neurons. Thus, only neurons with a grain density greater than the background \( \times 5 \) of the individual tissue section were included in the data analysis and are subsequently referred to as GAD67 mRNA-positive (+) neurons. The mean neuron density, mean grain density per neuron, and mean cross-sectional somal area of all GAD67 mRNA+ neurons were then determined for every layer of every subject. For each section, the values of the 3 dependent variables (neuron density, grain density per neuron, and somal size) were averaged across the 3 sampling frames for each cortical layer, with the value of each sampling frame weighted by the number of observations within that frame. Thus, in every layer of each subject, 4 section averages were obtained for each dependent variable. These 4 averages were treated as repeated measures with a compound symmetric covariance structure because the values were possibly correlated and were also exchangeable within a given subject. Pair effect was included to reflect the matching of subjects with schizophrenia with controls for sex, age, and postmortem interval. Postmortem brain pH was included as a covariate because it may reflect the integrity of some mRNA species.

Thus, the effect of diagnostic group on each of the 3 dependent variables in each layer was examined using a multivariate analysis of covariance model with the 4 section averages having a compound symmetric covariance matrix, with pair as a blocking effect and brain pH as a covariate.

For all comparisons, the Holm simultaneous inference procedure was used to identify which of the 6 layers showed a significant diagnostic group effect for that variable. The Holm procedure maintains the overall family-wise error rate at the .05 level and tests individual laminar diagnostic effects at certain prescribed significance levels. Specifically, the \( P \) values, \( P_i^* \), for diagnostic group effect are ordered from smallest (\( i^* = 1 \)) to largest (\( i^* = 6 \)) among the 6 layers. The layer corresponding to \( P_i^* \) is declared to have a significant diagnostic effect at the family-wise .05 level if \( P_i^* \leq 0.05/[(N + 1) - i^*] \), where \( N \) is the number of comparisons. For example, the laminar level corresponding to the smallest \( P \) value (\( i^* = 1 \)) is declared to have a significant diagnostic effect if that \( P \) value \( \leq 0.05/[(6 + 1) - 1] = .0083 \). To maintain consistency throughout the text, the prescribed significance level, and consequently the quoted \( P \) value, for each laminar diagnostic group have been adjusted to correspond with the family-wise error rate of .05 (ie, \( P_i^* \times [(N + 1) - 1^*] \)). For the haloperidol-treated monkeys, 2-paired t tests and the Holm correction were used to determine the effect of treatment group on each of the 3 dependent variables in each cortical layer.
through 5, at least 8 of 10 subject pairs showed a decrease in GAD	extsubscript{67} mRNA+ neuron density in the subject with schizophrenia (Figure 6).

In contrast to these differences in the density of labeled neurons, the mean grain density per GAD	extsubscript{67} mRNA+ neuron, a relative measure of GAD67 mRNA expression per neuron, did not differ (F1,8 \(= 4.1, P = .45\)) in any layer between subjects with schizophrenia and control subjects (Figure 5, bottom). The mean cross-sectional somal area of GAD	extsubscript{67} mRNA+ neurons also did not differ (F1,8 \(= 4.1, P = .43\)) in any layer between subjects with schizophrenia and control subjects. Furthermore, the mean (SD) thickness of the cortical gray matter for the subjects with schizophrenia (2.73 [0.33] mm) and control subjects (3.02 [0.47] mm) also did not differ (t\(_9\) = −1.65, P = .13).

GAD	extsubscript{67} mRNA EXPRESSION IN THE PFC OF MONKEYS

In the monkey tissue, silver grains were also selectively clustered over small- and medium-sized neurons, with the density of GAD	extsubscript{67} mRNA+ neurons greatest in layers 2 and 4. This laminar distribution matched that of GABA-immunoreactive neurons in the PFC of cynomolgus monkeys.27 However, in contrast to the observations in humans, neither neuron density nor grain density of the GAD	extsubscript{67} mRNA+ neurons differed significantly (t\(_3\) < 3.47, P > .24) in any layer between haloperidol-treated monkeys and controls (Figure 7).

We found that the density of GAD	extsubscript{67} mRNA+ neurons was significantly reduced in layers 3 through 5, with a trend (P = .06) toward a reduction in layer 2 of PFC area 9 in a new cohort of subjects with schizophrenia. In contrast, grain density per GAD67 mRNA+ neuron, a relative measure of the cellular level of GAD67 mRNA expression, did not differ between subject groups. Together, these observations suggest that in the PFC of subjects with schizophrenia, GAD67 mRNA expression is relatively unaltered in most GABA neurons but is reduced below a detectable level in a subset of GABA neurons. In addition, the results from the study of haloperidol-treated monkeys suggest that decreased GAD67 mRNA expression in the PFC of subjects with schizophrenia is not a consequence of long-term treatment with haloperidol.

Akbarian et al\(^\text{10}\) previously reported a 30% to 50% decrease in GAD	extsubscript{67} mRNA+ neuron density in layers 1 through 5 of PFC area 9 from the left hemisphere. In the present study, GAD	extsubscript{67} mRNA+ neuron density was also decreased by 25% to 35% in these same layers of area 9 from the right PFC, suggesting a common, bilateral de-
crease in GAD$_{67}$ mRNA+ neuron density in the PFC of subjects with schizophrenia. In addition, decreased GAD protein has been reported in the temporal cortex of subjects with schizophrenia. Together, these observations suggest that decreased GAD$_{67}$ mRNA expression in the association regions of the neocortex may be a frequent feature of schizophrenia.

Comparisons with other studies suggest that the decrease in the density of GAD$_{67}$ mRNA+ neurons was not due to a decrease in the number of PFC neurons in subjects with schizophrenia. Our finding of a decreased density of GAD$_{67}$ mRNA+ neurons is strikingly similar in magnitude and laminar distribution to the previous report by Akbarian et al, who also found that the density of GAD$_{67}$ mRNA+ neurons was decreased in layers 2 through 5, the primary location of chandelier neurons. However, the magnitude of the decrease in GAD$_{67}$ mRNA+ neuron density suggests that other subpopulations of GABA neurons may be affected as well.

The typical long-term exposure of subjects with schizophrenia to antipsychotic medications requires determining whether pharmacotherapy may contribute to the altered expression of GAD$_{67}$ mRNA in the PFC. Although long-term treatment with haloperidol and other dopamine D$_2$-like receptor antagonists can reportedly affect GAD$_{67}$ mRNA expression in the rat basal ganglia, it was unclear whether long-term use of antipsychotic medications could also affect GAD$_{67}$ mRNA expression in the PFC, where the density of D$_2$-like receptors is much lower. In our study, long-term treatment with haloperidol did not affect GAD$_{67}$ mRNA expression in the PFC of monkeys. Consistent with this observation, the 2 subjects with schizophrenia (cases 537 and 622) who were not receiving antipsychotic medications at the time of death had densities of GAD$_{67}$ mRNA+ neurons less than that of their matched

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controls. In addition, the 4 subjects who had received atypical antipsychotic agents (Table) showed a similar decrease in GAD67 mRNA+ neuron density compared with those who had been treated only with typical antipsychotics.

Although stereological approaches to determine absolute cell number are advantageous in many situations, they remain problematic in studies of individual cortical regions lacking clearly delineated boundaries and in studies using in situ hybridization. Thus, a 2-dimensional sampling technique was used to make relative comparisons of differences in neuron density and, importantly, was not confounded by cross-subject differences in somal size. In addition, the modest reduction of cortical gray matter in subjects with schizophrenia observed in many other studies would be expected to elevate neuronal density. Consistent with these reports, we found a 10% (but nonsignificant) decrease in cortical gray matter in subjects with schizophrenia. Thus, the observed 25% to 35% decrease in the relative density of GAD67 mRNA+ neurons may actually be an underestimate of the differences between subjects with schizophrenia and control subjects.

A threshold of grain density per neuron (≥5× the background) was identified to exclude nonspecifically labeled cells from analysis. The use of a lower threshold (≥3× the background) revealed similar differences in neuron density and grain density per GAD67 mRNA+ neuron. Thus, the level of GAD67 mRNA expression in a subset of GABA neurons appears to be so low that these neurons are still not detectable even when a less stringent threshold for specific labeling is used.

Premortem agonal state events may affect postmortem levels of some mRNA species. Brain pH, reportedly an inverse correlate of agonal state, did not differ between the subject groups in our study. Additionally, GAD67 mRNA was not generally degraded in the subjects with schizophrenia because most GAD67 mRNA+ neurons in subjects in the schizophrenic group had normal levels of GAD67 mRNA expression. Furthermore, a similar analysis of the cellular levels of synaptophysin mRNA revealed no differences between the same subjects with schizophrenia and control subjects examined in the present study.
Four subjects with schizophrenia in our study met criteria for a substance abuse disorder (Table), but the available data suggest that these comorbid conditions did not contribute to the decreased density of GAD67 mRNA+ neurons. First, the only 2 subjects with schizophrenia who, compared with their matched control subject, showed a similar or increased density of GAD67 mRNA+ neurons in several layers both met criteria for alcohol abuse (Table, pairs 6 and 9). Second, the only control subject (case 558) with alcohol abuse had a higher GAD67 mRNA+ neuron density than the matched subject with schizophrenia. Finally, the 3 control subjects with positive plasma alcohol levels (46-276 mmol/L) still had a higher density of GAD67 mRNA+ neurons than their matched subjects with schizophrenia.

Our study provides further insight into the potential pathophysiological mechanisms underlying altered PFC GABA neurotransmission in subjects with schizophrenia. The alteration in GAD67 mRNA expression may reflect an intrinsic defect in a subset of PFC GABA neurons. For example, in PFC area 9 in subjects with schizophrenia, the decreased density of chandelier neuron axon terminals immunoreactive for the GABA membrane transporter suggests that the uptake of GABA is impaired at these axon terminals. As a consequence, inhibitory GABA activity may be increased at postsynaptic sites. If the same relationships hold true for the GABA system, then GAD67 mRNA expression may be down-regulated in chandelier neurons as a compensatory response to excessive GABA activity.

Alternatively, an abnormality in afferents to the PFC may result in a reduced level of GAD67 mRNA expression in the PFC. For example, several studies of schizo-
phrenia have found decreased neuron number in and/or volume of the mediodorsal nucleus of the thalamus,\textsuperscript{51-54} a major source of excitatory input to the PFC. In addition, reports of fewer dendritic spines\textsuperscript{55,56} and axon terminals\textsuperscript{57} in PFC layers 3 and 4, the principal termination zone of projections from the thalamus, are consistent with a decrease in these afferents in subjects with schizophrenia. Monocular deprivation studies in monkeys indicate that a loss of thalamic input produces decreased GAD\textsubscript{67} mRNA expression in layer 4 and adjacent layers of visual cortex.\textsuperscript{58,59} Thus, the decreased expression of GAD\textsubscript{67} mRNA observed in our study may reflect a down-regulation of inhibition in the PFC to compensate for a decrease in excitatory drive from the mediodorsal nucleus of the thalamus. Further studies are needed to discriminate between these or other possible mechanisms underlying decreased GAD\textsubscript{67} mRNA expression in a subset of GABA neurons in subjects with schizophrenia.

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