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Markedly Lower Glutamic Acid Decarboxylase 67 Protein Levels in a Subset of Boutons in Schizophrenia

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ABSTRACT

BACKGROUND: Convergent findings indicate that cortical gamma-aminobutyric acid (GABA)ergic circuitry is altered in schizophrenia. Postmortem studies have consistently found lower levels of glutamic acid decarboxylase 67 (GAD67) messenger RNA (mRNA) in the prefrontal cortex (PFC) of subjects with schizophrenia. At the cellular level, the density of GABA neurons with detectable levels of GAD67 mRNA is ~30% lower across cortical layers. Knowing how this transcript deficit translates to GAD67 protein levels in axonal boutons is important for understanding the impact it might have on GABA synthesis. In addition, because reductions in GAD67 expression before, but not after, the maturation of GABAergic boutons results in a lower density of GABAergic boutons in mouse cortical cultures, knowing if GABAergic bouton density is altered in schizophrenia would provide insight into the timing of the GAD67 deficit.

METHODS: PFC tissue sections from 20 matched pairs of schizophrenia and comparison subjects were immunolabeled for the vesicular GABA transporter (vGAT) and GAD67.

RESULTS: vGAT+ bouton density did not differ between subject groups, consistent with findings that vGAT mRNA levels are unaltered in the illness and confirming that the number of cortical GABAergic boutons is not lower in schizophrenia. In contrast, in schizophrenia subjects, the proportion of vGAT+ boutons with detectable GAD67 levels (vGAT+/GAD67+ boutons) was 16% lower and mean GAD67 levels were 14% lower in the remaining vGAT+/GAD67+ boutons.

CONCLUSIONS: Our findings suggest that GABA production is markedly reduced in a subset of boutons in the PFC of schizophrenia subjects and that this reduction likely occurs after the maturation of GABAergic boutons.

Keywords: Development, GABAergic, GAD67, Gamma-aminobutyric acid, Quantitative microscopy, vGAT

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Cognitive deficits, such as impairments in working memory, are recognized as core clinical features in schizophrenia (1), and these impairments are thought to reflect, at least in part, disturbances in gamma aminobutyric acid (GABA)-releasing (GABAergic) neurons within the prefrontal cortex (PFC) (2). Perhaps the most widely and consistently reported finding in postmortem studies of subjects with schizophrenia is lower levels of messenger RNA (mRNA) for the GABA synthesizing enzyme, glutamic acid decarboxylase 67 (GAD67) (3–8). Although less well-studied, the deficit in GAD67 mRNA has been reported to be accompanied by lower GAD67 protein levels (6,7,9). The consistency of these findings suggests that lower GAD67 expression in schizophrenia is a common feature of the illness. Other findings indicate that it is not a consequence of illness chronicity or other factors frequently associated with the illness, such as use of antipsychotic medications (7,10).

However, not all GABAergic neurons exhibit lower GAD67 mRNA expression in schizophrenia. Specifically, ~30% of GABAergic neurons in schizophrenia PFC lack detectable levels of GAD67 mRNA, whereas the others express GAD67 mRNA at normal levels (3,5). The subset of GABAergic neurons with markedly lower GAD67 mRNA expression is prominent in PFC layers 2 through 5 (3,5). This deficit occurs in the absence of a change in total neuron density (5) or number (11), suggesting that all GABAergic neurons are present but that a subset have a markedly reduced capacity to synthesize GABA. Moreover, expression of some other gene products that can affect GABAergic neurotransmission are reported to be unaffected or only slightly altered in schizophrenia. For example, mRNA for the vesicular GABA transporter (vGAT), which packages GABA into synaptic vesicles, was reported to be unchanged or only modestly lower in the PFC of schizophrenia subjects (10,12), suggesting that the ability to load GABA into vesicles is preserved in the illness.

Although the timing of onset of the GAD67 mRNA deficit is unknown, dysfunction of the PFC in schizophrenia appears to be a late developmental event. For example, in children who are later diagnosed with schizophrenia, working memory performance appears to be intact until about 9 years of age

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and then subsequently declines (13). Because working memory relies on the coordinated firing of PFC pyramidal neurons (14) by GABAergic interneurons (15,16), these findings suggest that the GAD67 deficit may arise during childhood. Knowing if structural alterations in GABAergic axon boutons occur in schizophrenia could inform on the timing of the GAD67 deficit in schizophrenia. For example, genetic reduction of GAD67 expression in parvalbumin basket cells during early stages of development results in fewer boutons, whereas the same reduction later does not alter axonal architecture (17). Thus, it would be expected that a reduction in GAD67 expression during the prenatal or perinatal periods in individuals who are later diagnosed with schizophrenia would be accompanied by fewer GABAergic axon boutons.

In concert, these findings suggest the following testable hypotheses: 1) the density of all GABAergic axonal boutons is unaltered in the PFC of subjects with schizophrenia; and 2) GAD67 protein levels are markedly lower in a subset of these boutons. To test these hypotheses, we immunolabeled PFC tissue sections from 20 matched pairs of schizophrenia and comparison subjects for vGAT and GAD67 and assessed GABAergic bouton density and bouton protein levels across cortical layers using quantitative confocal microscopy techniques.

METHODS AND MATERIALS

Subjects

Brain specimens from 40 subjects were recovered during autopsies conducted at the Allegheny County Medical Examiner's Office (Pittsburgh, Pennsylvania) after obtaining consent from the next of kin. An independent committee of experienced research clinicians made consensus DSM-IV diagnoses or confirmed the absence of any diagnoses for each subject using the results of structured interviews conducted with family members and/or review of medical records (18). To reduce biological variance between groups and to control for experimental variance, each schizophrenia subject was matched to one comparison subject for sex and as closely as possible for age and postmortem interval (PMI) (Table 1; Supplemental Table S1). The length of the PMI can affect protein integrity (7,19) and aging can differentially affect gene expression (10). Consequently, to reduce the potential effects of these confounding variables, we selected all available subjects with PMI $<$ 16 hours and age $\leq$ 55 years. The University of Pittsburgh Committee for the Oversight of Research Involving the Dead and Institutional Review Board for Biomedical Research approved all procedures.

The left hemisphere of each brain was blocked coronally at 1 cm to 2 cm intervals, immersed in 4% paraformaldehyde for 48 hours at 4°C, and then washed in a series of graded sucrose solutions and cryoprotected. Tissue blocks containing the PFC were sectioned coronally at 40 μm on a cryostat and stored in a 30% glycerol/30% ethylene glycol solution at −30°C until processed for immunohistochemistry. Adjacent Nissl-stained sections were used to select sections that were cut perpendicular to the pial surface.

Immunohistochemistry

For each subject, four sections containing PFC area 9 spaced ~500 μm apart were used. To minimize experimental variance within and across subject pairs, two separate experimental runs consisting of two sections per subject (80 sections total per run) were performed with all sections within a run processed simultaneously. Sections were preprocessed (see Supplemental Methods and Materials) and then incubated for ~72 hours at 4°C in phosphate-buffered saline (PBS) containing 2% donkey serum and primary antibodies that recognize vGAT (mouse host; 1:500; product 131011, Lots 131011/41 and 131011/42; Synaptic Systems, Goettingen, Germany) and GAD67 (goat host; 1:100; product AF2086, Lot KRD0110031; R&D Systems, Minneapolis, Minnesota). The specificity of each antibody was verified by Western blot in our laboratory [data not shown and (20)] or other laboratories [vGAT (21); GAD67 (22,23)]. Sections were then rinsed for 2 hours in PBS and incubated for 24 hours in PBS containing 2% donkey serum and secondary antibodies (donkey host) conjugated to Alexa 488 (vGAT) or 647 (GAD67) (1:500 for all) (Invitrogen, Grand Island, New York) at 4°C. After washing, sections were mounted (ProLong Gold antifade reagent, Invitrogen) on slides that were coded to conceal diagnosis and subject number and stored at 4°C until imaged.

Microscopy

Data were collected on an Olympus IX81 inverted microscope equipped with an Olympus spinning disk confocal unit (Center Valley, Pennsylvania), Hamamatsu EM-CCD digital camera (Bridgewater, New Jersey), and high-precision BioPrecision2 XYZ motorized stage with linear XYZ encoders (Ludl Electronic Products Ltd., Hawthorne, New Jersey) using a 60X 1.40 numerical aperture super corrected oil immersion objective. The equipment was controlled by SlideBook 5.0 (Intelligent Imaging Innovations, Inc., Denver, Colorado), which was the same software used for postimage processing. Three-dimensional image stacks (two-dimensional images successively captured at intervals separated by .25 μm in the z-dimension) that were 512 × 512 pixels (~ 137 × 137 μm) were acquired over 50% of the total thickness of the tissue section starting at the coverslip. Importantly, imaging the same percentage of the tissue section thickness rather than the same number of microns controls for the potential confound of storage and/or mounting related volume differences (i.e., z-axis shrinkage). The stacks were collected using optimal exposure settings (i.e., those that yielded the greatest dynamic range with no saturated pixels), with differences in exposures normalized during image processing.

Sampling

As determined by measurements made in Nissl-stained sections (24), the boundaries of the six cortical layers can be estimated based on the distance from the pial surface to the white matter. For the presented studies, the cortical mantle was divided into layers as follows: 1 (pia to 10%), 2/superficial 3 (10% to 35%), deep 3/4 (35% to 60%), 5 (60% to 80%), and 6 (80% to gray/white matter border). Ten systematic randomly sampled image stacks were taken within each of these subdivisions using a sampling grid of 180 × 180 μm^2. Running means using pilot data indicated that 10 sites per subdivision were sufficient to limit intrasubject variability for intensity and density measures. The same investigator (BRR), who was blinded to subject and diagnosis, collected a total of 8000 image stacks. Both subjects within a pair were imaged on the same day.