N-Methyl-D-Aspartate Receptor and Calbindin-Containing Neurons in the Anterior Cingulate Cortex in Schizophrenia and Bipolar Disorder

Tsung-Ung W. Woo, Kevin Shrestha, Dorian Lamb, Martin M. Minns, and Francine M. Benes

Background: Glutamatergic modulation of γ-aminobutyric acid (GABA) interneurons via the NR2A subunit of the *N*-methyl-D-aspartate (NMDA) receptor in the cerebral cortex contributes to the pathophysiology of schizophrenia and bipolar disorder. Previously, we found that, in the anterior cingulate cortex (ACCx), the number of GABA cells that expressed the messenger RNA (mRNA) for the NMDA NR2A subunit was significantly decreased in subjects with schizophrenia and bipolar disorder and that this decrease occurred most prominently in layer 2. In this study, we hypothesized that the subset of GABA interneurons that contained the calcium-binding protein calbindin (CB), by virtue of their preferential localization to layer 2, might be particularly affected.

Methods: We simultaneously labeled the mRNA for the NMDA NR2A subunit with [^{35}S] and the mRNA for CB with digoxigenin with an immunoperoxidase procedure.

Results: We found that, in the normal human ACCx, only approximately 10% of all CB-containing cells expressed NR2A mRNA. However, compared with the normal control subjects and subjects with bipolar disorder, the density of CB+/NR2A+ neurons in layer 2 was increased by 41% to 44 % in subjects with schizophrenia, whereas the amount of NR2A mRNA/CB+ neurons was unchanged.

Conclusions: These observations suggest that, in schizophrenia, a number of CB-containing cells that normally do not express NR2A might become NR2A-expressing or, perhaps not mutually exclusively, the number of CB-expressing cells might be increased and these cells express NR2A. The findings of this study highlight the notion that glutamatergic innervation of subsets of GABA cells might be differentially altered in schizophrenia and bipolar disorder.

Key Words: Bipolar disorder, GABA, postmortem human brain, schizophrenia

Genetic, molecular, and clinical evidence increasingly implicates the *N*-methyl-D-aspartate (NMDA) class of glutamate receptors as playing a major role in the pathophysiology of schizophrenia (1–6). More specifically, it has been postulated that, in schizophrenia, hypofunction of the NMDA receptors that are located on γ-aminobutyric acid (GABA)ergic interneurons in the corticolimbic system might instigate a sequence of downstream events that might include neuronal injury and apoptosis (7,8). We recently reported that, consistent with this idea, in layer 2 of the anterior cingulate cortex (ACCx), the expression of the messenger RNA (mRNA) for the NR2A subunit of the NMDA receptor was decreased to an experimentally undetectable level in as many as 73% of the GABA interneurons in subjects with schizophrenia (9). Interestingly, similar changes were also observed in subjects with bipolar disorder (9), raising the possibility that altered NMDA receptor function on inhibitory interneurons might represent a shared pathophysiologic cascade for the two disorders.

Because connectionally and functionally distinct subpopulations of GABA interneurons regulate different aspects of information flow in the cerebral cortex (10–13), an important question that needs to be addressed to gain further insight into the pathophysiologic consequences of reduced NR2A expression is the identity of the GABA cells that are affected. Subpopulations of GABA neurons can be distinguished by their differential expression of distinct calcium binding proteins (13–18). For instance, the subset of GABA cells that express a high level of the calcium binding protein calbindin (CB) tends to be concentrated in the upper layers, especially layer 2, of the cortex, although cells that are weakly immunoreactive for CB, which might also include a small number of pyramidal cells, seem to spread across all layers (16,19,20). Because we previously found that, in the ACCx in schizophrenia and bipolar disorder, the decrease in the density of NR2A-expressing GABA cells occurred most prominently in layer 2 (9), we postulated that the expression of NR2A mRNA in CB-containing neurons in this layer would be decreased in these disorders. We instead found that, contrary to this hypothesis, the density of NR2A mRNA-expressing CB neurons might actually be increased in layer 2 of the ACCx in schizophrenia, but it was unchanged in bipolar disorder.

Methods and Materials

Subjects

A cohort of 60 human brains from 20 subjects with schizophrenia, 20 subjects with bipolar disorder, and 20 normal control subjects were obtained from the Harvard Brain Tissue Resource Center at McLean Hospital, Belmont, Massachusetts (Supplement 1). Each group of subjects with schizophrenia or bipolar disorder was matched to a normal control group on the basis of age, postmortem interval (PMI), and whenever possible, gender and side of hemisphere. The mean freezer storage time (days ± SD) of brains did not differ between the normal control (1407 ± 555), schizophrenia (1680 ± 630), and bipolar disorder (1500 ± 834) groups. The mean pH (± SD) also did not differ between the

From the Program in Structural and Molecular Neuroscience (T-UWW, KS, DL, MMM, FMB), McLean Hospital, Belmont; the Department of Psychiatry (T-UWW, FMB) and Program in Neuroscience (FMB), Harvard Medical School; and the Department of Psychiatry (T-UWW), Beth Israel Deaconess Medical Center, Boston, Massachusetts.

Address reprint requests to Tsung-Ung W. Woo, M.D., Ph.D., Program in Structural and Molecular Neuroscience, McLean Hospital, 115 Mill Street, Belmont, MA 02478; E-mail: wwoo@hms.harvard.edu.

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three groups of subjects (normal control: 6.51 ± .24; schizophrenia: 6.53 ± .23; bipolar: 6.48 ± .23).

Psychiatric diagnoses were established with a retrospective review of medical records and an extensive family questionnaire that included the medical, psychiatric, and social history of the subjects. For the diagnosis of schizophrenia, the criteria of Feighner *et al.* (21) were used and the diagnoses of schizoaffective and bipolar disorder were made according to DMS-III-R criteria. Four of the 20 subjects with schizophrenia were not taking antipsychotic medications at the time of death. In the bipolar group, 13 subjects were taking antipsychotic medications at the time of death, and the medication status of 2 subjects is not known. The average dosage of antipsychotic drugs that the bipolar subjects (213.3 ± 282.8 mg) were receiving (expressed as chlorpromazine-equivalent dose [CED]) was approximately one-half that of the schizophrenia subjects (412.2 ± 465.7 mg). Some subjects in both disease groups were also taking concomitant psychotropic medications, such as mood stabilizing, antidepressant, or anxiolytic drugs (Supplement 1). No subjects in the normal control group were receiving any psychotropic drugs at the time of death.

Tissue Preparation

Tissue blocks (3 mm in thickness) from Brodmann area 24 were removed from fresh brain specimens at the level of the rostrum of the anterior cingulate gyrus between the points at which the gyrus curves above and below the corpus callosum (22). The blocks were immediately fixed in .1% paraformaldehyde in ice-cold .1 mol/L phosphate buffer (pH 7.4) for 90 min, immersed in 30% sucrose in the same buffer overnight, and then frozen in Tissue Tek OCT (Sakura Finetek, Torrance, California) on dry ice. Tissue blocks were then sectioned at a thickness of 10 μm on a cryostat. Two sections/subject and therefore 6 sections/matched triplet were used for in situ hybridization. The six sections from each triplet were mounted on three slides as follows: 1) normal control + schizophrenia, 2) normal control + bipolar, and 3) schizophrenia + bipolar. This method of mounting sections controls for potential variability of hybridization signals between slides. All mounted sections were stored at −70°C until riboprobe labeling was performed.

Double In Situ Hybridization

Riboprobe Preparation. RADIOLABELED COMPLEMENTARY RNA PROBE FOR NR2A MRNA. The complementary RNA (cRNA) probes for the NR2A subunit (kindly provided by Dr. Christine Konradi) were transcribed in vitro from linearized complementary DNA (cDNA) subclones encoding the rat NMDA NR2A subunit, which is 89% identical to the human sequence. The probe was derived from a cDNA spanning nucleotides 1185–2154 (Genbank Accession #NM91561) within the coding region of the subunit. A corresponding sense probe was used as negative control. Radiolabeled cRNA probe was prepared by first drying down [^{35}S]UTP (500 μCi/mL of probe, New England Nuclear, Boston, Massachusetts) in a DNA Speed-Vac (Savant, Farmingdale, New York). Then, 100 ng/μL of the cDNA template, .1 mol/L dithiothreitol (DTT), 3 U/μL RNasin, 5 mmol/L NTPs, .8 U/μL of T3 or T7 RNA polymerases (for antisense and sense probe, respectively), and 5× transcription buffer were added. The transcription mixture was subsequently incubated at 37°C for 1 hour. The cDNA template was digested by incubating the mixture with R1Q DNase at 37°C for 15 min. Unincorporated NTPs were removed by running the mixture through a Stratagene Nuc-Trap (La Jolla, California) push column. The eluate was collected, and probe concentration was determined by scintillation counting. The probe was stored at −20°C until use.

DIGOXIGENIN-LABELED CB MRNA PROBE. The digoxigenin (DIG)-UTP-labeled cRNA probes were transcribed with 100 ng of linearized human cDNA subclones encoding nucleotides 289–937 (Genbank Accession #NM004929.2) of the human CB protein in the presence of .1 mol/L DTT; 3 U/μL Rnasin; .8 U/μL of T3 and T7 RNA polymerases; 10 mmol/L of ATP, CTP, and GTP; 6.5 mmol/L of UTP; and 3.5 mmol/L of DIG-labeled UTP (Boehringer Mannheim, Indianapolis, Indiana). The mixture was incubated at 37°C for 1 hour. The cDNA template was digested with RQ1 DNase. Probe concentration was determined with a standard with known concentrations. A corresponding sense probe was used as negative control.

Hybridization

Probes were reconstituted in a hybridization buffer consisting of 50% formamide, .1% yeast transfer RNA (tRNA), 10% dextran sulfate, 1× Dehardt's solution, .5 mol/L ethylenediamine tetraacetic acid (EDTA), .02% sodium dodecyl sulfate (SDS), 4× saline-sodium citrate (SSC) buffer, 10 mmol/L DTT, and .1% single-stranded DNA (ssDNA), at a final concentration of .4 ng probe/μL hybridization buffer. Before hybridization, mounted tissue sections were air dried and warmed to room temperature. They were then post-fixed in 4% paraformaldehyde for 10 min and incubated in .1 mol/L tetraethylammonium (TEA) for 5 min at room temperature before being dehydrated in a graded series of ethanol. Probes were then added to slides for hybridization in a prewarmed, humidified dish. Sections were covered with coverslips and incubated at 57°C for 12 hours. At the end of hybridization, coverslips were soaked off in 4× SSC in the presence of 100 μL of βMer. Tissue was then incubated in .5 mol/L sodium chloride (NaCl)/.05 mol/L PB for 10 min, .5 mol/L NaCl with .025 mg/mL RNaseA at 37°C for 30 min, followed by a high-stringency wash with a solution containing 50% formamide, .5 mol/L NaCl/.05 mol/L PB, and 100 μL βMer at 63°C for 30 min. Sections were finally washed overnight in .5× SSC with 20 mmol/L βMer ethanol at room temperature.

Visualization of DIG Labeling

After incubation in blocking buffer (100 mmol/L Tris-HCL, 150 mmol/L NaCl pH 7.5, 3% normal goat serum (NGS), .3% Triton X100, sections were incubated overnight at 4°C in buffer containing 1:200 dilution of sheep anti-DIG antibody (Roche Diagnostics, Indianapolis, Indiana). Processing was then completed by using the Vectastain ABC Elite Kit (Vector Laboratories, Burlingame, California) and diaminobenzidine (DAB).

Emulsion Autoradiography

It was determined that sufficient autoradiographic signal had developed after the slides were apposed to X-ray film (Kodak Biomax MS, Rochester, New York) for 5 days. The slides were then dipped in emulsion (Kodak NTB-2), air dried, and stored at 4°C in dark for 3 weeks. After development in the dark with Kodak D-19 developer, slides were counterstained with cresyl violet and coverslipped.

Quantification of NR2A mRNA Expression

All microscopic analyses were conducted under strictly blind condition by one investigator (D.L.) as previously described (9,23). The entire quantification process was completed in 10 weeks. The [^{35}S]-labeling of NR2A mRNA appeared as clusters of silver grains after processing for emulsion autoradiography. The