Molecular Determinants of Dysregulated GABAergic Gene Expression in the Prefrontal Cortex of Subjects with Schizophrenia

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Background: Prefrontal deficits in gamma-aminobutyric acid (GABA)ergic gene expression, including neuropeptide Y (NPY), somatostatin (SST), and parvalbumin (PV) messenger RNAs (mRNAs), have been reported for multiple schizophrenia cohorts. Preclinical models suggest that a subset of these GABAergic markers (NPY/SST) is regulated by brain-derived neurotrophic factor (BDNF), which in turn is under the inhibitory influence of small noncoding RNAs. However, it remains unclear if these mechanisms are important determinants for dysregulated NPY and SST expression in prefrontal cortex (PFC) of subjects with schizophrenia.

Methods: Using a postmortem case-control design, the association between BDNF protein, NPY/SST/PV mRNAs, and two BDNF-regulating microRNAs (miR-195 and miR-30a-5p) was determined in samples from the PFC of 20 schizophrenia and 20 control subjects. Complementary studies were conducted in cerebral cortex of mice subjected to antipsychotic treatment or a brain-specific ablation of the *Bdnf* gene.

Results: Subjects with schizophrenia showed deficits in NPY and PV mRNAs. Within-pair differences in BDNF protein levels showed strong positive correlations with NPY and SST and a robust inverse association with miR-195 levels, which in turn were not affected by antipsychotic treatment or genetic ablation of *Bdnf*.

Conclusions: Taken together, these results suggest that prefrontal deficits in a subset of GABAergic mRNAs, including NPY, are dependent on the regional supply of BDNF, which in turn is fine-tuned through a microRNA (miRNA)-mediated mechanism.

Key Words: BDNF, microRNAs, NPY, prefrontal cortex, schizophrenia, SST

Schizophrenia is a complex psychiatric disorder with genetic (1,2) and epigenetic (3,4) factors potentially contributing to its pathophysiology, which has been linked among others to aberrant inhibitory synaptic function in the prefrontal cortex (PFC) (5,6). Interestingly, multiple studies have revealed deficits in the expression of gamma-aminobutyric acid (GABA)ergic transcripts such as neuropeptide Y (NPY), somatostatin (SST), parvalbumin (PV), and glutamic acid decarboxylase 67 (GAD67) in the prefrontal cortex of patients with schizophrenia (7–11). Two interneuron subtypes, including PV-positive fast-spiking neurons forming synapses with perisomatic domains of pyramidal neurons and nonfast-spiking NPY-positive and SST-positive neurons targeting pyramidal neuron distal dendrites (5,6), are pivotal for the synchronization of prefrontal neuronal networks, which are disrupted in schizophrenia (12–14). Furthermore, brain-derived neurotrophic factor (BDNF), a potential schizophrenia susceptibility gene (15–18), and its receptor tropomyosin related kinase B (TRK-B) could be important regulators of the GABAergic transcriptome in mammalian cerebral cortex (19–21). Based on studies in *Bdnf* mutant mice (21), NPY and SST messenger RNA (mRNA) expression is dependent on BDNF, but this link has not yet been explored in schizophrenia postmortem studies.

MicroRNAs (miRNAs) are small noncoding RNAs that are evolutionarily conserved and are predicted to target at least one third of protein coding genes (22,23). They are derived from longer precursor molecules through a combined action of the nuclear microprocessor complex and the cytoplasmic RNAase III enzyme Dicer (22,23). The mature product of approximately 20 nucleotides (nts) in length is loaded to the RNA-induced silencing complex (RISC) and targets areas predominantly in the 3' untranslated region (UTR), mediating translational repression or mRNA degradation, depending on the degree of complementarity (22,23). The emerging important role of miRNAs in various cellular processes and their implication in a plethora of human diseases (24) has made them a new promising field of molecular epigenomics (25). Furthermore, miRNAs have been proposed to account for part of the variability in gene expression in human cerebral cortex (26); to display remarkable resistance to the effects of temperature, pH, and prolonged storage (27); and to be stable and consistent biomarkers in postmortem studies (28). We have previously shown that the expression of BDNF in adult human PFC is inversely correlated to a subset of miRNAs predicted to target conserved regions within human BDNF 3' UTR, with two species in particular, miR-30a-5p and miR-195, having the most pronounced inhibitory effect on BDNF translation (29).

In this case-control study, we determined the expression and potential interactions of NPY, SST, and PV mRNAs with BDNF protein levels in 20 subjects with schizophrenia, including the putative influence of miR-30a-5p and miR-195 microRNAs. Our results show for the first time that alterations in NPY and SST, but not PV, mRNA in PFC of schizophrenia subjects are modulated by BDNF protein, which in turn is negatively regulated by miR-195. Furthermore, we show that the cerebral cortex of mice with a

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Received October 3, 2008; revised November 17, 2008; accepted November 18, 2008.

0006-3223/09/$36.00
doi:10.1016/j.biopsych.2008.11.019

central nervous system (CNS)-specific conditional ablation of BDNF exhibits deficits in NPY and SST mRNAs without concomitant changes in miR-195 levels. Therefore, a small noncoding RNA, miR-195, could be an important modifier of BDNF-related GABAergic deficits in schizophrenia.

Methods and Materials

Postmortem Brains

A total of 40 postmortem brain samples from 20 subjects diagnosed with schizophrenia and 20 control subjects were used in this study. See Supplement 1 and Table 1 for more details.

BDNF Immunoassay

Protein was extracted with the mirVANA PARIS Kit (Ambion, Austin, Texas) according to manufacturer's instructions, and after centrifugation, the supernatants were used for estimation of total protein with BCA Micro-kit (Pierce, Rockford, Illinois). Brain-derived neurotrophic factor levels were essayed with enzyme-linked immunosorbent assay (ELISA) and with the use of BDNF ELISA Kit (Chemicon, Temecula, California) according to manufacturer's instructions.

RNA Isolation

Total RNA was isolated by using RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, California) and then treated with DNase I (Ambion). Small RNAs (<200 nts) were isolated by using the mirVANA PARIS Kit (Ambion), according to the manufacturer's instructions and as described before (29). For isolation of <40 nts RNA, the flashPAGE Fractionator System (Ambion) was used according to manufacturer's instructions. Briefly, 5 μg of total RNA was run for 12 minutes at 75 mV and RNA from the lower running buffer was purified using flashPAGE Reaction Clean-Up Kit (Ambion).

RNA Quantification

The mirVana qRT-PCR miRNA Detection Kit (Ambion) was used for measuring human miR-195 in samples of <200 nts and <40 nts RNA. For each sample and amplicon, cycle thresholds were averaged from triplicate reactions and normalized to either 5S ribosomal RNA (rRNA) (<200 nts RNA) or miR-191 (<40 nts RNA). The miRCURY LNA microRNA PCR System (Exiqon, Vedbaek, Denmark) was used for quantification of miRNA expression in mouse RNA samples. In this case, duplicate reactions were used and data were normalized again to 5S rRNA. TaqMan One-Step RT-PCR (Applied Biosystems, Foster City, California) was used according to manufacturer's instructions for human and mouse NPY, SST, PV, beta 2 microglobulin (B2M), BDNF, and 18S rRNA with primers shown in Supplement 2. Custom primers (Applied Biosystems) were used for human β-glucuronidase (*GUSB)* quantitative reverse transcription polymerase chain reaction (qRT-PCR).

Genotyping

Neuropeptide Y single nucleotide polymorphism (SNP) genotyping was performed using direct sequencing and also matrix-assisted laser desorption/ionization mass spectrometry (Sequenom, San Diego, California), in conjunction with SpecroDesign software (Sequenom) for polymerase chain reaction (PCR) and MassEXTEND primers (Sequenom).

Chromatin Immunoprecipitation in Postmortem Tissue

Postmortem tissue (70 mg to 100 mg) was subjected to chromatin immunoprecipitation as described before (3) and histone methylation levels at specific promoter sequences measured by qRT-PCR, using the primers shown in Supplement 2.

Animal Studies

For antipsychotic drug studies, adult male C57BL/6 mice, 10 to 15 weeks of age, were treated for 21 days with once daily intraperitoneal injections of saline or haloperidol (.5 mg/kg) or clozapine (5 mg/kg) (Sigma, St. Louis, Missouri) and then killed 1 hour after the last treatment. The Nestin-Cre transgenic line was used for a CNS-wide conditional ablation of *Bdnf* before E14.5 (30). The mutant genotype was Nestin-Cre+, $BDNF^{2lox/2lox}$, and the control animals from the same outbred colony had the genotype Nestin-Cre+, $BDNF^{+/+}$. Brains were harvested at E19.5 and postnatal weeks 14 to 15.

Statistical Analyses

See Supplement 1 for information on statistical analyses.

Results

Altered Expression of GABAergic Transcripts in PFC of Subjects with Schizophrenia

To determine if the reported deficits in a subset of GABAergic transcripts in PFC of patients with schizophrenia (10,11) could be recapitulated in our cohort of 20 matched pairs (Table 1), we measured with qRT-PCR mRNA levels for NPY, SST, and PV. Our results revealed significant deficits (NPY, PV) or a trend for decrease (SST) in mRNA levels of the schizophrenia subjects (Figure 1A–C). To rule out that these alterations were due to differences in the level of the normalization gene, B2M, we reanalyzed NPY transcript changes for eight randomly selected matched pairs using two additional reference genes (18S rRNA and GUSB) (31). The within-pair differences in NPY levels based on each of these two additional reference genes were highly correlated with the B2M-based values (18S rRNA: $r = +.87$, $p = .005$; GUSB: $r = +.85$, $p = .008$, with r = Pearson correlation coefficient).

Disease-Specific Changes in Prefrontal NPY and SST mRNAs Are Related to Within-Pair Differences in BDNF Protein

Next, we wanted to explore the molecular mechanisms that could underlie the observed deficits in NPY, SST, and PV expression in the schizophrenia cohort of this study. Based on studies in genetically engineered mice, expression of NPY and SST in cerebral cortex is dependent on BDNF (21), but it is not known whether a similar mechanism plays a role in schizophrenia. To address this issue, we measured BDNF protein by ELISA in our cohort and determined BDNF protein and NPY, SST, and PV mRNA levels in schizophrenia subjects relative to their matched control subjects (S/C). Although no significant changes in BDNF protein in this cohort were observed (data not shown), there were significant positive correlations between within-pair differences in BDNF and NPY or SST mRNA levels (Figure 1D and 1E). However, there was no correlation between BDNF and PV S/C ratios (Figure 1F). Therefore, changes in BDNF protein levels in schizophrenia preferentially affect prefrontal NPY and SST but not PV gene expression.

MiR-195, a BDNF-Targeting microRNA, Is an Upstream Effector of BDNF and BDNF-Regulated GABAergic Gene Transcripts in Schizophrenia

From the above findings, one could draw two conclusions. First, prefrontal BDNF levels had a significant effect on a subset of GABAergic gene transcripts. Second, within-pair differences in