Alterations in Somatostatin mRNA Expression in the Dorsolateral Prefrontal Cortex of Subjects with Schizophrenia or Schizoaffective Disorder

Alterations in the inhibitory circuitry of the dorsolateral prefrontal cortex (DLPFC) in schizophrenia include reduced expression of the messenger RNA (mRNA) for somatostatin (SST), a neuropeptide present in a subpopulation of γ-aminobutyric acid (GABA) neurons. However, neither the cellular substrate nor the causal mechanisms for decreased SST mRNA levels in schizophrenia are known. We used in situ hybridization to quantify the compartmental, laminar, and cellular levels of SST mRNA expression in the DLPCF of 23 pairs of schizophrenia or schizoaffective disorder and control subjects. We also explored potential causal mechanisms by utilizing similar methods to analyze SST mRNA expression in 2 animal models. The expression of SST mRNA was significantly decreased in layers 2–superficial 6 of subjects with schizophrenia, but not in layer 1, deep 6 or the white matter. At the cellular level, both the density of cortical SST mRNA-positive neurons and the expression of SST mRNA per neuron were reduced in the subjects with schizophrenia. These alterations were not due to potential confounds and appeared to be a downstream consequence of impaired neurotrophin signaling through the TrkB receptor. These findings support the hypothesis that a marked reduction in SST mRNA expression in a subset of GABA neurons contributes to DLPCF dysfunction in schizophrenia.

Keywords: BDNF/TrkB, GABA, human, interneurons, NPY

Introduction

Alterations in the inhibitory circuitry of the dorsolateral prefrontal cortex (DLPFC) appear to be a common feature of schizophrenia (Torrey et al. 2005; Akbarian and Huang 2006). For example, reduced levels of the messenger RNA (mRNA) that encodes for the 67-kDa isoform of glutamic acid decarboxylase (GAD67), an enzyme for γ-aminobutyric acid (GABA) synthesis, have been consistently found in the DLPCF of individuals with schizophrenia (Akbarian et al. 1995; Guidotti et al. 2000; Mirnics et al. 2000; Volk et al. 2000; Hashimoto et al. 2005; Straub et al. 2007). Furthermore, this decrease is due to a marked reduction in GAD67 mRNA expression in a minority (~25–35%) of GABA neurons, with apparently normal levels of expression in the remaining neurons (Volk et al. 2000). The affected neurons include the ~25% of GABA neurons that express the calcium-binding protein parvalbumin (PV), whereas the ~50% of GABA neurons that express calretinin (CR) appear to be unaffected (Hashimoto et al. 2003). However, the deficits in PV expression are restricted to layers 3 and 4, whereas the alterations in GAD67 mRNA are found in layers 2–5; these laminar differences suggest that an additional subset of GABA neurons is affected in schizophrenia.

In a recent study utilizing a custom-designed microarray of GABA-related transcripts, the most robust expression difference in the DLPCF of subjects with schizophrenia was a reduction in the levels of somatostatin (SST) mRNA (Hashimoto et al. 2007), which is expressed in a subpopulation of GABA neurons that do not contain either PV or CR (Kubota et al. 1994; González-Albo et al. 2001; Gonchar and Burkhalter 2003; Sugino et al. 2006). The reduction in SST mRNA expression in schizophrenia was confirmed by both real-time qPCR and in situ hybridization in the same subjects (Hashimoto et al. 2007). However, neither the cellular substrate nor the causal mechanisms for decreased SST mRNA expression in schizophrenia have been explored.

SST neurons are present in all layers of the cortex as well as in the underlying white matter. SST neurons in layer 1, deep layer 6, and the white matter are generated early in development and represent residual neurons from the embryonic preplate, whereas those generated later during the development of the cortical plate reside in layers 2–superficial 6 (Kostovic and Rakic 1980; Chun and Shatz 1989b; Bayer and Altman 1990; for review see Allendoerfer and Shatz 1994). Within the gray matter of the adult monkey and human DLPCF, the greatest densities of SST neurons are found in layers 2–superficial 3 and layer 5 (Lewis et al. 1986; Hayes et al. 1991; Da Cunha et al. 1995). In addition, subsets of SST neurons with different membrane properties and morphological features tend to differ in their laminar locations (Kawaguchi and Kubota 1996; Ma et al. 2006). Thus, it is important to determine if the expression deficit in SST mRNA is 1) restricted to the gray matter compartment, 2) pronounced in certain cortical layers, and 3) confined to a subset of SST neurons.

Consequently, in this study we used in situ hybridization and autoradiographic analyses to quantify the compartmental, laminar, and cellular levels of SST mRNA expression in DLPCF area 9 of 23 matched pairs of schizophrenia and control subjects. In addition, we explored potential causal mechanisms of decreased SST mRNA expression in schizophrenia by utilizing similar methods to analyze SST mRNA expression in 2 animal models.

Materials and Methods

Human Subjects

With the consent of the surviving next-of-kin, brain tissue specimens were obtained from the Allegheny County Medical Examiner’s Office at the time of routine autopsy. Twenty-three subjects with schizophrenia (Table 1) were each matched with 1 control subject for sex, and as closely as possible for age, and postmortem interval (PMI). Subject groups did not differ in mean age, PMI, brain pH, RNA integrity number (RIN; as determined from the Agilent Bioanalyzer 2100) or tissue storage time at ~80 °C (for all $k_{22} < 1.84; P > 0.08$).
<table>
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**Table 1** Characteristics of subjects

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<tr>
<th>Case</th>
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<th>Sex/race</th>
<th>Age</th>
<th>PMI</th>
<th>RIN</th>
<th>Storage time</th>
<th>Cause of death</th>
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<td>7.4</td>
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<td>Heat Stroke</td>
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<td>M/W</td>
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<td>ASCVD</td>
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<td>M/W</td>
<td>48</td>
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<td>7.9</td>
<td>102</td>
<td>Accidental combined drug overdose</td>
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<tr>
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<td>8.3</td>
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<td>Myocarditis</td>
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PMI indicates postmortem interval in hours.
Storage time (months) at −80°C.
ASCVD indicates arteriosclerotic cardiovascular disease.
Indicates prescribed antipsychotic medications at time of death.
Alcohol abuse, in remission at time of death.
Alcohol dependence, current at time of death.
Other substance abuse, current at time of death.
Other substance abuse, in remission at time of death.
Alcohol dependence, in remission at time of death.
Other substance abuse, in remission at time of death.
History of posttraumatic stress disorder, in remission 39 years at time of death.
An independent committee of experienced research clinicians made consensus DSMIV (American Psychiatric Association, 1994) diagnoses based on medical records and structured interviews conducted with family members of the deceased. One control subject (987) had a history of posttraumatic stress disorder that had been in remission for 39 years at the time of death. For the subjects with schizophrenia, the mean (SD) age of illness onset was 25.2 (8.0) years and the mean duration of illness was 23.3 (13.3) years. We define the age of onset as the 1st episode of psychotic symptoms, as indicated by the available information from medical records and the subject’s family. Fifteen subjects with schizophrenia had a history of substance (including alcohol) abuse and/or dependence disorder, although only 8 met criteria for dependence at time of death. Four subjects with schizophrenia (537, 622, 621, and 829) were free of antipsychotic medications at time of death for 9.6 months, 1.2 months, 8.2 years, and unknown length of time, respectively. Toxicology of all subjects detected positive plasma alcohol levels (0.01% and 0.06%) in 2 control subjects (516 and 685) and 1 subject with schizophrenia (0.09%; 656).

All procedures were approved by the University of Pittsburgh’s Institutional Review Board for Biomedical Research and Committee for Oversight of Research Involving the Dead.

**Tissue Preparation**

The right frontal cortex from each brain was blocked coronally, immediately frozen, and stored at −80 °C. Serial sections with a thickness of 20 μm containing the superior frontal gyrus were cut, thaw-mounted onto the middle portion of glass slides, 1 section per slide, and stored at −80 °C until processed. The location of DLPCF area 9 was identified by cytoarchitectonic criteria in Nissl-stained sections as previously described (Glanz et al. 2000; Volk et al. 2000). Three sections per subject, at intervals of approximately 300 μm, were matched for anterior-posterior location within subject pairs, and used to assess SST mRNA expression.

**In Situ Hybridization**

Templates for the synthesis of the antisense and sense riboprobes for human and mouse SST mRNA were 1st generated by polymerase chain reaction (PCR). The specific primers amplified a 337 and 439 base pair (bp) fragment of human and mouse SST, respectively. The fragment was then subcloned into a plasmid (pBluescript II). The antisense and sense riboprobes were transcribed in the presence of 35S-CTP (Amersham Biosciences, Piscataway, NJ) using T7 and SP6 RNA polymerase, respectively. DNase I was used to digest the DNA template. The riboprobes were purified using RNaseasy mini spin columns (Qiagen, Valencia, CA). One section from each pair was processed during a single run with the sections from each pair processed side by side, and with the location of the slides in the hybridization container counterbalanced between diagnostic groups during each run.

Prior to the hybridization reaction, tissue sections were fixed with 4% paraformaldehyde in phosphate-buffered saline solution, acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine/0.9% NaCl for 10 min, dehydrated with a graded ethanol series, and air dried. The sections, as well as carbon-14 radioactive standards, were exposed on the same BioMax MR film (Kodak, Rochester, NY) for 3 days. Afterward, sections were coated with NTB2 emulsion (Kodak) diluted 2:1 with water. The consistency of the thickness of the emulsion was maintained with use of a mechanical dipper (Auto-dip Emulsion Coater, Ted Pella, Redding, CA) at a constant withdrawal speed (64 mm/min) and temperature (43 °C). Utilizing DLPCF sections from control subjects, different emulsion exposure times were systematically evaluated in order to achieve an optimal signal to noise ratio. The emulsion was exposed for 18 days at a constant temperature of 4 °C. The slides were developed with D-19 (Kodak) and counterstained with Cresyl violet.

**Quantification of mRNA Expression Levels**

Each section was randomly coded, so that subject number and diagnosis were unknown to the single rater (H.M.M.). Autoradiographic films were trans-illuminated and captured on video camera under controlled conditions, digitized, and analyzed with a Microcomputer Imaging Device (MCID; Imaging Research, Inc., London, Ontario, Canada). Digitized images of adjacent sections stained with cresyl violet were superimposed onto autoradiographic images to draw contours of the full cortical thickness of the zones of area 9 that were cut perpendicularly to the pial surface. Optical density (OD) measures within each sampled area were calibrated to radioactive carbon-14 standards (ARC, Inc., St Louis, MO), exposed on the same autoradiographic film, and expressed as nanocuries per gram (nCi/g) of tissue. The mean (SD) total area of gray matter sampled in each subject was 381 (111) mm² for control subjects and 353 (101) mm² for subjects with schizophrenia. OD measures in the superficial white matter were determined in a zone 800 μm below, and with a contour that followed, the layer 6/white matter border of the previously sampled gray matter zones. The mean total areas of sampled superficial white matter per subject were 32 (14) mm² for control subjects and 30 (10) mm² for subjects with schizophrenia. Total white matter was determined by outlining the gray matter/white matter border and including all white matter on the section. The mean total areas of the sampled total white matter per subject were 280 (117) mm² for control subjects and 277 (105) mm² for subjects with schizophrenia.

SST mRNA expression as a function of cortical layer was determined in a series of cortical traverses (1-2 mm in width) extending from the pial surface to the white matter. Three cortical traverses were sampled for each section (9 traverses per subject) (Fig. 1A). Each traverse was divided into 50 equal bins parallel to the pial surface and the OD was determined for each bin. These bins were then combined into zones that approximated laminar boundaries based on previous studies (Akbarian et al. 1995; Pierri et al. 1999). These zones (i.e., bins 1-5, 6-10, 11-15, 16-20, 21-25, 26-30, 31-35, 36-40, and 41-45) corresponded to layers 1, 2, 3a/3b, superficial 3 (2/3s), deep 3 (3d), 4, 5, and 6, respectively (Fig. 1B). The mean OD was calculated for each zone. Background measures were sampled from deep white matter where no specific expression of SST mRNA was observed. All sampled areas were corrected by subtracting the corresponding background measure from the same slide.

Evaluation of mRNA expression at the cellular level was performed by determining silver grain accumulation on emulsion-dipped, Nissl counterstained sections. Using the MCID system coupled to a microscope equipped with a motor-driven stage, 2 regions of interest (ROIs) (Fig. 1B) were defined in 1-mm-wide cortical traverses (3/section; 9/subject). The superficial ROI extended from 10% to 30% of the distance from the pial surface to the white matter (corresponding to layer 2-superficial 3), and the deep ROI extended from 60% to 80% (corresponding to layer 5). Four sampling frames (120 × 170 μm) were placed in each ROI such that the edges of the frames were equidistant from the border of the ROI and the edge of the next sampling frame. Within each frame, a circle with a fixed diameter of 22 μm (380 μm²) was placed over each Nissl-stained nucleus under brightfield illumination using an unbiased inclusion and exclusion rule (Fig. 1C), and then the number of grains within the circle were counted in the corresponding darkfield image (Fig. 1C). Because RNase A treatment during the in situ hybridization procedure degrades Nissl-stainable substances within the cytoplasm, it was not possible to draw contours around the soma of neurons. In a previous study, we determined that the largest cross-sectional area of human DLPCF GABA neurons is −100 μm² (Volk et al. 2000). Thus, in order to include the maximal number of...
grains/neuron, we utilized a fixed 22 μm diameter (380 μm²) circle, which would include most grains from the largest interneurons as previously described (Hashimoto et al. 2003). The use of fixed diameter sampling does not account for potential differences in somal size across subject groups. However, this confound is unlikely because the somal size of GAD 67 mRNA-positive neurons has been reported to be unchanged in subjects with schizophrenia (Akbarian et al. 1995; Volk et al. 2000). Background grain density was measured in each sampling frame by using the same sampling circle to count grains over 4 glial nuclei in each sampling frame. The smaller size and intense cresyl violet staining of glial nuclei distinguished them from the larger, more faintly stained neuronal nuclei (Fig. 1C). Total neuron numbers sampled in the superficial ROI were 13,791 and 14,593 for control and subjects with schizophrenia, respectively. Deep ROI total neuron numbers sampled were 13,981 and 14,356 for control and subjects with schizophrenia, respectively.

Grain density per neuron (i.e., number of grains within the 22 μm diameter circle) was calculated for all neurons. Specifically labeled neurons were determined by creating a threshold of grains per neuron. For both subject groups, histograms (natural log transformed) of the grain density of all sampled neurons had a bimodal distribution representing nonspecifically and specifically labeled neuron populations (Gerfen et al. 1991). A point of rarity between these populations was used to calculate the threshold above background needed to distinguish the labeled neuron population. Using this threshold, which was equal to 7× background grain density, histograms (natural log transformed) of the grain density of all sampled neurons appeared unimodal and normal in both subject groups. Therefore, grain densities of ≥ 7× background were considered to be specifically labeled and are referred to as SST mRNA+ cells.

Haloperidol-Exposed Monkeys
To mimic the treatment of schizophrenia with high doses of haloperidol, 4 pairs of young adult, male macaque monkeys (Macaca fascicularis), matched for age and weight, were chronically exposed to haloperidol decanoate (mean [SD] trough plasma level, 4.3 [1.1] ng/mL) and benztropine mesylate (1 mg b.i.d.) to treat extrapyramidal symptoms for 9–12 months, as previously described (Pierri et al. 1999). Processing of monkey brain tissue was conducted as previously described (Hashimoto et al. 2003). Briefly, coronal sections with a thickness of 16 μm were cut from fresh-frozen tissue blocks containing the middle one-third of the principal sulcus. Two serial sections from each animal were processed for SST mRNA expression utilizing the35S-labeled riboprobe as described above. The OD of the cortex was determined for the full cortical thickness of areas 9 and 46 cut perpendicular to the pial surface similar to the methods in the human study. Background measures were sampled from deep white matter where no specific expression of SST mRNA was observed. All sampled areas were corrected by subtracting corresponding background measures from the same section.

Genetically Engineered Mice
To test the effect of decreased expression of the neurotrophin receptor tyrosine kinase B (trkB) on the expression of SST mRNA, we used trkB hypomorphic mice (Xu et al. 2000) in which the 1st coding exon of the trkB gene is replaced with a trkB cDNA unit flanked by 2 loxP sites (fbZ locus). These mice were generated with 129 strain mice-derived embryonic stem cells and C57BL/6 mice-derived blastocysts (Xu et al. 2000) and back-crossed into C57BL/6 mice for at least 5 generations. Wild-type C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were
used as control. Homozygous (fBZ/fBZ) and heterozygous (fBZ/+)
animals were reported to express ~25% and ~62%, respectively, of the
trbk protein levels present in the wild-type animals (Xu et al. 2000;Rohrer 2001; Rico et al. 2002). Animals with the fBZ/fBZ genotype do
not express the truncated isoforms of trkB (Xu et al. 2000).
Heterozygous and homozygous mice for the fBZ locus and wild-type
controls (n = 3 for each group) were euthanized at 8 weeks of age.
The OD from the autoradiographic film was measured for SST mRNA
expression of the cortex in the PFC, including the cingulate and
prelimbic cortices, as described for the human study. All density
measures were corrected by subtracting background measures in the
corpus callosum.

Statistical Analyses
Analyses were performed on SPSS (SPSS, Inc., Chicago, IL). Analysis of
covariance (ANCOVA) models were used to test differences in SST
mRNA expression between control subjects and subjects with
schizophrenia. The data were averaged across the 3 sections for each
subject before statistical analysis. The 1st ANCOVA model used
diagnostic group as the main effect, pair as a blocking effect, and
storage time and RIN as covariates. The pair effect reflects the matching
of individual subject pairs for sex, age, and PMI. RIN was included as a
covariate because it reflects mRNA integrity (Stan et al. 2006). Subject
pairing may be considered an attempt to balance the 2 diagnostic
groups with regard to the experimental factors instead of a true
statistical paired design. Thus, to validate the 1st model, a 2nd ANCOVA
model was performed with a main effect of diagnostic group and
covariates of sex, age, PMI, RIN, and storage time. Storage time as
a covariate was not significant in either model and thus was excluded
in the reported analyses. Both models produced comparable results
for diagnostic group effect; however, because the effect of age on
SST mRNA expression was significant, the results of the 2nd model are
reported.

In order to correct for multiple comparisons in the laminar analyses,
significance of the diagnostic group effect was determined for
individual layers using the Holm simultaneous inference procedure
(Holm 1979) as previously described (Volk et al. 2002). To maintain
consistency, the reported P values for each laminar comparison have
been adjusted to correspond to the family-wise error rate of 0.05.

The potential influence of sex, diagnosis of schizoaffective disorder,
lifetime history of any substance abuse/dependency, diagnosis of
alcohol abuse/dependency at time of death, use of antidepressant
medication at time of death, use of benzodiazepines/valproate at time
of death, or suicide on the within-pair percentage of differences in
mRNA expression was assessed by 2-sample t-test analyses. Correlations
between age and mRNA expression were assessed by Pearson’s
correlation analyses.

For the haloperidol-treated monkeys, paired t-test analyses were used
to assess the effects of treatment group on SST mRNA expression.
For the trkB hypomorphic mice, the effects of genotype on SST
mRNA expression were determined by a single-factor ANOVA. Tukey’s
multiple comparison test was used in post hoc comparisons across
genotypes.

Results

Specificity of SST Riboprobe
Several lines of evidence confirm the specificity of the
riboprobe for SST mRNA used in this study. First, the distinctive
laminar distribution of SST mRNA expression is very similar to
that previously reported for both SST-immunoreactive cell
bodies in monkey and human prefrontal cortex (Lewis et al.
1986; Hayes et al. 1991) and SST mRNA expression in monkey
prefrontal cortex (Da Cunha et al. 1995). Specifically, the
density of SST mRNA+ neurons was lowest in layer 1, highest in
layers 2 and superficial 3, moderate in deep layer 3 and layer 4,
high in layer 5, and moderate in layer 6 (Figs 1 B and 2A).
Second, the presence of intensely SST mRNA+ neurons in the
superficial white matter (Figs 1 B and 2A) is consistent with
previous studies of SST-immunoreactive cell bodies in human
and monkey prefrontal cortex (Lewis et al. 1986; Hayes et al.
1991) and SST mRNA expression in the monkey prefrontal
cortex (Da Cunha et al. 1995). Third, sense riboprobes for SST
mRNA showed an absence of signal above background (data
not shown).

Compartmental Expression of SST in DLPFC Area 9 of
Control Subjects and Individuals with Schizophrenia
We previously reported reduced SST mRNA expression by
microarray, quantitative PCR (qPCR), and in situ hybridization in
the DLPFC of subjects with schizophrenia (Hashimoto et al.
2007). However, whether this expression deficit was specific
to the gray matter or also present in the white matter was not
examined. Consistent with qualitative impressions (Fig. 2),
quantitative measures of SST mRNA expression in the gray
matter of DLPFC area 9 confirmed that the mean (±SD) OD of
gray matter was significantly (F(1,40) = 18.07; P < 0.001)
decreased by 36% in subjects with schizophrenia (55.8 ± 31.9

Figure 2. Representative film autoradiograms from 1 pair of subjects illustrating SST mRNA expression in human DLPFC. The densities of hybridization signals are represented in a pseudocolor manner according to the calibration scale (center). The solid and dashed lines indicate the pial surface and the gray/white matter border, respectively. Expression of SST mRNA in the cortical gray matter is reduced in the subject with schizophrenia (B) relative to the matched control subject (A).
nCi/g) compared with control subjects (87.5 ± 22.9 nCi/g). OD measurements in the gray matter were lower in the subjects with schizophrenia for 20 of the 23 subject pairs (Fig. 3A). In contrast, mean film OD from the total white matter did not significantly differ (F(1,40) = 0.72; P = 0.40) between control subjects (18.8 ± 5.6 nCi/g) and subjects with schizophrenia (17.0 ± 5.3 nCi/g) (Fig. 3B). Because SST mRNA expression was most dense in the superficial white matter (Fig. 2), the OD was also determined from contours in the 800 μm of white matter immediately below the layer 6-white matter border. These measures also did not differ (F(1,40) = 1.74; P = 0.20) between control subjects (33.8 ± 10.9 nCi/g) and subjects with schizophrenia (28.3 ± 10.1 nCi/g) (Fig. 3C).

**Examination of Factors that May Affect Cortical Expression of SST mRNA**

In the 2nd ANCOVA model, age was a significant (F(1,40) = 19.24; P < 0.0001) determinant of SST mRNA levels in the gray matter. Indeed, OD measures in the gray matter contours were negatively correlated with age in both control subjects (r = −0.74; P < 0.0001) and subjects with schizophrenia (r = −0.51; P = 0.01) (Fig. 4). However, the within-subject pair percent differences in SST mRNA expression in the gray matter did not differ as a function of sex, diagnosis of schizoaffective disorder, lifetime history of any substance abuse or dependency, diagnosis of alcohol abuse or dependency at time of death, use of benzodiazepines/valproate at time of death, or suicide (all t(21) < 1.52, all P > 0.14) (Fig. 5).

In a previous study, the expression of SST mRNA in DLPFC areas 9 and 46 of monkeys did not differ among monkeys chronically exposed to haloperidol, olanzapine, or sham (Hashimoto et al. 2007). However, the steady-state trough plasma levels (~1.5 ng/mL) in the haloperidol-treated monkey group might be considered relatively low compared with levels likely achieved in at least some of the subjects with schizophrenia included in this study. Thus, in order to determine if chronic exposure to higher levels of haloperidol could affect SST mRNA expression, we examined the OD measures by layer (Fig. 7). Because of the high density of SST mRNA+ neurons in layers 2–superficial 3 (2/3s), these layers were combined and distinguished from deep layer 3 (3d). SST mRNA expression was significantly decreased in all layers (F(1,40) > 4.34; P < 0.04, for all layers), with the exception of layer 1 (F(1,40) = 0.64; P = 0.23). Of the layers with a significant diagnostic effect, the largest percentage difference was in layer 2/3s (36.6%) and the smallest difference in layer 3d (29.6%).

**Cellular Levels of SST mRNA Expression**

In order to determine if all or a subset of SST cortical neurons were affected, we determined the expression level per neuron by counting the silver grains deposited over Nissl-stained neurons in layers 2/3s and 5. The mean (±SD) number of grains per positive neuron in layers 2/3s was significantly (F(1,40) = 9.72; P = 0.003) 31% lower in the subjects with schizophrenia (104.4 ± 49.1) than in the control subjects (150.4 ± 53.4). Similarly, the mean number of grains per positive neuron in layer 5 was significantly (F(1,40) = 6.96; P = 0.012) decreased by 25% in subjects with schizophrenia (121.5 ± 67.3) compared with controls (162.7 ± 45.7) (Fig. 8A,B). Furthermore, the mean density of SST mRNA+ neurons in layers 2/3s was significantly (F(1,40) = 10.79; P = 0.002) decreased by 26% in subjects with schizophrenia (63.3 ± 25.7 neurons/mm²) relative to control subjects (85.3 ± 16.2 neurons/mm²), and in layer 5 the 23% decrease in mean density of SST mRNA+ neurons in the subjects with schizophrenia (52.5 ± 27.2 neurons/mm²) compared with control subjects (67.7 ± 15.4 neurons/mm²) showed a trend (F(1,40) = 3.93, P = 0.05) toward statistical significance (Fig. 8C,D).

**Figure 3.** SST mRNA expression levels assessed by autoradiographic film OD measures in DLPFC area 9 of subjects with schizophrenia (gray circles) and control subjects (black circles). Subjects in each pair are connected by black lines and the mean expression levels for each subject group are represented by a horizontal line. The expression of SST mRNA is significantly reduced in the gray matter (A), but not in the total white matter (B) or the superficial white matter (C) of the schizophrenia subjects.
Correlation of Altered Expression of SST mRNA with Changes in Other Transcripts

Within-pair percentage differences in SST mRNA expression were significantly correlated ($r = 0.72, P < 0.001$; Fig. 9A) with those found for GAD67 mRNA expression in a previous study of the same subjects (Hashimoto et al. 2005). These reductions in GAD67 mRNA expression in schizophrenia were also strongly correlated with changes in the expression of receptor trkB mRNA, the principal receptor for brain-derived neurotrophic factor (BDNF), and to a lesser extent with those in BDNF mRNA expression (Hashimoto et al. 2005). Thus, because trkB is expressed in ~50% of SST neurons (Gorba and Wahle 1999), we examined whether reduced BDNF–trkB signaling might be associated with the reduced SST mRNA expression in schizophrenia. The within-pair percentage differences in SST mRNA were significantly correlated with within-pair percentage differences in both BDNF mRNA expression ($r = 0.77, P < 0.001$; Fig. 9B), and trkB mRNA expression ($r = 0.74, P < 0.001$; Fig. 9C).

SST mRNA Expression in Genetically Engineered Mice

We have previously shown that mice with reduced expression of BDNF mRNA exhibit decreased SST mRNA expression (Glorioso et al. 2006), suggesting that the correlations observed in the human subjects might represent cause and effect. In order to determine if the level of trkB mRNA expression also regulates SST mRNA expression, we examined SST mRNA expression in the PFC of trkB hypomorphic mice (Xu et al. 2006).

Figures 4 and 5. OD measures of SST mRNA expression in human area 9 as a function of age. SST mRNA expression levels are significantly negatively correlated with age in both subject groups. The regression line for subjects with schizophrenia (gray line) is parallel to and shifted downward from that for control subjects (black line), suggesting that the decreased expression of SST mRNA is similar in magnitude across adult life.
Levels of trkB mRNA in the frontal cortex of fBZ/+ and fBZ/fBZ genotype mice were decreased by 42% and 75%, respectively, as compared with wild-type animals (Hashimoto et al. 2005). TrkB genotype was significantly ($F_{(2,6)} = 6.03, P = 0.037$) related to the expression level of SST mRNA (Fig. 10). SST mRNA levels in wild-type mice ($596.9 \pm 31.4$ nCi/g) were significantly greater ($P = 0.045$) than in the fBZ/fBZ genotype ($406.3 \pm 55.6$ nCi/g), and SST mRNA expression in the fBZ/+ genotype ($426.6 \pm 110.5$ nCi/g) was intermediate, showing a trend to lower values as compared with wild-type mice ($P = 0.068$).

**Discussion**

The expression levels of SST mRNA were significantly decreased in layers 2–6 of DLPFC area 9 in subjects with schizophrenia compared with matched controls. This decrease was evident in 20 of the 23 subject pairs studied; determining whether this heterogeneity reflects interindividual variability in SST mRNA expression or diversity in the underlying disease process (i.e., reduced SST mRNA expression identifies a subtype of schizophrenia) will require studies in larger cohorts of subjects. In contrast, SST mRNA expression in layer 1 and the white matter did not differ between subject groups. At the cellular level, both the density of cortical SST mRNA+ neurons and the expression of SST mRNA per neuron were reduced in the subjects with schizophrenia. These alterations appear to reflect the disease process of schizophrenia and not to be the consequence of potential confounds. Observations in both humans and genetically engineered mice suggest that these changes are a downstream consequence of impaired neurotrophin signaling in schizophrenia. Together, these findings indicate that reduced SST mRNA expression in the DLPFC of...
subjects with schizophrenia is restricted to the gray matter, confined to a subset of SST neurons, and the consequence of a plausible pathogenetic mechanism.

Altered SST mRNA Expression in Schizophrenia is not due to Confounds
Several lines of evidence indicate that the reduction in SST mRNA expression is due to the disease process of schizophrenia, and is not the consequence of the methods employed or the result of other factors commonly associated with the illness. First, in a subset of the subject pairs included in this study, SST mRNA was found to be significantly decreased in the subjects with schizophrenia using DNA microarray and real-time quantitative PCR with probes that recognize different sequences of SST mRNA than the probe used in the present study (Hashimoto et al. 2007). In addition, the within-subject pair differences in SST mRNA expression as determined by the in situ hybridization probe used in the present study were significantly correlated with those determined by microarray ($r = 0.78; P < 0.002$) and real-time qPCR ($r = 0.91; P < 0.001$). Second, all 4 subjects with schizophrenia off antipsychotic medications at the time of death demonstrated a decrease in SST mRNA expression relative to their matched controls. Third, SST mRNA expression was unaltered in the DLPFC of monkeys chronically exposed to either haloperidol or olanzapine with mean trough plasma levels in the therapeutic range (~1.5 and ~15 ng/mL, respectively) for humans (Hashimoto et al. 2007). Fourth, in the present study the gray matter expression of SST mRNA was not altered in monkeys chronically exposed to higher mean trough plasma levels of haloperidol (~4.3 ng/mL) that produced marked extrapyramidal symptoms requiring treatment with benzotropine mesylate, a manner of treatment likely achieved in at least some of our human subjects. Fifth, neither SST mRNA (Marcus et al. 1997) nor protein (Sakai et al. 1995) levels were decreased in the frontal cortex of rats exposed to haloperidol. Sixth, neither sex, lifetime history of substance abuse or dependency, diagnosis of alcohol abuse or dependency at time of death, treatment with antidepressant medications or benzodiazepines/valproate, nor suicide accounted for the decreased expression of SST mRNA in the subjects with schizophrenia (see Fig. 5). However, it should be noted that the within-subject pair differences in SST mRNA expression did not differ between subjects with schizophrenia or schizoaffective disorder, leaving open the possibility that this reduction is common to psychotic illnesses.

A significant negative correlation between age and SST mRNA gray matter expression was seen in both controls and individuals with schizophrenia, consistent with previous findings of an inverse relationship between age and SST mRNA or protein levels in rat hippocampus and monkey frontal cortex (Hayashi et al. 1997; Vela et al. 2003). The regression line for the subjects with schizophrenia was parallel to and shifted downward from that of the control subjects (Fig. 4), indicating that the decreased expression of SST mRNA in the subjects with schizophrenia was present across adult life and is thus unlikely to be a consequence of illness chronicity. Furthermore, this observation suggests that the SST mRNA expression deficit is present early in the course of the illness and thus could contribute to the pathophysiology underlying the clinical features of the illness.

A Subset of SST Neurons is Affected in Schizophrenia
Our results demonstrate that SST mRNA expression in layers 2–6, but not in layer 1 or the white matter, was reduced in subjects with schizophrenia. During development of the cerebral cortex, early germinal zones proliferate over successive rounds of cell division and give rise to postmitotic migratory neurons. $^3$H-thymidine birth-dating and autoradiographic analyses have demonstrated that the earliest generated cells comprise the preplate which, later in development, is split into the marginal zone (adult layer 1) and the subplate (adult deep layer 6 and superficial white matter) by the later born neurons of the cortical plate (adult layers 2–superficial 6)

![Correlations of within-subject pair percent differences in SST mRNA expression with GAD67 (A), BDNF (B), and TrkB (C) mRNA expression.](image-url)
The combination of birth-dating techniques and immunohistochemistry revealed that a subpopulation of the early generated preplate neurons expresses SST (Chun and Shatz 1989a). Our data suggest that the early generated SST mRNA+ neurons which reside in layer I and the superficial white matter are not affected in schizophrenia, whereas the later developing SST mRNA+ neurons which migrate to the cortical plate are affected. Additional analyses support this interpretation. For example, the rat cortex sublayer 6b also contains residual neurons of the embryonic preplate (Valverde et al. 1989), suggesting that SST+ neurons in layer 6b might be less affected in schizophrenia than those present in the more superficial layer 6a. Consistent with this prediction, the mean decrease in OD measures of SST mRNA in subjects with schizophrenia was significantly greater ($t_{22} = -5.08; P < 0.0001$) in the superficial (~37.4 nCi/g) than in the deep (~14.0 nCi/g) half of layer 6. Given that the cortical plate forms during the 2nd trimester of gestation, these findings raise the possibility that the alterations in SST neurons reflect the effect of adverse environmental events during that time frame (e.g., maternal influenza; Brown 2006) that have been associated with an increased risk for schizophrenia. However, given how common the alterations in SST neurons appear to be from the present study (i.e., 20/23 pairs), other causal factors must also be contributory. For example, both SST and PV-containing cortical GABA neurons are affected in schizophrenia, whereas those that contain CR appear to be unaffected. Thus, factors shared by SST and PV neurons that differ from CR neurons (e.g., place and timing of neuron birth, transcription factors regulating cell fate, etc.; see Wonders and Anderson 2006, for review) might also contribute to cell type-specific vulnerability. Of course, other features intrinsic to, or associated with the connectivity of, adult SST neurons in layers 2–superficial 6 might contribute to their greater vulnerability relative to other SST neurons.

In layer 2–superficial 3, we observed a significant reduction in the mean density of SST mRNA+ neurons, but only a trend toward a decrease in layer 5, raising the possibility that the severe reduction in SST mRNA expression within individual neurons is restricted to layer 2/3s. The mean expression level of SST mRNA per neuron was significantly reduced. In subjects with schizophrenia, total neuron number in the frontal lobe is unchanged (Thune et al. 2001) and the density of nonpyramidal neurons in the DLPFC is slightly increased (Selemon et al. 1995) or unchanged (Akbarian et al. 1995). Thus, it appears that SST neurons are still present in the DLPFC of subjects with schizophrenia, but that the expression of SST mRNA per neuron is reduced, with the reduction so great in some neurons that SST mRNA levels fall below the threshold of detection. This pattern of change in SST mRNA expression contrasts with the alterations in GAD$_{67}$ and PV mRNA expression in the DLPFC of subjects with schizophrenia. The expression levels of GAD$_{67}$ mRNA were reduced below detectable levels in ~25–35% of GABA neurons, whereas the remaining GABA neurons expressed normal levels of GAD$_{67}$ mRNA, suggesting that a subpopulation of GABA neurons is affected in the illness (Volk et al. 2000). The reduced expression of PV mRNA was found to be due to a decrease in expression level per neuron rather than to a decrease in the density of PV mRNA+ neurons suggesting that most PV mRNA+ neurons are affected (Hashimoto et al. 2003). Because the

![Figure 10](image_url)
reduction in SST mRNA expression was due to both a decrease in positive neuron density and in expression per neuron, these findings suggest that a majority of SST neurons express reduced levels of SST mRNA and that a subset of severely affected SST neurons express undetectable levels of SST mRNA. The severely affected neurons might include the approximately 50% of SST+ neurons that express trkB (Gorba and Wätle 1999), the principal receptor of BDNF. Consistent with this interpretation, mice with genetically engineered reductions in the expression of BDNF mRNA have significantly lower levels of cortical SST mRNA and protein (Grosse et al. 2005; Glorioso et al. 2006). In addition, the significant reduction in the expression of SST mRNA in the PFC of homozygote trkB hypomorphic mice indicates that signaling via trkB is also involved in regulating the expression of SST mRNA, even in the face of conserved levels of BDNF. Given that the expression levels of BDNF (Weickert et al. 2003; Hashimoto et al. 2005) and trkB mRNAs (Hashimoto et al. 2005; Weickert et al. 2005) are reduced in the DLPFC of subjects with schizophrenia, our findings imply that reduced BDNF-trkB signaling in schizophrenia is an “upstream” event that contributes to reduced SST mRNA expression. This interpretation is consistent with studies demonstrating that the addition of BDNF to cultured cortical cells or the intraventricular administration of BDNF in rats increased SST mRNA and protein levels (Nawa et al. 1994; Villuendas et al. 2001). Also, previous studies have reported that BDNF can influence the expression of GAD67 (Yamada et al. 2000; Cotrufo et al. 2003; Wirth et al. 2003; Patz et al. 2004; Palizvan et al. 2004). Therefore, altered BDNF-trkB signaling in schizophrenia may be a conserved mechanism driving the highly correlated reductions in SST and GAD67 mRNA expression in schizophrenia. However, the present study cannot exclude the possibilities that reduced SST mRNA expression is due to other unknown upstream mechanisms or is a secondary consequence of cortical dysfunction.

Functional Implications of Alterations in SST Neurons in Schizophrenia

In rat and monkey frontal cortex, ~40% of SST neurons also express neuropeptide Y (NPY) (Hendry et al. 1984; Kubota et al. 1994). Interestingly, expression deficits in SST and NPY mRNAs are strongly correlated in schizophrenia (r = 0.81; P < 0.001) (Hashimoto et al. 2007) and both are similarly reduced in BDNF knockout mice (Glorioso et al. 2006), suggesting that the NPY-containing subclass of SST neurons are affected in schizophrenia. Interneurons that contain both SST and NPY include the Martinotti cells (Kawaguchi and Kubota 1997; Reyes et al. 1998; Gibson et al. 1999; Ma et al. 2006). The axons of Martinotti cells project to layer 1 where they synapse on the apical dendrites of pyramidal neurons, and in rodent and monkey neocortex, SST interneurons predominately innervate the dendrites of pyramidal neurons (Hendry et al. 1984; DeLima and Morrison 1989; Kawaguchi and Kubota 1996; Melchitzky and Lewis 2005). Thus, disturbances in the SST/NPY-containing Martinotti class of GABA neurons could contribute to the dysfunction of DLPFC circuitry associated with working memory impairments in schizophrenia (Weinberger et al. 1986; Goldman-Rakic 1994; Cannon et al. 2005; Tan et al. 2005). How the observed alterations in SST-containing GABA neurons could contribute to cognitive dysfunction in schizophrenia requires further study, but the existing literature suggests the following mechanistic hypotheses.

First, in individuals with schizophrenia, disturbances in sensory-gating have been correlated with reductions in working memory performance (Silver and Feldman 2005), suggesting that the inability to filter distracting stimuli disrupts working memory. Interestingly, in computational modeling of cortical microcircuits and working memory, inhibitory interneurons that target the dendritic domain of pyramidal neurons provide resistance against distracting stimuli by sending enhanced inhibition to dendrites of nearby pyramidal neurons that are selective for other stimuli (Wang et al. 2004). Furthermore, in rats, high frequency trains from pyramidal neurons produce facilitating excitatory inputs to Martinotti cells that, via synapses onto the dendrites of neighboring pyramidal neurons, cause disynaptic inhibition (Silberberg and Markram 2007). Therefore, Martinotti interneurons, by mediating the disynaptic inhibition of neighboring pyramidal neurons selective for other stimuli, may filter distracting stimuli during working memory tasks. Thus, alterations in SST/NPY-containing Martinotti cells in schizophrenia may contribute to altered working memory performance.

Second, Martinotti interneurons also exhibit low threshold-spiking membrane properties (Kawaguchi and Kubota 1997; Reyes et al. 1998; Gibson et al. 1999; Ma et al. 2006). These low threshold-spiking, SST-containing interneurons are extensively electrically coupled into networks that robustly synchronize their spiking activity (Gibson et al. 1999, 2005) in the theta range (4–7 Hz) producing synchronized inhibitory postsynaptic potentials in neighboring pyramidal neurons (Beierlein et al. 2000). Although a direct connection between the synchronized theta spiking frequency in these neuronal networks and theta band electroencephalography (EEG) oscillations has not been demonstrated, EEG studies in humans and monkeys have demonstrated that theta band EEG oscillations increase in power during working memory tasks (Krause et al. 2000; Raghavachari et al. 2001; Lee et al. 2005). Furthermore, subjects with schizophrenia demonstrate altered frontal theta oscillations during working memory tasks (Schmidt et al. 2005). Thus, disturbances in SST/NPY-containing, low threshold-spiking Martinotti interneurons might contribute to alterations in theta oscillations and ultimately to impaired working memory performance in subjects with schizophrenia.

Funding

National Institute of Health grants (MH043784 and MH1045156).

Notes

Conflict of Interest D.A.L. currently receives research support from the BMS Foundation, Merck and Pfizer and in 2007–2007 served as a consultant to Bristol-Meyer Squibb, Pfizer, Roche, Sepracor and Wyeth.

Address correspondence to David A. Lewis, MD, W1650 BST, Department of Psychiatry, 3811 O’Hara St, University of Pittsburgh, Pittsburgh, PA 15213, USA. Email: lewisda@upmc.edu.

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