Lamina-Specific Alterations in Cortical GABA_A Receptor Subunit Expression in Schizophrenia

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Dysfunction of the dorsolateral prefrontal cortex (DLPFC) in schizophrenia is associated with lamina-specific alterations in particular subpopulations of interneurons. In pyramidal cells, postsynaptic γ-aminobutyric acid (GABA_A) receptors containing different α subunits are inserted preferentially in distinct subcellular locations targeted by inputs from specific interneuron subpopulations. We used in situ hybridization to quantify the laminar expression of α1, α2, α3, and α5 subunit, and of β1-3 subunit, mRNAs in the DLFPC of schizophrenia, and matched normal comparison subjects. In subjects with schizophrenia, mean GABA_A α1 mRNA expression was 17% lower in layers 3 and 4, α2 expression was 14% higher in layer 2, α5 expression was 15% lower in layer 4, and α3 expression did not differ relative to comparison subjects. The mRNA expression of β2, which preferentially assembles with α1 subunits, was also 20% lower in layers 3 and 4, whereas β1 and β3 mRNA levels were not altered in schizophrenia. These expression differences were not attributable to medication effects or other potential confounds. These findings suggest that GABA neurotransmission in the DLPFC is altered at the postsynaptic level in a receptor subunit- and layer-specific manner in subjects with schizophrenia and support the hypothesis that GABA neurotransmission in this illness is predominantly impaired in certain cortical microcircuits.

Keywords: inhibition, in situ hybridization, interneurons, postmortem, prefrontal cortex

Introduction

Fast synaptic inhibition in the cerebral cortex is mediated by the interaction of the neurotransmitter γ-aminobutyric acid (GABA) with heteropentameric postsynaptic GABA_A receptors that form ligand-gated chloride channels (Semyanov et al. 2004; Kullmann et al. 2005). The majority of GABA_A receptors result from the assembly of a pair of α subunits and a pair of β subunits, in combination with a fifth, either γ or δ, subunit (Mohler 2006). Native GABA_A receptors exhibit a preferential assembly for specific αβ pairings. The major combinations that have been identified in the central nervous system are α1β2γ2, α2β3γ2, and α3β3γ2, with α5β3γ2 considered to be highly probable (Mohler 2006; Olsen and Sieghart 2009). Specific α subunits confer distinct kinetic and pharmacological properties to the receptor and are associated with particular sites of subcellular localization. For example, α1-containing receptors show faster kinetics of the GABA-activated Cl⁻ current than those with α2, α3, or α5 subunits (Farrant and Nusser 2005); in pyramidal cells, GABA_A receptors containing different α subunits are targeted to specific subcellular locations and contribute to the characteristic nature of the inhibitory response evoked by the class of interneuron innervating that location (Nusser et al. 1996; Nyiri et al. 2001; Klausberger et al. 2002).

Convergent evidence indicates that schizophrenia is associated with alterations in presynaptic markers of specific interneuron subpopulations in the dorsolateral prefrontal cortex (DLPFC) (Lewis et al. 2008b). For example, lower levels of the transcript encoding the 67-kDa isoform of glutamic acid decarboxylase (GAD67), the principal enzyme for GABA synthesis, have been consistently found in the DLPFC of individuals with schizophrenia (Akbarian et al. 1995b; Guidotti et al. 2000; Mirnics et al. 2000; Volk et al. 2000; Straub et al. 2007; Hashimoto et al. 2008a). Furthermore, this deficit is particularly prominent in the subset of GABA cells that express the calcium-binding protein parvalbumin (PV) (Hashimoto et al. 2003) and may also be present in interneurons that contain the neuropeptide somatostatin (SST) (Morris et al. 2008). PV-positive cells in the DLPFC include basket cells, which target synapses enriched in α1-containing GABA_A receptors subunits in the soma (and proximal dendrites) of pyramidal neurons and chandelier cells whose terminals contact pyramidal neuron axon initial segments that contain α2 or α3 subunits (Nusser et al. 1996; Loup et al. 1998, 2006; Nyiri et al. 2001). On the contrary, SST-containing Martinotti cells make contacts preferentially onto the apical dendrites of pyramidal neurons and produce inhibitory postsynaptic currents that are mediated by α5-containing GABA_A receptors (Serwanski et al. 2006; Ali and Thomson 2008).

Interestingly, alterations in expression of both PV and SST mRNAs in the DLPFC of subjects with schizophrenia appear to be lamina specific. For example, levels of PV mRNA are lower in layers 3 and 4, with no difference in layers 2 and 5 (Hashimoto et al. 2003); the density of PV-positive varicosities (putative basket cell axon terminals) is lower in layers 3 and 4, but not in layer 2 (Lewis et al. 2001; Lewis and Gonzalez-Burgos 2008); and GAD67 mRNA expression is lower in PV-positive neurons in layers 3 and 4 (Hashimoto et al. 2003). On the other hand, SST mRNA expression in the DLPFC is lower principally in layers 2, 3, and 5 in subjects with schizophrenia (Morris et al. 2008), and mRNA expression of the SST2 receptor, which is localized to pyramidal cell apical dendrites, is lower in layer 5 neurons in the same subjects (Morris et al. 2006). Given that GABA_A receptors enriched in specific α subunits have a preferential subcellular distribution and that they are particularly targeted by interneurons whose altered expression in the DLPFC in schizophrenia is lamina specific, we used in situ hybridization to analyze the laminar expression of the postsynaptic GABA_A receptor subunits α1, α2, α3, and α5 in schizophrenia and matched normal comparison subjects. Due

to the preferred assembly of each α subunit with a specific β subunit in cortical GABA$_A$ pentamers (Olsen and Sieghart 2009), we also analyzed the expression levels of β1-3 subunits in the same subjects. Our findings support the hypothesis that GABA neurotransmission in the DLPFC is in predominantly in certain cortical microcircuits in schizophrenia.

Material and Methods

Human Subjects

Brain specimens from 46 subjects were obtained after consent from the next-of-kin during autopsies conducted at the Allegheny County Medical Examiner's Office (Pittsburgh, PA). All procedures were approved by the University of Pittsburgh's Committee for the Oversight of Research Involving the Dead and Institutional Review Board for Biomedical Research.

In order to control for experimental variance and to reduce biological variance between groups, each subject with schizophrenia ($n = 23$) was matched for sex and, as closely as possible, for age and PMI, with one normal comparison subject (for demographic details, see Table 1). Subject groups did not differ in mean age, postmortem interval (PMI), RNA integrity number (RIN), brain pH, or tissue storage time at –80 °C (Table 1). Samples from these same subjects have been used in previous studies of mRNA expression levels for presynaptic markers of GABA neurons by in situ hybridization (Eggan et al. 2008; Morris et al. 2008), and a subset of these subject pairs ($n = 14$) was also included in a previous quantitative polymerase chain reaction (PCR) study in which GABA$_A$ α1 subunit mRNA was found significantly reduced in schizophrenia (Hashimoto et al. 2008a).

Tissue Preparation

The right hemisphere of each brain was blocked coronally, immediately frozen, and stored at –80 °C (Volk et al. 2000). Cryostat sections (20 μm) from the anterior–posterior level corresponding to the middle portion of the superior frontal sulcus were cut serially and collected into tubes containing Trizol reagent (Invitrogen) for RNA isolation and RIN determination (Eggan et al. 2008), or mounted on Super frost plus glass slides (VWR International) for Nissl staining or in situ hybridization. The location of DLPFC area 9 was determined by cytoarchitectonic criteria from the Nissl-stained sections (Rajkowska and Goldman-Rakic 1995).

In Situ Hybridization

Histological analysis of gene expression was performed by in situ hybridization performed as described previously (Hashimoto et al. 2003, 2005; Morris et al. 2008) (see Supplementary Methods for additional details).

Table 1
Summary of subject characteristics

Parameter	Comparison	Schizophrenia	t-Test
Sex	17 Male, 6 female	17 Male, 6 female	
Race	18 White, 5 black	15 White, 8 black	
Age (years)	48.0 (15.5)	47.9 (14.1)	$t_{22} = 0.16$; $P = 0.88$
PMI (h)	18.0 (5.5)	17.8 (9.3)	$t_{22} = 0.22$; $P = 0.83$
Brain pH	6.9 (0.2)	6.8 (0.3)	$t_{22} = 0.62$; $P = 0.54$
RIN	8.7 (0.4)	8.4 (0.7)	$t_{22} = 1.84$; $P = 0.08$
Storage time (months at −80 °C)	113.6 (23.5)	117.8 (23.5)	$t_{22} = -0.96$; $P = 0.35$

Note: Values are mean ($\pm$SD).

Quantification of GABA$_A$ Receptor Subunit mRNAs

Trans-illuminated autoradiographic film images were captured by a video camera under precisely controlled conditions, digitized, and quantified using a Microcomputer Imaging Device (MCID) system (Imaging Research Inc.). Quantification was performed without knowledge of subject diagnosis by random coding of the sections. Images of Nissl-stained sections were also captured and superimposed onto the autoradiographic images to draw contours of the full thickness of the cortex where the cortex exclusively was cut perpendicular to the pial surface. Optical density (OD) was measured within the contours and expressed as nCi/g of tissue by reference to radioactive ^{14}C standards (ARC Inc.) exposed on the same autoradiographic film. The mean (standard deviation [SD]) total area of gray matter sampled in each subject was 140 (64) mm^2 for normal comparison subjects and 128 (51) mm^2 for subjects with schizophrenia.

Levels of mRNA expression in cortical layers 2 and 6 were determined for those subunits for which the total gray matter analysis detected a significant difference between groups, as well as for those subunits that previous reports suggested were altered in a lamina-specific manner in schizophrenia (Volk et al. 2002). Laminar expression was calculated using a series of cortical traverses (1-2 mm in width) extending from the pial surface to the white matter, as previously described (Morris et al. 2008) (see Supplementary Methods for additional details).

Evaluation of mRNA expression at the cellular level was performed for GABA$_A$ α1 and β2 transcripts in a subset of 12 subject pairs that showed >15% decrease in mRNA expression. Silver grain accumulation on emulsion-dipped, Nissl-counterstained sections was conducted as previously described (Hashimoto et al. 2003; Beneyto and Meador-Woodruff 2006, 2008; Morris et al. 2008). Briefly, using the MCID imaging software and a Zeiss microscope with a motorized stage, four 1-mm-wide cortical traverses extending from the pial surface to the white matter were placed on each tissue section in locations where area 9 was cut perpendicular to the cortical surface. In each of the cortical traverses, 4 sampling frames (120 × 170 μm) were systemically and randomly placed in deep layer 3 (defines as 35–50% of the distance from the pial surface to the white matter border), corresponding to the laminar distribution of the major change in mRNA expression observed for both GABA$_A$ α1 and β2 subunits. The edges of the frames were equidistant from the border of each traverse and the edge of the next sampling frame.

As RNase-A treatment during the in situ hybridization procedure degrades Nissl-stainable substances within the cytoplasm, it is not possible to draw contours of the neuronal soma. Thus, the number of grains/cell was counted in each frame by placing circles over nuclei of cells in a bright-field image. As previously described, grain clusters confined within a 22-μm diameter circle were considered to be interneurons and those within a 30-μm diameter circle were considered to be pyramidal cells (Benes et al. 1986; Rajkowska et al. 1998; Hashimoto et al. 2003; Beneyto and Meador-Woodruff 2006, 2008; Morris et al. 2008). In the corresponding dark-field image, the software determined the number of grains in each circle. Background grain density was measured in each slide by counting the grains in a sampling frame placed over the white matter. The smaller size and intense Nissl staining of glial nuclei distinguished them from the larger, more faintly stained neuronal nuclei. An average of 90 large cells and 60 small cells were sampled per slide (total number, α1: 1552 small cells, 819 from control vs. 732 from schizophrenia subjects, and 2232 large cells, 1120 from control vs. 1112 from schizophrenia subjects; β2: 1320 small, 715 for control vs. 605 for schizophrenia subjects, and 2088 large cells, 996 from control vs. 1092 from schizophrenia subjects). For both subject groups, frequency histograms of the number of grains of all sampled neurons normalized by the background for each slide showed unimodal and normal distribution for both large and small cells in both subject groups.

Antipsychotic-Exposed Monkeys

As described previously, 18 experimentally naive, male macaque monkeys (*Macaca fasicularis*), 4.5-5.3 years of age, were chronically exposed to twice daily oral doses of haloperidol, olanzapine, or placebo ($n = 6$ monkeys per group) for 17–27 months (Dorph-Petersen et al. 2005). Trough plasma levels for haloperidol and olanzapine were within the range associated with clinical efficacy in humans.

Animals were euthanized in triads, and serial coronal sections (16 μm) from the right frontal lobe were cut from blocks containing the anterior one-third of the principal sulcus. In situ hybridization and quantification of mRNA levels were performed using methods similar to those described above (see Supplementary Methods for additional details).

All studies were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Statistical Analysis

Two analysis of covariance (ANCOVA) models were used to test group differences for total, laminar, and cellular expression of each mRNA. The first ANCOVA model used diagnostic group as the main effect, pair as a blocking effect, and RIN, brain pH, and storage time as covariates. The pair effect reflects the matching of individual subject pairs for sex, age, and PMI. A second unpaired ANCOVA model was performed to validate the first model using diagnostic group as the main effect and sex, age, PMI, pH, RIN, and storage time as covariates. For the covariates that showed a significant effect on the OD for any of the transcripts analyzed, Person's coefficient analyses were performed and the resulting r values are reported. The results from both models are presented. Because both models produced comparable results for diagnostic group effect, only the unpaired model is reported for analysis of expression differences within layers.

The influences of potential confounding variables on the OD values in subjects with schizophrenia were assessed with ANCOVA models using each variable (sex; schizoaffective disorder; suicide; antidepressants, benzodiazepines or sodium valproate, or antipsychotics at the time of death; diagnosis of substance abuse or dependence at the time of death) as the main effect and sex, age, PMI, brain pH, RIN, and storage time as covariates. A one-way analysis of variance model, with OD as the dependent variable and treatment group as the main effect, was used to compare mRNA expression levels in the DLPFC of antipsychotic-exposed monkeys.

Results

Specificity of Riboprobes and Laminar Expression Patterns

The specificity of the riboprobes was confirmed by the following observations. First, each riboprobe showed a distinctive laminar pattern of expression in the DLPFC (Figs 1 and 2, and Supplementary Fig. 1) that was consistent with previous studies of human prefrontal cortex (Akbarian et al. 1995a; Loup et al. 2000, 2006; Petri et al. 2006). Second, no signal above background was found in sections hybridized with sense riboprobes (data not shown). Third, silver grain clusters in emulsion-dipped sections were located exclusively over neuronal nuclei (Supplementary Fig. 2).

In normal comparison subjects, α1 mRNA expression was high across layers 3 to superficial 5, intermediate in layers 2 and deep 5, lower in layer 6, and lowest in layer 1 (Figs 1A and 3A-B). GABA_A α2 subunit mRNA levels were high in layers 2 and superficial 3, intermediate in layers deep 3, 4, and superficial 5, low in layers deep 5 and 6, and lowest in layer 1 (Figs 1C and 3C-D). Consistent with previous reports in humans (Loup et al. 1998, 2006), but in contrast to rodents (Pirker et al. 2000), the highest expression levels of α3 mRNA were in layers 2 and 5 (Fig. 1E and Supplementary Fig. 3A); however, the overall expression levels were very low compared with the other α subunits (Fig. 1E,F). GABA_A α5 subunit mRNA expression was highest in layer 4, moderate in layer 5, low in layers 2, 3, and 6, and lowest in layer 1 (Figs 1G and 3E-F). Although cross-probe comparisons must be made with caution, α1 mRNA levels were relatively higher than those for the other α subunits, consistent with previous reports of the relative abundance of these subunits (Mohler 2006).

GABA_A β2 subunit mRNA expression was high in deep layer 3 and layer 4, intermediate in layer 2 and superficial layer 3, and low in layers 5 and 6 (Figs 2C and 3G-H). This laminar distribution, and the relative expression level of the β2 subunit, were very similar to those for the α1 subunit (compare Fig. 3A,G), consistent with the observation that the α1β2γ2 assembly constitutes ~60% of GABA_A receptors in the adult mammalian brain (Mohler 2006). In contrast, expression levels of β1 mRNA were high in all layers except layer 1 (Fig. 2A; Supplementary Fig. 3B), and expression of β3 mRNA was the highest in layer 2, followed by intermediate expression levels in layers 3–6, and low expression in layer 1 (Fig. 3E; Supplementary Fig. 3C).

GABA_A α Subunit mRNA Expression in Schizophrenia

The mean (±SD) α1 mRNA expression level, as measured in the full thickness of the gray matter, was significantly decreased by 18% (paired: $F_{1,19} = 10.58$, $P = 0.004$; unpaired: $F_{1,38} = 16.64$, $P < 0.001$) in the schizophrenia subjects (302.6 ± 53.8 nCi/g) relative to the normal comparison group (368.3 ± 46.0 nCi/g) (Fig. 1B). Laminar analysis (Fig. 3A,B) revealed a significantly lower mRNA expression in the schizophrenia group in layer 3 (–17%; $F_{1,38} = 5.07$, $P = 0.03$) and layer 4 (–17%; $F_{1,38} = 4.77$, $P = 0.035$), with trends in the same direction in layer 5 (–17%; $F_{1,38} = 3.87$, $P = 0.056$) and layer 6 (–22%; $F_{1,38} = 3.84$, $P = 0.057$). Group differences in layers 1 and 2 did not reach statistical significance (both $F_{1,38} < 2.20$, both $P > 0.15$).

Total gray matter OD levels for α2 mRNA expression in the schizophrenia subjects did not significantly differ (paired: $F_{1,19} = 2.86$, $P = 0.12$; unpaired: $F_{1,38} = 0.19$, $P = 0.66$) from the matched comparison group (Fig. 1D). However, laminar analysis showed a significant ($F_{1,38} = 6.86$, $P = 0.013$) 14% higher expression of α2 subunit mRNA in layer 2 of the schizophrenia subjects (Fig. 3C,D), consistent with a previous immunocytochemistry study of α2 protein (Volk et al. 2002). Levels of α2 mRNA expression did not differ between diagnostic groups in the remaining cortical layers (all $F_{1,38} < 1.82$, all $P > 0.19$) (Fig. 3C,D).

For GABA α3 mRNA, one comparison subject had an OD value > 2.4 SD from the mean; consequently, this subject and the matched schizophrenia subject were removed for the statistical analyses. The overall expression of α3 mRNA in DLPFC gray matter was not different in the subjects with schizophrenia relative to comparison subjects (paired: $F_{1,18} = 2.74$, $P = 0.12$; unpaired: $F_{1,36} = 1.62$, $P = 0.21$) (Fig. 1F).

Analysis of the OD values for the α5 subunit mRNA revealed a significant (paired: $F_{1,19} = 5.51$, $P = 0.03$; unpaired: $F_{1,38} = 6.98$, $P = 0.01$) 16% lower expression level in the subjects with schizophrenia (118.9 ± 27.4 nCi/g) relative to comparison subjects (140.8 ± 26.3 nCi/g) (Fig. 1H). Laminar analysis (Fig. 3E,F) showed a significant 15% lower expression of α5 subunit mRNA in layer 4 ($F_{1,38} = 5.55$, $P = 0.024$), with trends in the same direction in layer 5 (–14%; $F_{1,38} = 3.4$, $P = 0.073$) and layer 6 (–15%; $F_{1,38} = 3.67$, $P = 0.063$). Expression of α5 mRNA did not differ between diagnostic groups in layers 1-3 (all $F_{1,38} < 2.3$, all $P > 0.14$).

GABA_A β Subunit mRNA Expression in Schizophrenia

Total gray matter expression levels of the β1 subunit did not differ between schizophrenia and normal comparison subjects (paired: $F_{1,19} = 1.94$, $P = 0.18$; unpaired: $F_{1,38} = 3.68$, $P = 0.07$) (Fig. 2B). In contrast, total gray matter expression for β2 was significantly 20% lower in schizophrenia relative to comparison subjects (paired: $F_{1,19} = 10.11$, $P = 0.005$; unpaired: $F_{1,38} = 14.21$, $P = 0.001$)
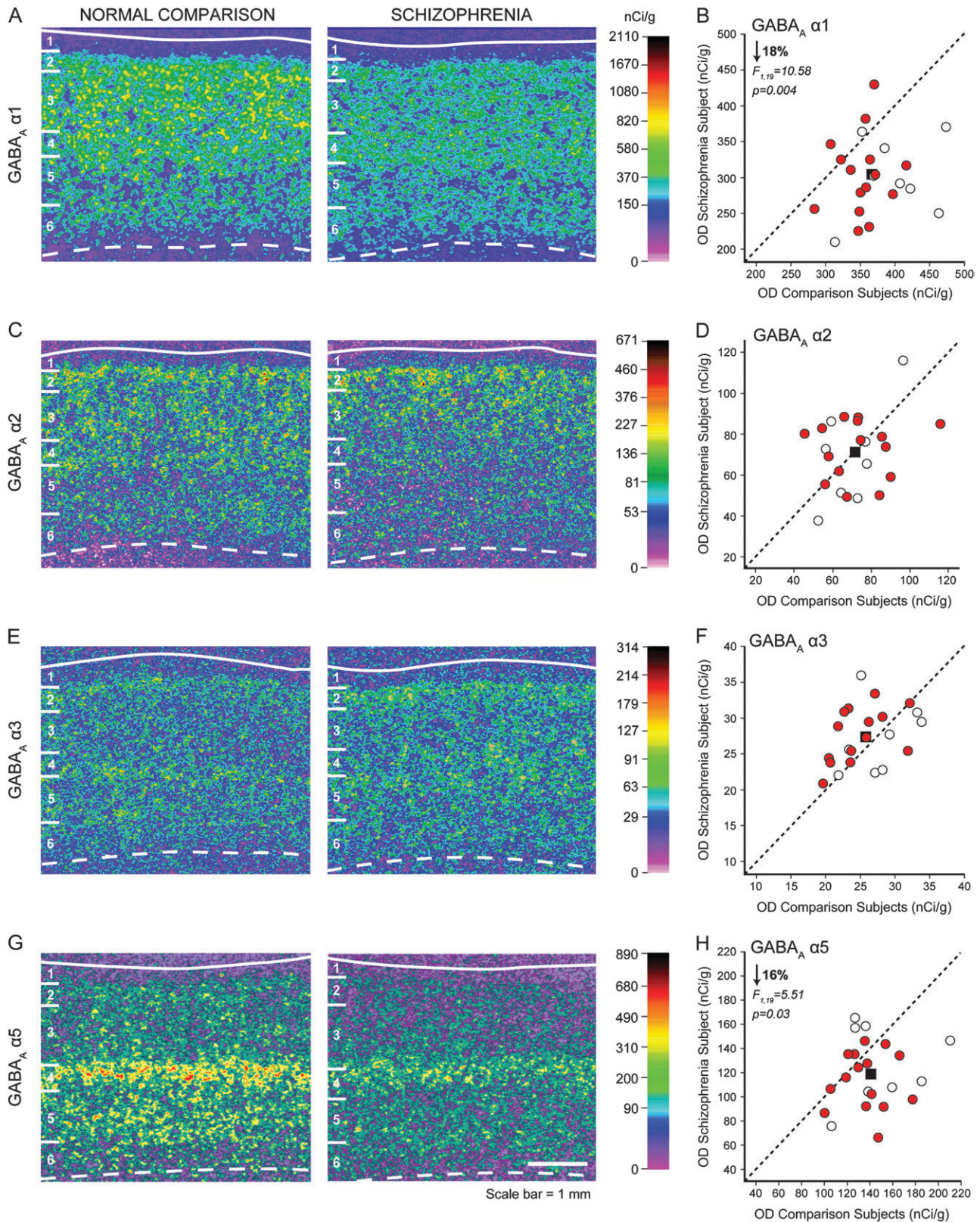
Figure 1. Representative autoradiograms illustrating expression of GABA$_A$ α1 (*A*), α2 (*C*), α3 (*E*), and α5 (*G*) subunits mRNA in DLPFC area 9 of a comparison subject (left) and a matched subject with schizophrenia (right). The density of hybridization signal is represented in pseudocolor according to the calibration scale (nCi/g) from the ^{14}C standards. Solid and broken lines denote the pial surface and the gray matter–white matter border, respectively. The 6 cortical layers, identified by Nissl staining, are indicated on the left of each panel. Scale bar = 1 mm. Comparison of film autoradiogram OD measures for α1 (*B*), α2 (*D*), α3 (*F*), and α5 (*H*) in total gray matter of DLPFC in matched pairs of normal comparison subjects and subjects with schizophrenia (red circles) and schizoaffective disorder (open circles). Mean value for the group difference is indicated by the black square. Labels below the dashed unity line indicate pairs for which the subject with schizophrenia or schizoaffective disorder had a lower mean expression level than the matched comparison subject.
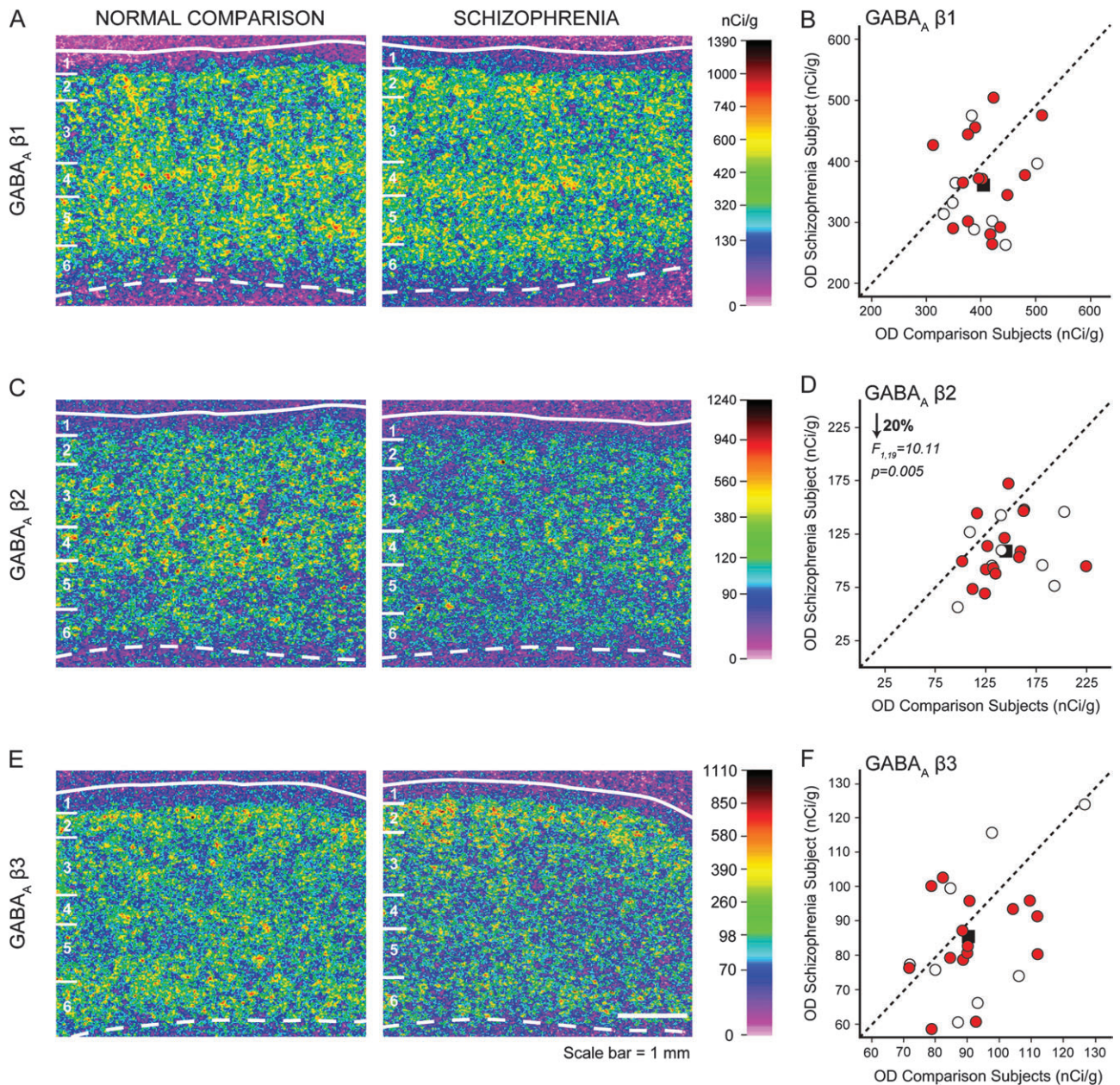
Figure 2. Representative autoradiograms illustrating expression of GABA$_A$ β1 (*A*), β2 (*C*), and β3 (*E*) subunits mRNA in DLPFC area 9 of a comparison subject (left) and a matched subject with schizophrenia (right). For details, refer to Figure 1. Scale bar = 1 mm. Comparison of film autoradiogram OD measures for β1 (*B*), β2 (*D*), and β3 (*F*) in total gray matter of DLPFC in matched pairs of normal comparison subjects and subjects with schizophrenia (red circles) and schizoaffective disorder (open circles). For details, refer to Figure 1.

(Fig. 2*D*). Laminar analysis (Fig. 3*G,H*) revealed that this difference in β2 expression was due to significantly lower expression in layer 3 (−16%; $F_{1,38}$ = 8.39, P = 0.006), layer 4 (−20%; $F_{1,38}$ = 15.15, P < 0.001), layer 5 (−20%; $F_{1,38}$ = 15.99, P < 0.001), and layer 6 (−23%; $F_{1,38}$ = 11.37, P = 0.002). The overall expression of the β3 subunit in the total gray matter was not significantly different between schizophrenia and comparison subjects (unpaired: $F_{1,38}$ = 2.38, P = 0.131; paired: $F_{1,19}$ = 4.35, P = 0.102) (Fig. 2*F*).
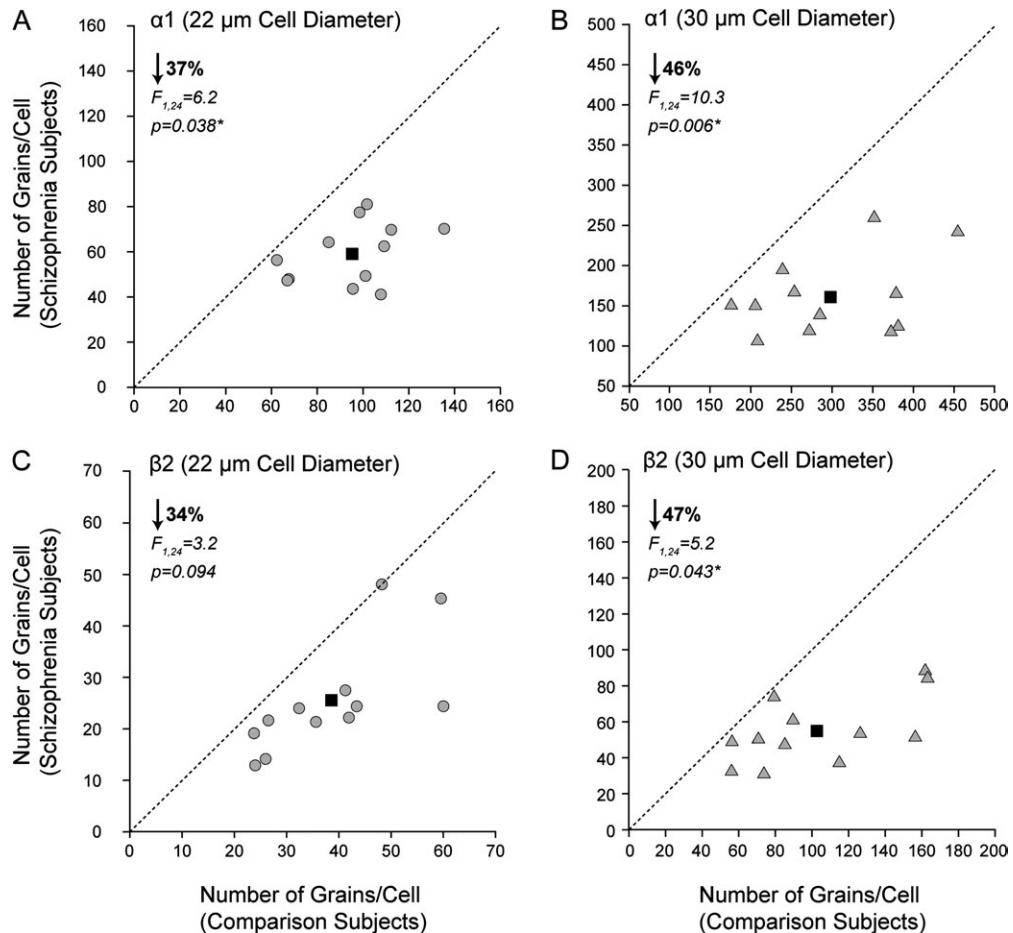
Cellular mRNA Expression Levels of GABA$_A$ Subunits α1 and β2

Given that α1 is known to be located at both the synapses from PV-positive basket cells on pyramidal cells as well as in the

reciprocal connections between PV-positive basket cells (Nusser et al. 1996; Fritschy et al. 1998a, 1998b; Loup et al. 1998), we decided to analyze if putative pyramidal cells and interneurons exhibited lower α1 subunit expression in layer 3 in schizophrenia. The mean (±SD) number of grains per putative interneuron (i.e., 22-μm diameter sampling circle) was significantly decreased by 37% in subjects with schizophrenia (59.2 ± 13.5) relative to controls (95.3 ± 21.5) ($F_{1,24}$ = 6.2, P = 0.038) (Fig. 4*A*). Likewise, the number of grains per putative pyramidal neuron (i.e., 30-μm diameter sampling circle) was significantly ($F_{1,24}$ = 10.3, P = 0.0061) 46% lower in the subjects with schizophrenia (160.7 ± 48.8) than in the control subjects (298.3 ± 87.4) (Fig. 4*B*). We also measured the
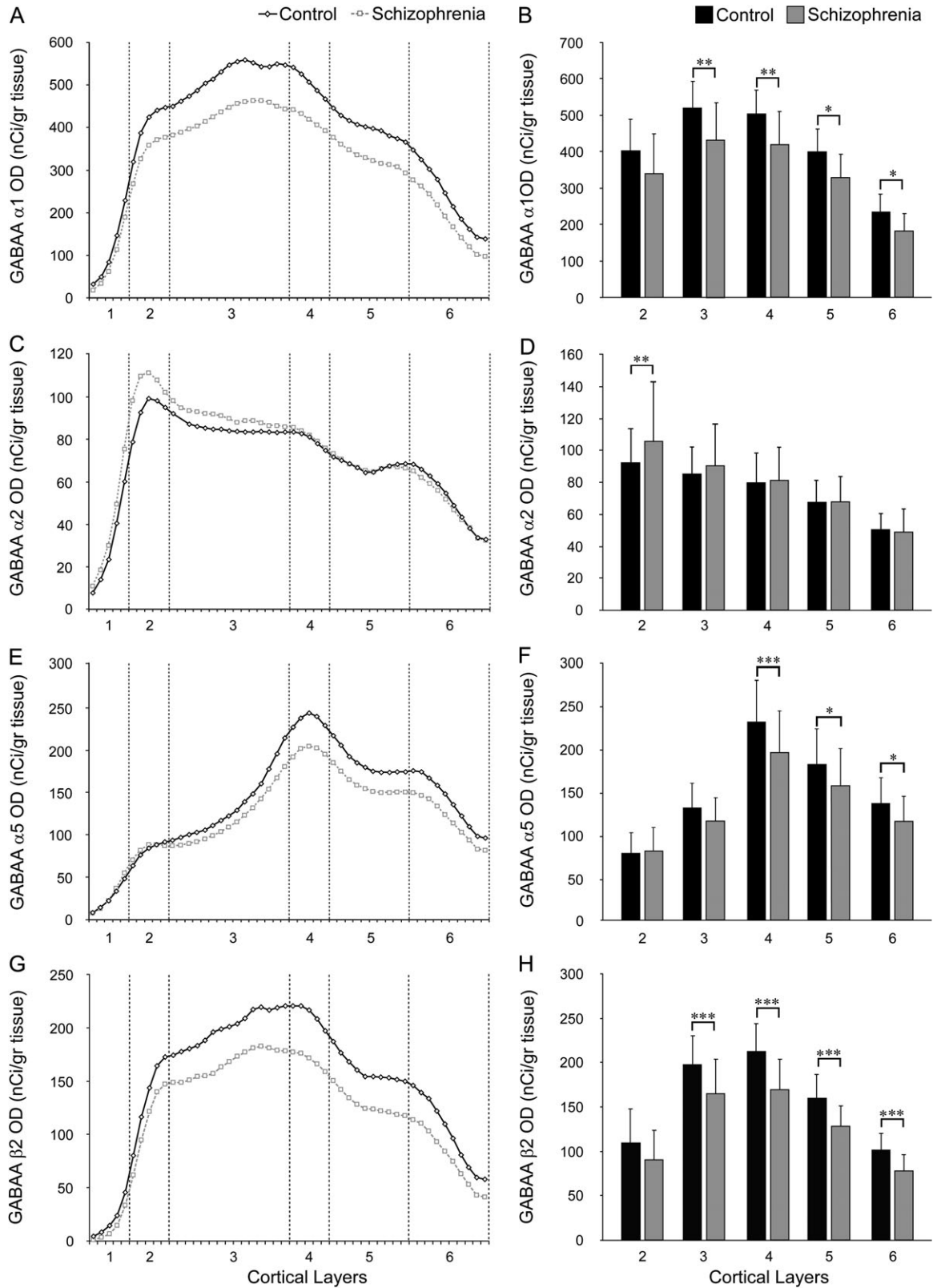
Figure 3. Laminar expression of the mRNAs for GABA$_A$ receptor α1 (*A, B*), α2(*C, D*), α5 (*E, F*), and β2 (*G, H*) subunits. Left panels: Mean mRNA OD across cortical layers from the pial surface to the white matter border in schizophrenia (gray) and comparison groups (black). Right panels: Mean (SD) film OD for mRNA expression in each cortical layer between comparison and schizophrenia groups. *$P < 0.1$, **$P < 0.05$, ***$P < 0.01$.

Figure 4. Cellular level analysis of the difference in GABA$_A$ subunits α1 and β2 mRNA expression in deep layer 3 of the DLPFC. Comparison of number of grains per cell measures for α1 (*A, B*) and β2 (*C, D*) in small (circles) (*A, C*) and large cells (triangles) (*B, D*) in matched pairs of normal comparison subjects and subjects with schizophrenia. Mean value for the group difference is indicated by the black square. The dashed unity line indicates no difference in expression levels.

grain density per neuron for the β2 subunit given its preferential assembly with α1 subunits (Mohler 2006). Consistent with the findings from the film analysis, the mean (±SD) number of grains per large neuron was significantly ($F_{1,24}$ = 5.2, P = 0.043) 47% lower in the subjects with schizophrenia (55.9 ± 18.7) relative to control subjects (102.8 ± 40.4) (Fig. 4*D*). Finally, the difference in β2 mRNA expression in small neurons, although similar in magnitude to the observed for α1, showed a trend level ($F_{1,24}$ = 3.2, P = 0.094) 34% reduction in schizophrenia (25.4 ± 10.8) compared with control subjects (38.6 ± 12.9) (Fig. 4*C*).

Analysis of Potential Confounding Factors

Pearson's correlation analyses revealed significant associations between RIN and α1 (r = 0.58, P < 0.001), α2 (r = 0.48, P = 0.001), α5 (r = –0.51, P < 0.001), β1 (r = 0.51, P < 0.001), β2 (r = 0.61, P < 0.001) and β3 subunit mRNA expression (r = 0.52, P < 0.001), but not with α3 subunit mRNA expression. Correlation analyses also showed inverse associations between PMI and α1 (r = –0.44, P = 0.002) and β1 subunit mRNA expression (r = –0.41, P = 0.05). Finally, we also observed significant inverse relationships between age and α5 (r = –0.51, P < 0.001) and β3 subunit expression (r = –0.44, P = 0.002). Each of these confounding variables was included as a covariate in the ANCOVAs performed for each GABA$_A$ receptor subunit.

The mean OD values for α1, α2, α5, and β2 subunit mRNAs in the subjects with schizophrenia did not differ as function of diagnosis of schizoaffective disorder; suicide; use of antidepressants, benzodiazepines or sodium valproate, or antipsychotics at the time of death; or diagnosis of substance abuse or dependence at the time of death (all $F_{1,16}$ < 5.1, all P > 0.07; Supplementary Fig. 4). Mean OD values for α1, α5, and β2 in subjects with schizophrenia did not differ by sex (Supplementary Fig. 4*A,C–D*); but α2 OD levels were greater in male than female subjects with schizophrenia in layer 2 ($F_{1,17}$ = 4.43, P = 0.05) (Supplementary Fig. 4*B*). Consistent with these findings, α2 subunit expression in layer 2 was significantly higher in male subjects with schizophrenia than male normal comparison subjects ($F_{1,30}$ = 9.19; P = 0.005) (Supplementary Fig. 4*B*). Although no difference in α2 mRNA in layer 2 was observed between female subjects with schizophrenia and female normal comparison subjects ($F_{1,5}$ = 1.17; P = 0.2), the small sample (n = 6 pairs) of females in our cohort does not provide enough power to draw conclusions regarding the sex specificity of these findings.

To further test for potential effects of antipsychotic medication on the expression of GABA$_A$ receptor subunits, we examined the mRNA expression of α1, α2, α5, and β2 subunits in male macaque monkeys with long-term exposure to haloperidol, olanzapine, or placebo. The mean OD values in total gray matter and in each layer did not differ (all $F_{2,15}$ < 1.1;

all $P > 0.4$) across the subject groups for any of the subunits studied (Supplementary Fig. 5).

Discussion

Our results suggest that GABA neurotransmission in the DLPFC of subjects with schizophrenia is altered at the postsynaptic level in a receptor subunit- and layer-specific manner. These findings do not appear to be attributable to a number of potential confounds, such as antipsychotic treatment. For example, none of the transcripts ($\alpha1$, $\alpha2$, $\alpha5$, or $\beta2$) that were altered in schizophrenia showed differences in expression in monkeys chronically exposed to either a typical (haloperidol) or atypical (olanzapine) antipsychotic medication relative to sham-treated animals (Supplementary Fig. 5). Although this approach cannot exclude a potential interaction between illness and medications, we also did not find a difference in the effect of illness in subjects with schizophrenia who were on versus those who were off antipsychotic medications at the time of death. Similarly, the effect of diagnosis did not differ between subjects who were on or off antidepressant medications, benzodiazepines, or valproate at the time of death (Supplementary Fig. 4). Thus, the combination of 1) distinctive laminar pattern of differences between schizophrenia and control subjects for some GABA$_A$ receptor subunits, 2) the lack of group differences for other subunits, and 3) the apparent absence of effects for potential confounds suggests that the disease process of schizophrenia alters GABA neurotransmission in specific DLPFC microcircuits.

Potential Relationships between Pre- and Postsynaptic Alterations in Markers of GABA Neurotransmission in Schizophrenia

GABA$_A$ Receptors Containing $\alpha1$ Subunits and PV-Containing Basket Cell Inputs

The inputs from the PV-positive basket cell class of GABA neurons to pyramidal neuron cell bodies are mediated predominately by $\alpha1$-containing GABA$_A$ receptors in the adult cerebral cortex, whereas contacts made by PV-negative basket cell terminals have very low levels of this subunit (Nusser et al. 1996; Fritschy et al. 1998a, 1998b; Loup et al. 1998). Interestingly, the significantly lower expression of $\alpha1$ mRNA predominantly in layers 3 and 4 of schizophrenia subjects is similar to that previously reported for the following presynaptic markers of PV-positive interneurons in the DLPFC of subjects with schizophrenia: 1) lower PV mRNA expression in layers 3 and 4, with no difference in layers 2 and 5 (Hashimoto et al. 2003); 2) lower density of PV-positive varicosities (putative basket cell axon terminals) in layers 3 and 4, but not in layer 2 (Lewis et al. 2001; Lewis and Gonzalez-Burgos 2008); and 3) lower GAD67 mRNA expression in PV-positive neurons in layers 3 and 4 (Hashimoto et al. 2003). Together, these findings suggest a coordinated pre- and postsynaptic reduction of phasic GABA neurotransmission from PV-positive basket cells to pyramidal cells and/or other interneurons in the middle cortical layers in schizophrenia (Fig. 5). For example, $\alpha1$-containing GABA$_A$ receptors are present postsynaptic to PV-positive basket cells in both pyramidal neurons and other PV-positive basket cells (Klausberger et al. 2002). Our single-cell-level analysis of both $\alpha1$ and $\beta2$ expression by emulsion grain counting showed that both mRNAs are lower in both large cells, putative pyramidal

neurons, and in small cells, putative interneurons. However, due to the technical limitations for cell type classification associated with the use of Nissl-stained, RNase-treated samples, confirmation of lower $\alpha1$ and $\beta2$ mRNA in both pyramidal and interneurons awaits dual label in situ hybridization studies with cell type–specific markers.

Consistent with the interpretation of a coordinated pre- and postsynaptic reduction of phasic GABA neurotransmission from PV-positive basket cells in the middle layers of the DLPFC in schizophrenia, mRNA levels for the GABA$_A$ receptor $\beta2$ (present study) and $\gamma2$ (Akbarian et al. 1995a; Huntsman et al. 1998, 2008a) subunits, which typically assemble with $\alpha1$ subunits in postsynaptic receptors mediating phasic GABA neurotransmission (Farrant and Nusser 2005), are also reduced in the DLPFC in schizophrenia. Indeed, we detected strong positive correlations between the within-subject expression of $\alpha1$ and $\beta2$ subunit mRNAs ($r = 0.758$, $P < 0.001$) and between their within-pair percentage differences ($r = 0.601$, $P = 0.002$) in the DLPFC. Furthermore, this correlation was also observed at the cellular level, indicating that the reduction in $\alpha1\beta2$-containing GABA$_A$ receptors likely affects both pyramidal cells and interneurons (large and small cells) in layers 3 and 4 of the DLPFC in schizophrenia. Schizophrenia-associated alterations in expression of $\alpha1$ and $\gamma2$ subunit mRNAs are also positively correlated (Hashimoto et al. 2008a). Given that $\alpha1$, $\beta2$, and $\gamma2$ subunits coassemble to form ~60% of GABA$_A$ receptors in the adult cortex (Mohler 2006), a coordinated difference in their expression levels would be expected. The biological relevance of these correlations is further supported by the similar magnitude and identical laminar pattern of the expression alterations in $\alpha1$ and $\beta2$ mRNAs in schizophrenia subjects and by the absence of expression alterations in either $\beta1$ or $\beta3$ subunits, which do not assemble with $\alpha1$ subunits, and accordingly have different laminar patterns of expression. Thus, this constellation of findings suggests that the total number of $\alpha1\beta2\gamma2$ GABA$_A$ receptors is lower in the middle layers of the DLPFC in subjects with schizophrenia.

Interestingly, $\alpha1$ subunits can also coassemble with a δ (instead of γ) subunit to form functional receptors (Mertens et al. 1993; Saxena and Macdonald 1994; Bianchi and Macdonald 2003). Cortical GABA$_A$ receptors containing δ subunits are extrasynaptic, have a high affinity for GABA, and mediate tonic inhibition, defined as the constant activation of extrasynaptic receptors that, by increasing input conductance, reduces the probability of generating an action potential (Farrant and Nusser 2005). In primate DLPFC, both $\alpha1$ and δ subunits have similar laminar patterns of expression and undergo similar developmental trajectories, which are distinct from those of GABA$_A$ $\alpha2$ and $\alpha4$ subunits (Hashimoto et al. 2009; Maldonado-Aviles et al. 2009). In the present study, the laminar pattern of altered $\alpha1$ mRNA expression matches that exhibited by the δ subunit transcript in the same subjects (Maldonado-Aviles et al. 2009), and the within-subject pair differences in $\alpha1$ and δ mRNA levels were significantly correlated ($r = 0.74$, $P < 0.0001$). Together, these findings suggest that schizophrenia might be associated with a reduced complement of $\alpha_1\beta_x\delta$ GABA$_A$ receptors in the DLPFC. Thus, our findings raise the possibility that both synaptic, phasic inhibition from PV-positive basket cells (via $\alpha_1\beta_2\gamma_2$ receptors) and tonic inhibition (via $\alpha_1\beta_x\delta$ receptors) are altered in the DLPFC of subjects with schizophrenia (Maldonado-Aviles et al. 2009).

Deficits in both $\alpha1$ and $\beta2$ transcript expression, similar in magnitude and laminar pattern to those observed in the
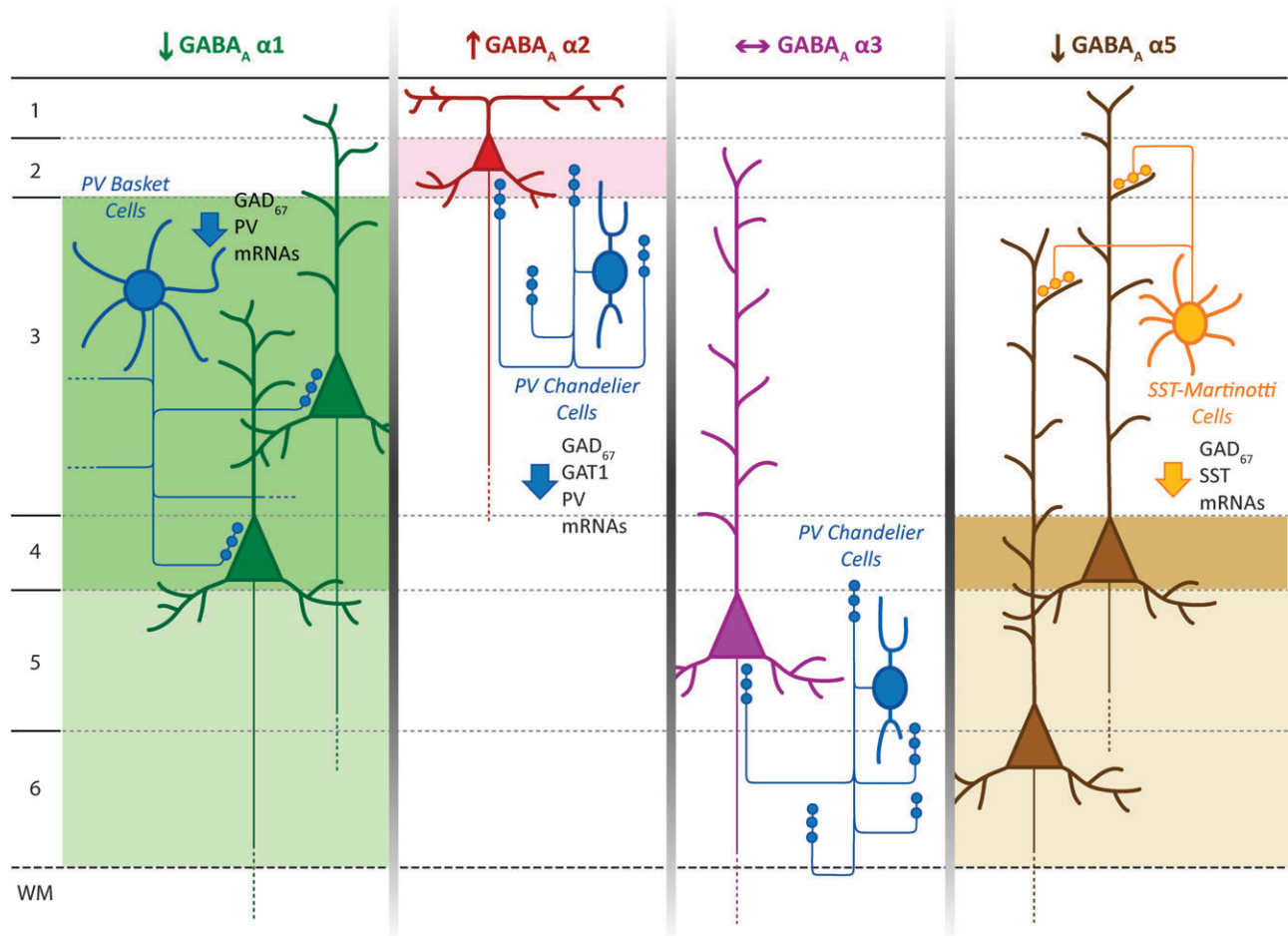
Figure 5. Schematic summary of hypothesized circuit-specific transcript alterations in pre- and postsynaptic markers of GABA neurotransmission in the DLPFC of subjects with schizophrenia. For each $GABA_A$ α subunit, the background shading marks the cortical layers where the indicated change in expression of that subunit was found. The laminar specificity of the decrease in $\alpha1$ expression matches that of the alterations in GAD67 and PV mRNAs thought to be present in PV-positive basket cells. The increase in $\alpha2$ expression in layer 2 is consistent with previous findings of pre- and postsynaptic alterations in chandelier cell inputs to the axon initial segment of pyramidal cells in this location. In contrast, the absence of alterations in $\alpha3$ subunit expression, which is present postsynaptic to chandelier cells in deep layer pyramidal neurons matches the failure to find significant changes in chandelier cell inputs in these layers. The decrease in $\alpha5$ was observed in deeper layers of DLPFC where the somata of pyramidal neurons whose apical dendrites are known to be innervated by SST+ Martinotti cells, also affected in schizophrenia, are predominantly located.

present study, were previously reported in the DLPFC in schizophrenia, although those findings did not achieve statistical significance. Such due to a smaller sample size (Akbarian et al. 1995a). Lower $\alpha1$ transcript levels in the DLPFC in schizophrenia have also been observed by microarray and quantitative PCR (Hashimoto et al. 2008a, 2008b). However, some studies have reported higher cortical levels of $\alpha1$ transcript (Impagnatiello et al. 1998; Ohnuma et al. 1999) and protein (Ishikawa et al. 2004) in schizophrenia. These earlier studies utilized subject groups that were not as well matched for age and sex and did not assess RNA integrity markers, such as RIN or brain pH, raising the possibility that the group differences reflect factors other than the disease process of schizophrenia. However, additional studies are required to reconcile the differences in the literature.

$GABA_A$ Receptors Containing $\alpha2$ Subunits and PV-Positive Chandelier Cell Inputs

Our finding of greater expression of $GABA_A$ $\alpha2$ subunit mRNA in DLPFC layer 2 (and superficial layer 3; Fig. 3C,D) from schizophrenia subjects parallels the laminar distribution of alterations in both pre- and postsynaptic markers of inputs from PV-positive chandelier cells to the axon initial segment of pyramidal neurons (Fig. 5). For example, increased $\alpha2$ subunit protein immunoreactivity (Volk et al. 2002) and decreased ankyrin-G immunoreactivity in pyramidal neuron axon initial segments (Cruz et al. 2009), as well as decreased GABA membrane transporter (GAT-1) immunoreactivity in chandelier neuron axon cartridges (Woo et al. 1998; Pierri et al. 1999), are all preferentially or selectively found in DLPFC layers 2 and superficial 3 of subjects with schizophrenia. Thus, these findings suggest that alterations in chandelier cell-pyramidal cell connectivity differ in laminar location from those found in basket cell-pyramidal cell connectivity. Consistent with this idea that an interaction between GABA cell type and laminar location confers vulnerability in schizophrenia, we did not observe altered mRNA expression of the $\alpha3$ subunit, which in contrast to the $\alpha2$ subunit, is enriched in the axon initial segment of pyramidal cells in deeper cortical layers (Nusser et al. 1996; Loup et al. 1998, 2006) where presynaptic alterations chandelier neuron axon cartridges are less prominent (Woo et al. 1998; Pierri et al. 1999). Interestingly, the

expression of GAD67 mRNA is not altered in DLPFC layer 6 of subjects with schizophrenia (Akbarian et al. 1995a; Volk et al. 2000), supporting the idea of laminar and circuit specificity of altered GABA neurotransmission in schizophrenia (Fig. 5).

In the hippocampus, α2 subunit–containing GABA$_A$ receptors are also present in pyramidal cell bodies postsynaptic to axon terminals from PV-negative basket neurons that express the neuropeptide cholecystokinin (CCK) and that contain the cannabinoid receptor 1 (CB1R) on their axon terminals (Nyiri et al. 2001). Although we cannot exclude an upregulation of α2 subunits postsynaptic to these CCK/CB1R inputs, their upregulation in layers 2 to superficial 3 does not match the high density of CCK/CB1R axon terminals in layers 4 and 6 (Oeth and Lewis 1993; Eggan and Lewis 2007) or the findings that CB1R-positive axons in the DLPFC of subjects with schizophrenia are significantly altered in those layers, but not in layers 2 and superficial 3 (Eggan et al. 2008).

GABA$_A$ Receptors Containing α5 Subunits and SST–Martinotti Cell Inputs

Our findings of a 15% reduction in α5 mRNA expression in DLPFC layers 4-6 in schizophrenia are consistent in magnitude and laminar distribution with a previous report showing a trend decrease of ~22% for α5 mRNA expression (Akbarian et al. 1995a). However, higher expression for α5 subunit transcript expression has also been observed in schizophrenia (Impagnatiello et al. 1998). As for α1 subunit expression, these discrepancies might reflect differences across studies in the extent to which the potential impact of various confounds were controlled, but further studies are warranted.

GABA$_A$ receptors containing α5 subunits were previously thought to be almost exclusively extrasynaptic, contributing to the tonic inhibition of pyramidal cells (Caraiscos et al. 2004). However, recent studies conclusively identified the presence of GABA$_A$ α5 subunits on pyramidal cell apical dendrites postsynaptic to GABA inputs from SST-containing Martinotti cells (Ali and Thomson 2008). Interestingly, SST mRNA expression is lower in the DLPFC in schizophrenia, principally in layers 2, 3, and 5 (Morris et al. 2008), and mRNA expression of SST2 receptor, which is localized to pyramidal cell apical dendrites, is lower in layer 5 neurons in the same subjects (Morris et al. 2006). Together, these findings suggest a correlated alteration in schizophrenia of GABA and SST inputs to the apical dendrites, present in layers 2 and 3, of pyramidal neurons whose cell bodies are present in the deeper layers of the DLPFC (Fig. 5).

Functional Implications of Lamina-Specific Alterations in GABA$_A$ α Subunits

In concert with previous investigations, our findings suggest that cortical GABA neurotransmission in altered in schizophrenia in certain DLPFC microcircuits, defined by the laminar location of coordinated pre- and postsynaptic changes in the inputs from specific subclasses of GABA neurons (Fig. 5). Specifically, the findings suggest altered inhibitory regulation of different domains (cell body, axon initial segment, or dendrites) in separate populations of pyramidal neurons. In contrast, although we cannot exclude alterations in the inhibitory regulation of GABA neurons, neither the results of this study nor of previous studies of the calretinin-containing GABA neurons that provide the majority of such inputs (Woo et al. 1997; Cotter et al. 2002; Hashimoto et al. 2003) provide evidence of such a disturbance. Thus, the composite data suggest

a circuit-specific dysregulation of the inhibition, but not of disynaptic disinhibition, of DLPFC pyramidal neurons in schizophrenia.

The synaptic inhibition provided by the affected GABA inputs serves to coordinate the timing of neuronal activity by synchronizing the firing of pyramidal cells at different frequencies. For example, gamma band (30–80 Hz) oscillations appear to require the fast decay of the GABA-mediated Cl⁻ current through α1-containing GABA$_A$ receptors; in particular, α1-containing synaptic inputs from PV-positive basket cells to the perisomatic region of pyramidal cells are critical for rhythmic gamma activity (Mann and Paulsen 2005; Bartos et al. 2007), whereas α1-mediated connections between PV-positive basket cells are not (Wulff et al. 2009). Thus, if our findings are indicative of reduced inhibitory transmission in basket cell inputs to pyramidal cells, then one would expect to see a reduction in gamma band oscillations in schizophrenia, which has, in fact, been observed over the DLPFC (Lewis et al. 2005; Cho et al. 2006). Whether this impairment can be attributed specifically to the laminar-specific alteration in PV-positive basket cell-pyramidal cell connectivity remains to be determined, but layer-specific factors in the generation of oscillations have been observed in vitro (Cunningham et al. 2003).

In vivo data (Cardin et al. 2009; Sohal et al. 2009) clearly indicate the dependence of gamma oscillations on PV neurons, but these studies were not able to discriminate between the contributions of basket versus chandelier cells. In studies of hippocampal oscillations in vivo, the firing pattern of chandelier cells strongly suggests a potent inhibitory effect on pyramidal cells, but recent in vitro studies indicate that chandelier neurons can, under certain conditions, powerfully depolarize pyramidal neurons (Szabadics et al. 2006; Khirug et al. 2008). Thus, how pre- and postsynaptic alterations in chandelier cell inputs contribute to the observed deficits in prefrontal gamma oscillations in schizophrenia remains to be determined, but their involvement is supported by the finding that a positive allosteric modulator for α2-containing GABA$_A$ receptors increased prefrontal gamma band power during a working memory task in individuals with schizophrenia (Lewis et al. 2008a). Similarly, theta oscillations (4–7 Hz) may depend on GABA inputs from SST-containing Martinotti cells (Fanselow et al. 2008) that are mediated by the slower kinetics of α5-containing GABA$_A$ receptors (Ali and Thomson 2008; Zarnowska et al. 2009). Interestingly, gamma oscillations are thought to be embedded in theta frequency oscillations in the context of certain cognitive tasks, such as working memory (Lisman and Idiart 1995), raising the possibility that the working memory disturbances in schizophrenia reflect the behavioral outcome of convergent disturbances in different GABA circuits in the DLPFC.

Although the findings of this study are consistent with earlier data in supporting circuit-specific alterations in GABA neurotransmission in schizophrenia, they do not suggest a simple hypothesis regarding the pathogenetic mechanisms underlying the alterations. For example, prior studies suggested that the upregulation of postsynaptic α2-containing receptors in pyramidal neuron axon initial segments represented a compensatory response to a presynaptic deficiency in GABA release from chandelier neurons due to lower levels of GAD67 (Volk et al. 2002; Lewis et al. 2005). If so, why are α1-containing receptors not upregulated postsynaptic to PV basket cells, given the likelihood that GAD67 mRNA expression is reduced in these neurons as well? One possibility is suggested by

findings in mice that GAD67-mediated GABA synthesis is required for the formation of basket cell axon terminals and synapses (Chattopadhyaya et al. 2007). If GAD67 expression is low during postnatal development in individuals who later develop schizophrenia, then fewer basket cell synapses would form on pyramidal cell bodies, reducing the need for postsynaptic GABA$_A$ receptors containing α1 subunits. Alternatively, the disease-related differences in the expression of α1 and α2 subunits might reflect disturbances in their opposed patterns of development. For example, in monkey DLPFC, the expression of α2 is high at birth and progressively declines through adolescence, whereas α1 levels are low at birth and progressively increase with age (Hashimoto et al. 2009). Thus, schizophrenia might involve a disruption, or arrest, in the normal developmental trajectories of GABA$_A$ α1 and α2 subunit expression. This hypothesis suggests new avenues of investigation for GABA circuit-specific interventions in individuals at high risk for schizophrenia.

Supplementary Material

Supplementary Methods and Figures 1–5 can be found at: http://www.cercor.oxfordjournals.org/

Notes

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