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ORIGINAL ARTICLE

Selective epigenetic alteration of layer I GABAergic neurons isolated from prefrontal cortex of schizophrenia patients using laser-assisted microdissection

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Among the most consistent results of studies of post-mortem brain tissue from schizophrenia patients (SZP) is the finding that in this disease, several genes expressed by GABAergic neurons are downregulated. This downregulation may be caused by hypermethylation of the relevant promoters in affected neurons. Indeed, increased numbers of GABAergic interneurons expressing DNA methyltransferase 1 (DNMT1) mRNA have been demonstrated in the prefrontal cortex (PFC) of SZP using *in situ* hybridization. The present study expands upon these findings using nested competitive reverse transcription-polymerase chain reaction combined with laser-assisted microdissection to quantitate the extent of DNMT1 mRNA overexpression in distinct populations of GABAergic neurons obtained from either layer I or layer V of the PFC of SZP. In a cohort of eight SZP and eight non-psychiatric subject (NPS) post-mortem BA9 tissue samples, DNMT1 mRNA was found to be selectively expressed in GABAergic interneurons and virtually absent in pyramidal neurons. DNMT1 mRNA expression was approximately threefold higher in GABAergic interneurons microdissected from layer I of SZP relative to the same neurons microdissected from NPS. GABAergic interneurons obtained from layer V of the same samples displayed no difference in DNMT1 mRNA expression between groups. In the same samples, the GABAergic neuron-specific glutamic acid-decarboxylase$_{67}$ (GAD$_{67}$) and reelin mRNAs were underexpressed twofold in GABAergic interneurons isolated from layer I of SZP relative to GABAergic interneurons microdissected from layer I of NPS, and unaltered in GABAergic interneurons of layer V. These findings implicate an epigenetically mediated layer I GABAergic dysfunction in the pathogenesis of schizophrenia, and suggest novel strategies for treatment of the disease.
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Introduction

DNA methylation within CpG-rich gene promoters is used by various types of mammalian cells to prevent transcriptional initiation and to ensure silencing of individual genes.[1–3] At least three independently encoded DNA methyltransferase enzymes catalyze the methylation of carbon 5 of cytosine residues in CpG dinucleotides.[2–4] DNA methyltransferase 1 (DNMT1) is considered to be the cell's maintenance DNA methyltransferase acting at replication foci in dividing cells, whereas DNMT3a and DNMT3b are considered the *de novo* DNA methyltransferases active in the methylation of cytosine residues during the establishment of genomic imprinting in early development.[3,4] The expression and activity of DNMT1 is generally restricted to dividing cells and is very high during development, but in most cell types, DNMT1 expression is greatly diminished once terminal differentiation occurs.[3–6] However, previous studies have reported that adult mammalian brains, which mainly consist of postmitotic, terminally differentiated neurons and glial cells, express high levels of DNMT1 mRNA and enzyme activity.[7–9]

In recent *in situ* and immunohistochemical studies of DNMT1 cellular localization in adult human brains, we provided evidence that DNMT1 is below the limit of detection in cortical pyramidal neurons and in glial cells, but is expressed at unexpectedly high levels in cortical GABAergic interneurons.[10,11] As cortical GABAergic neurons are postmitotic, terminally differentiated cells, the observation that these neurons express relatively high levels of DNMT1 mRNA and protein suggests that in these cells, DNMT1 possesses additional functions beyond the maintenance of DNA methylation at replication foci.[5]

The concept of a non-maintenance role for DNMT1 in cortical GABAergic interneurons is supported by

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recent studies, in which we demonstrated that the number of DNMT1 mRNA-positive neurons is increased in the prefrontal cortex (PFC) of schizophrenia and bipolar disorder patients.[10,11] This increase is associated with *RELN* promoter CpG island hypermethylation[12,13] and with the downregulation of the GABAergic interneuron-specific genes, *RELN* and *GAD1*, coding for the reelin and glutamic acid decarboxylase (GAD_{67}) proteins, respectively.[14–17] Recent *in vitro* and *in vivo* animal studies[18,19] provide compelling evidence that *RELN* and *GAD1* promoter hypermethylation brought about by increased DNMT1 catalytic activity is a dynamic and drug-reversible process that may be part of the core dysfunctions of schizophrenia and bipolar disorder.

Downregulation of reelin and GAD_{67} mRNAs is among the most robust and consistent findings of studies of post-mortem schizophrenia brains.[20–23] Reelin and GAD_{67} are known to be critically associated with dendritic spine expression,[20,23,24] neuronal plasticity[25–28] and cognition.[29–32] Hence, it has been suggested that the reelin and GAD_{67} deficit elicited by promoter hypermethylation associated with DNMT1 overexpression in cortical GABAergic interneurons may be a key pathogenetic factor expressed in these neurons of significance in the generation of psychotic symptoms in schizophrenia and bipolar disorder.

In the study presented here, we further tested the hypotheses that (a) cortical GABAergic interneurons are unique among differentiated neurons in that they express high levels of DNMT1, and (b) in the PFC of schizophrenia patients (SZP), the upregulation of DNMT1 expression is associated with the dysfunction of a specific subpopulation of GABAergic interneurons. To determine the neurons affected by this dysfunction among the many distinct cell subtypes of the GABAergic system, we employed laser-assisted microdissection. Using this method, we obtained pure samples of various cell types from a cohort of post-mortem BA9 tissue from SZP and non-psychiatric subjects (NPS). In these samples of GABAergic interneuron populations, we quantified the differences in expression of the DNMT1, GAD_{67} and reelin mRNAs between groups using quantitative nested competitive reverse transcription-polymerase chain reaction (RT-PCR). Our findings indicate that DNMT1 mRNA is easily detectable in pure samples of GABAergic interneurons and is below the limit of detection in pyramidal neurons. Additionally, we have demonstrated a threefold overexpression of DNMT1 mRNA and a twofold downregulation of both the GAD_{67} and reelin mRNAs in the PFC of SZP, and these changes are specific to the GABAergic interneurons of the superficial cortical layers.

Materials and methods

Subjects

Fresh-frozen brain tissue from eight SZP and eight NPS was obtained from the Stanley Foundation (Brain Bank Neuropathology Consortium, Bethesda, MD, USA). Originally, this cohort[33] included 15 NPS and 15 SZP samples, but some of the tissue had been expended in previous studies of GAD_{67} and reelin mRNA and protein expression and *RELN* promoter methylation.[12,15] Available demographic data on the 16 tissue samples analyzed in this study are listed in Table 1.

Laser-assisted microdissection

Brain tissue from each subject was sliced to 20 μm at -23°C in a Microm HM 550 cryostat, mounted on polyethylene terephthalate membrane frame slides (Leica, Wetzlar, Germany), fixed in cold 70% ethanol for 2 min, and stained briefly with 0.1% toluidine blue. This staining method allows differentiation of neurons from glial cells using morphological criteria such as size, the presence or absence of visible cytoplasm and the distribution of chromatin within the nucleus.[34,35] The slices were then microdissected using the Application Solution Laser Microdissection system (Leica Microsystems) to collect pools of 100–250 cells or 40 tissue sections approximately $3 \times 10^5\ \mu m^2$ in area from layer I or layer V. Collections of tissue sections were assembled from 10 fields from each of four consecutive tissue slices, resulting in a total of $2.5 \times 10^8\ \mu m^3$ of layer I or layer V tissue. Samples were collected into the cap of a 200 μl microcentrifuge tube containing 40 μl of RNA extraction buffer with β-mercaptoethanol. Collection of individual neurons and cortical layers is shown in Figure 1.

RNA extraction

RNA was extracted from the microdissected samples using the RNeasy Micro Kit from Qiagen (Valencia, CA, USA) according to the manufacturer's directions. This kit was designed specifically for use with tissue samples obtained using the ASLMD system and it greatly increases the yield and improves the quality of RNA, and optimizes the reproducibility of experiments when working with small quantities of tissue.

Nested competitive RT-PCR

Competitive RT-PCR involves the use of an internal standard (IS), a molecule of RNA that has been engineered to be identical to the target mRNA in the region between the PCR primers, except for a specific deletion mutation introduced to allow the products of these two templates to be differentiated by agarose gel electrophoresis. At the beginning of the assay, a known amount of IS is added to the reaction and is amplified along with the target mRNA by a single primer pair after reverse transcription. At the end of the PCR, the products are electrophoresed on an ethidium bromide-containing agarose gel and visualized using a Kodak 1D Image Analysis System.

To increase reliability, each measurement included four reactions, each containing a constant amount of sample mRNA and differing amounts of IS, diluted twofold between reactions. At the end of the assay,

Table 1 Summary of clinical and demographic data available on subjects and tissue samples

Case no.	*Group*	*Sex*	*Age (years)*	*PMI*	*Laterality*	*pH*	*Storage*	*DO*	*Flu*	*Cause of death*
1	SZP	M	31	14	Left	5.8	3177	18	4000	Suicide, jumped
2	SZP	M	44	50	Right	6.5	3078	17	1000	Cardiac
3	SZP	M	25	32	Left	6.6	3120	20	4000	Suicide, hanged
4	SZP	F	30	60	Right	6.2	3494	22	6000	Suicide, jumped
5	SZP	F	60	40	Left	6.2	3363	15	0	Cardiac
6	SZP	M	32	19	Left	6.1	3232	27	15 000	Acute alcohol intoxication
7	SZP	M	60	31	Right	6.2	3239	27	80 000	Accidental drowning
8	SZP	M	44	29	Left	5.9	2979	21	130 000	Pulmonary disease
Average			40.8	34.4		6.2	3210			
s.d.			13.6	15.3		0.3	163			
9	NPS	M	52	28	Left	6.5	3329			Cardiac
10	NPS	M	58	27	Left	6	2686			Cardiac
11	NPS	F	44	25	Right	6.3	3220			Cardiac
12	NPS	M	52	8	Left	6.1	3128			Cardiac
13	NPS	F	57	26	Right	6	2586			Motor vehicle accident
14	NPS	M	41	11	Right	6	2793			Pulmonary embolus
15	NPS	F	35	40	Left	5.8	2724			Pulmonary embolus
16	NPS	M	52	22	Right	6.2	2935			Cardiac
Average			48.9	23.4		6.1	2925			
s.d.			8.1	10.1		0.2	273			
P-value			0.169	0.11		0.6	0.024			

Abbreviations: DO, disease onset (years of age); Flu, lifetime quantity of fluphenazine or equivalent (mg); PMI, post-mortem interval (h); storage, freezer storage duration (days).

the ratio of products in each reaction was plotted against the original quantity of IS. The point of equivalence of the line of regression indicated the amount of target mRNA present in the original sample. The plot also served as a quality control for the measurement, as the quality of the data is reflected by the R^2 value of the line of regression. Assays yielding R^2 values <0.95 were repeated. A representative nested competitive RT-PCR for DNMT1 mRNA is depicted in Figure 2 (for further details of the method, see Grayson and Ikonomovic[36] and Gabbott and Somogyi[37]).

DNMT1, GAD_{67}, neuron-specific enolase (NSE), glyceraldehyde 3 phosphate dehydrogenase (G3PDH) and reelin mRNA content of microdissected samples were quantitated using nested competitive RT-PCR. The reactions were carried out using the following conditions and primers designed to anneal to the following sites: DNMT1: forward 2421–2444, reverse 2907–2930, forward nested 2446–2469 and reverse nested 2883–2906. DNMT1 first PCR cycle conditions were 94°C for 5 min followed by 30 cycles of 94°C for 30 s, 65°C for 30 s and 72°C for 30 s, followed by 72°C for 7 min. At the end of the first PCR, the reaction products were diluted 300-fold for use in a nested PCR performed as above but with only 25 cycles. NSE: forward 382–405, reverse 769–792, forward nested 446–467 and reverse nested 711–734. NSE PCR cycling conditions were the same as for DNMT1. GAD_{67}: forward 1855–1878, reverse 2246–2269, forward nested 1886–1909 and reverse nested 2189–2212. GAD_{67} PCR cycling conditions were the same as for DNMT1 with an annealing temperature of 60°C. G3PDH: forward 231–254, reverse 891–914, forward nested 257–280 and reverse nested 816–839. G3PDH PCR cycling conditions were the same as for DNMT1. Reelin: forward 9211–9234, reverse 9549–9572, forward nested 9241–9264 and reverse nested 9518–9541. Reelin PCR cycling conditions were the same as for DNMT1 with an annealing temperature of 60°C.

Internal standard construction

For use in competitive RT-PCR experiments, ISs were generated for the NSE, G3PDH, DNMT1, GAD_{67} and reelin mRNAs using site-directed mutagenesis with PCR-overlap extension using the PCR conditions listed above for the first PCR of each mRNA's nested competitive PCR assay.[36] A deletion mutation was created at the center of each IS using the following internal primers: NSE – 5′-AATGGTGGCTCTCATGCTTCTACCATACACTCAAG-3′, G3PDH – 5′-GTATGACAACAGCCTCAACATCATCCCTGCCTCTAC-3′, DNMT1 – 5′-CGGGCAGATGTTTCACGAGGCATGGATCCCGAGT-3′, GAD_{67} – 5′-CCAGATCAACAAATGCCGAGCACACCAACGTCTG-3′, reelin – 5′-GAGCAGAAATCAATCCCCATCCTTTCACCTCTAT-3′.

Nested non-competitive RT-PCR

The glial fibrillary acidic protein (GFAP) and vesicular glutamate transporter 2 (VGlut2) mRNAs were assayed by nested RT-PCR without the use of an IS. These reactions were carried out using the following

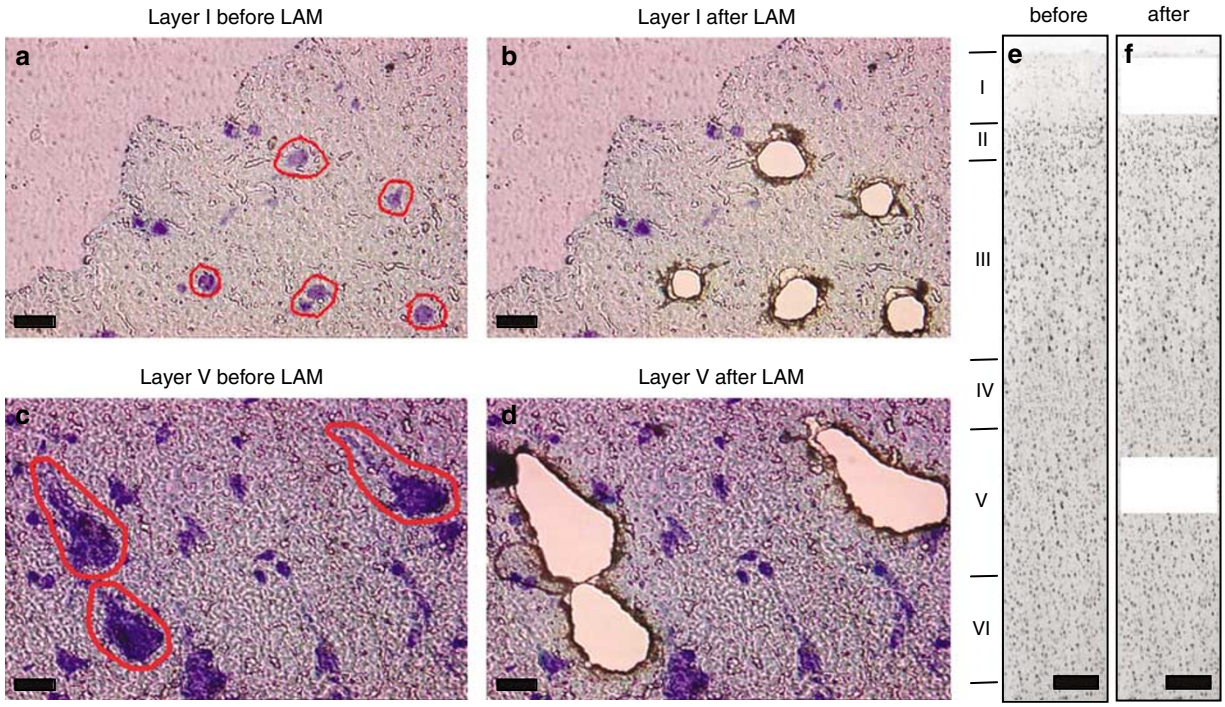

Figure 1 Laser-assisted microdissection (LAM) of neurons and tissue sections from layers I and V of post-mortem human PFC BA9. Tissue slices (20 μm) were stained with toluidine blue and microdissected using a Leica ASLMD system. Layer I is easily identified by its proximity to the tissue border and by its low cell density, and the cells there are a relatively homogenous population of GABAergic interneurons.[37–40] Layer V is a heterogenous mixture of cell types, but identification and collection of pyramidal neurons is straightforward owing to their characteristic morphology. A field of view of layer I before (**a**) and after (**b**) microdissection of a group of GABAergic interneurons, and a field of view of layer V before (**c**) and after (**d**) microdissection of a group of pyramidal neurons, is shown. Shown in box (**e**) are all six cortical layers. Cortical layer I or V was identified (box **f**) and microdissected from approximately 10 fields of view across four consecutive tissue slices, for a total of 40 collected sections per sample. Each collected section was approximately 3×10^5 μm^2 in area, resulting in an approximate total of 2.5×10^8 μm^3 of layer-specific cortical tissue per sample. Scale bars in (**a–d**) represent 25 μm and in boxes (**e** and **f**) represent 200 μm.

conditions and primers designed to anneal to the following sites: GFAP: forward 2041–2064, reverse 2563–2586, forward nested 2111–2134 and reverse nested 2515–2538. GFAP PCR cycling conditions were the same as for DNMT1. VGlut2: forward 920–943, reverse 1501–1534, forward nested 964–987 and reverse nested 1457–1480. VGlut2 PCR cycling conditions were the same as for DNMT1. Although the sensitivity of nested PCR varies between primer sets, based on our experience with both nested competitive and nested non-competitive RT-PCR, we estimate the limit of detection of this assay to be on the order of 1 amol of target mRNA per reaction.

Double immunohistochemistry studies

For double DNMT1 and $GAD_{65/67}$ immunolabeling, 20 μm floating sections were taken from BA9 samples of NPS fixed in 4% formaldehyde (three subjects 40, 60 and 70 years of age, who died from ski accident, respiratory failure and cancer, respectively, and with a post-mortem interval (PMI) of 28, 15.3 and 22.5 h, respectively) obtained from the McLean 66 Cohort Collection (Harvard Brain Tissue Research Center, Belmont, MA, USA). The sections were preincubated in 3% normal goat serum (NGS) and 3% normal rabbit serum (NRS) in phosphate-buffered saline (PBS) at room temperature for 30 min. After several rinses in PBS, sections were incubated with an anti-$GAD_{65/67}$ monoclonal antiserum (Chemicon, Temecula, CA, USA) and anti-DNMT1 rabbit antiserum (New England Biolabs, Ipswich, MA, USA) for 72 h. The primary antibodies were diluted 1:500 in PBS with 1% NGS and 1% NRS. Afterward, sections were washed in buffer and incubated in biotinylated goat anti-mouse antiserum diluted 1:250 (Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. After the sections were washed several times in PBS, they were incubated with Cy-5-conjugated goat anti-rabbit IgG (Amersham Biosciences, Piscataway, NJ, USA, 1:1000) to label the antibodies reacted with DNMT1. Cy2-conjugated streptavidin (1:1000) was used to label $GAD_{65/67}$ immunoreactivity. After several rinses, the sections were incubated in 2 mM $CuSO_4$ and 50 mM CH_3COONH_4 for 10 min to eliminate lipofuscin-mediated autofluorescence and were mounted and

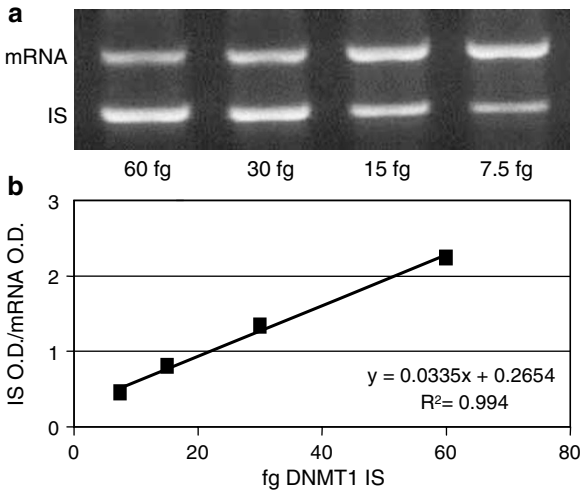

Figure 2 Nested competitive RT-PCR for DNMT1 mRNA in a sample of layer I tissue microdissected from NPS BA9. (**a**) The result of ethidium bromide gel electrophoresis with four lanes, each of which contains the products of a separate PCR. All reactions contain identical amounts of sample RNA, and before the beginning of the RT step, 60, 30, 15 or 7.5 fg of DNMT1 IS was added to the reactions. (**b**) The ratio of IS optical density (OD) to DNMT1 mRNA OD is plotted against the amount of DNMT1 IS added at the beginning of the assay. Solving the equation of the line of regression for $y=1$ indicates the amount of DNMT1 mRNA present in each reaction at the beginning of the assay (21.9 fg in this example), and the R^2 value is a reflection of the quality of the assay.

coverslipped. Controls omitting the primary antibody showed a lack of immunostaining. Sections were analyzed, and images were captured with a confocal microscope (Leica, Wetzlar, Germany) at a magnification of $\times 40$. The specificity of the DNMT1 antibody was ascertained by preadsorption of the antiserum with recombinant human DNMT1 protein (New England Biolabs). The DNMT1 antibody was tested against human brain extracts homogenized directly in sodium dodecyl sulfate loading buffer (200 μl/10 mg tissue). After electrophoresis on a 10–20% SDS-polyacrylamide gel electrophoresis (PAGE) gel, proteins were blotted onto a nitrocellulose membrane (Amersham Biosciences) and incubated with anti-DNMT1 (diluted 1:3000) or anti-DNMT1 (diluted 1:3000) preadsorbed with 20 U of DNMT1 control protein for 12 h at 4°C in 0.005 M $NaPHO_4$ buffer (pH 7.2), 0.2 M NaCl and 5% bovine serum albumin (BSA). Two immunoreactive bands, one of $\sim$180 kDa and one of $\sim$70 kDa, are recognized in human brain extracts with this DNMT1 antibody. However, when the antibody was preadsorbed with human recombinant DNMT1 protein, labeling of the two bands was virtually obliterated (Figure 3).

Nucleotide sequences

The mRNAs studied have the following GenBank accession numbers: DNMT1 – XM_049501; GAD_{67} – M81883; reelin – U79716; NSE – M22349; G3PDH – BC029340; Vglut2 – NM_020346; GFAP – BC062609.

Results

Colocalization of DNMT1 and GAD_{67}

Using double DNMT1 and GAD_{67} immunohistochemistry, we first studied the colocalization of these two markers in GABAergic neurons of layers I/II and layer V in slices of PFC BA9 from NPS. Figure 3 shows that GAD_{67} (a, green fluorescence) and DNMT1 (b, red fluorescence) are colocalized (c, merge) in BA9 layer I/II GABAergic interneurons. GAD_{67} (d, green fluorescence) and DNMT1 (e, red fluorescence) are also colocalized (f, merge) in GABAergic neurons of layer V, but there is no detectable DNMT1 or GAD_{67} immunoreactivity in pyramidal neurons (arrowheads in Figure 3d–f).

DNMT1 is expressed at high levels in GABAergic interneurons

These experiments were designed to compare, with nested RT-PCR, the difference in DNMT1 mRNA expression between GABAergic interneurons of layer I and glutamatergic pyramidal neurons of layer V in the PFC (BA9) of NPS. The neurons of layer I were chosen because they represent a relatively homogenous population of GAD_{67}-positive GABAergic interneurons[37–40] that express DNMT1 (Figure 3). Layer V is by no means a homogenous neuronal population; in fact, this layer contains pyramidal neurons and GABAergic neurons in addition to glial cells, but the distinct morphology of the pyramidal neurons (after Nissl staining) makes their identification and collection straightforward (Figure 1).

The comparison of DNMT1 expression between layer I GABAergic interneurons and layer V pyramidal neurons was made in pools of 100–250 neurons isolated using laser-assisted microdissection. Consistent with the histological data of Figure 3, we found that DNMT1 mRNA is expressed at much higher levels in the pools of GABAergic interneurons of layer I than in pools of pyramidal cells of layer V, where DNMT1 mRNA is virtually below detection limits (Figure 4). In these pools, we controlled for the quantity of neuronal material collected by assaying the NSE mRNA, which was easily detected by nested RT-PCR in all samples (Figure 4). As a further control, we determined the amount of tissue collected by measuring G3PDH mRNA content (not shown). Figure 4c shows the results of nested RT-PCR for DNMT1 mRNA referred to NSE mRNA varying the number of cycles of nested PCR. The difference in expression of DNMT1 mRNA between our two populations of neurons (GABAergic neurons of layer I versus pyramidal neurons of layer V) is robust both across a wide range of amplification and during the linear range of amplification.

To confirm the type and the homogeneity of the neurons contained in the microdissected cell collections, samples from NPS1 and NPS2, which are

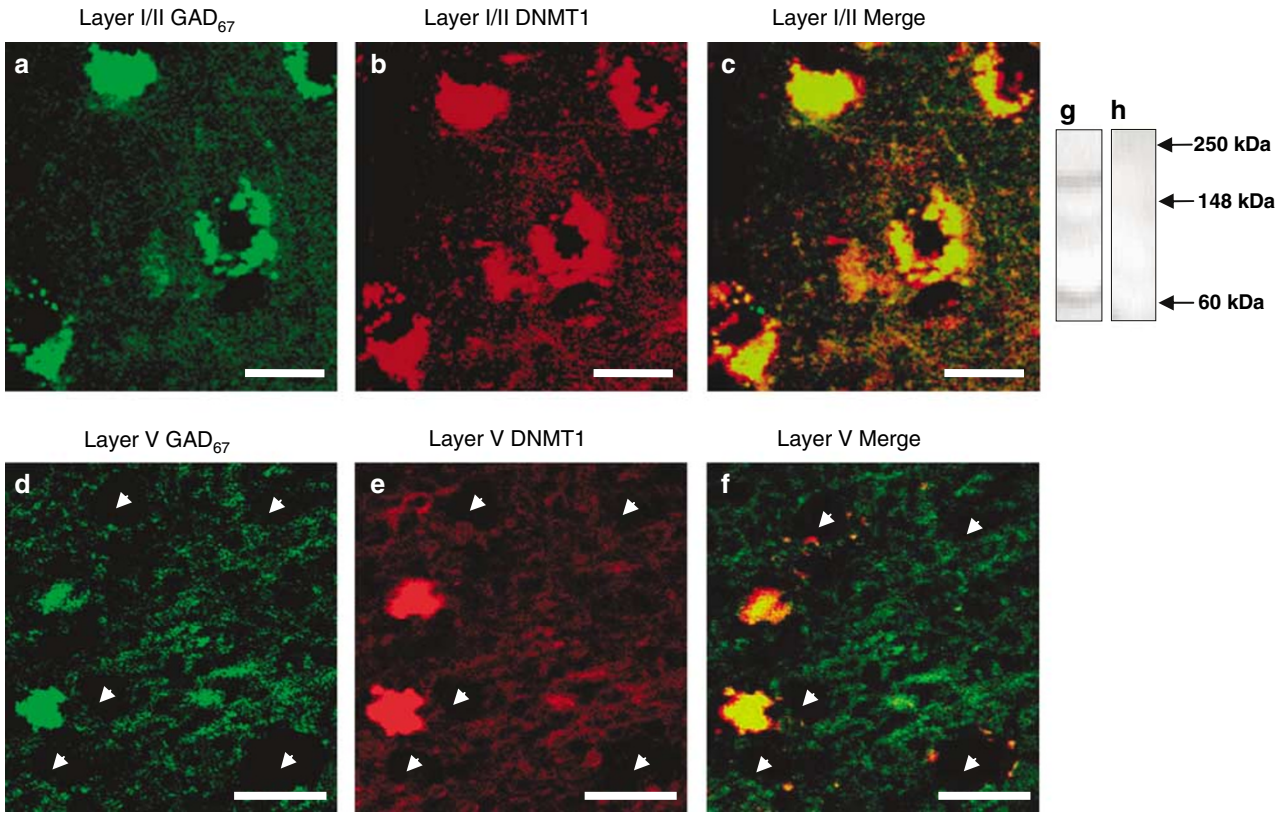

Figure 3 Colocalization of GAD_{67} and DNMT1 in BA9 layers I/II (**a–c**) and V (**d–f**) of one NPS. (**a**) GAD_{67} immunoreactivity, color-coded in green. (**b**) DNMT1 Immunoreactivity, color-coded in red. (**c**) Merge of (**a**) and (**b**). (**d**) GAD_{67} immunoreactivity, color-coded in green. (**e**) DNMT1 immunoreactivity, color-coded in red. (**f**) Merge of (**d**) and (**e**). Scale bars, 20 μm. Arrowheads in (**d–f**) indicate presumable locations of layer V pyramidal neurons unstained for both GAD_{67} and DNMT1 immunoreactivity. To demonstrate the specificity of the anti-DNMT1 antibody, Western blots were run using extracts of human brain. In panel **g**, the blot was incubated with the anti-DNMT1 antibody, and in panel **h**, the blot was incubated with anti-DNMT1 antibody that had been preadsorbed with human recombinant DNMT1 protein.

shown in Figure 4, were characterized by the measurement of four mRNAs – NSE mRNA as a marker of neuronal cells in general, GFAP mRNA as a marker of glial cells, GAD_{67} mRNA as a marker of GABAergic interneurons and VGlut2 mRNA as a marker of pyramidal neurons. As shown in Figure 5a, the pools of neurons dissected from layer I represent a neuronal population highly enriched in GAD_{67}, with virtually no contamination by glutamatergic neurons, as revealed by the almost complete absence of VGlut2 mRNA. The pools of neurons taken from layer V, on the other hand, represent a population of neurons greatly enriched in VGlut2 mRNA, with little or no contamination with GABAergic neurons, as shown by the absence of GAD_{67} mRNA. Figure 5b shows the results of nested RT-PCRs for GAD_{67} and VGlut2 mRNAs referred to NSE mRNA with varying numbers of cycles of nested PCR. Again, across a wide range of amplification and during the linear range of amplification, the difference in expression of these two mRNAs between our two populations of neurons is robust.

Before microdissection, tissue sections were stained with toluidine blue, allowing us to dissect neuronal collections with minimal contamination by glial cells. In fact, GFAP mRNA was virtually undetectable by nested RT-PCR in our collections of cells taken from layers I and V (Figure 5a). This, coupled with the fact that glial cells do not express NSE or GAD_{67} and express DNMT1 only at very low levels,[10] leads us to conclude that glial contamination is inconsequential to our study of DNMT1 and GAD_{67} mRNAs. Therefore, our cell collection procedure generated distinct samples of GABAergic interneurons enriched in DNMT1 from layer I, and glutamatergic pyramidal neurons from layer V showing little or no DNMT1 expression and no cross-contamination with GABAergic interneurons.

Layer I GABAergic interneurons of SZP PFC overexpress DNMT1 mRNA and underexpress GAD_{67} and reelin mRNA

To compare the expression levels of DNMT1 and GAD_{67} mRNAs in GABAergic neurons of NPS and SZP PFC, we sampled distinct populations of

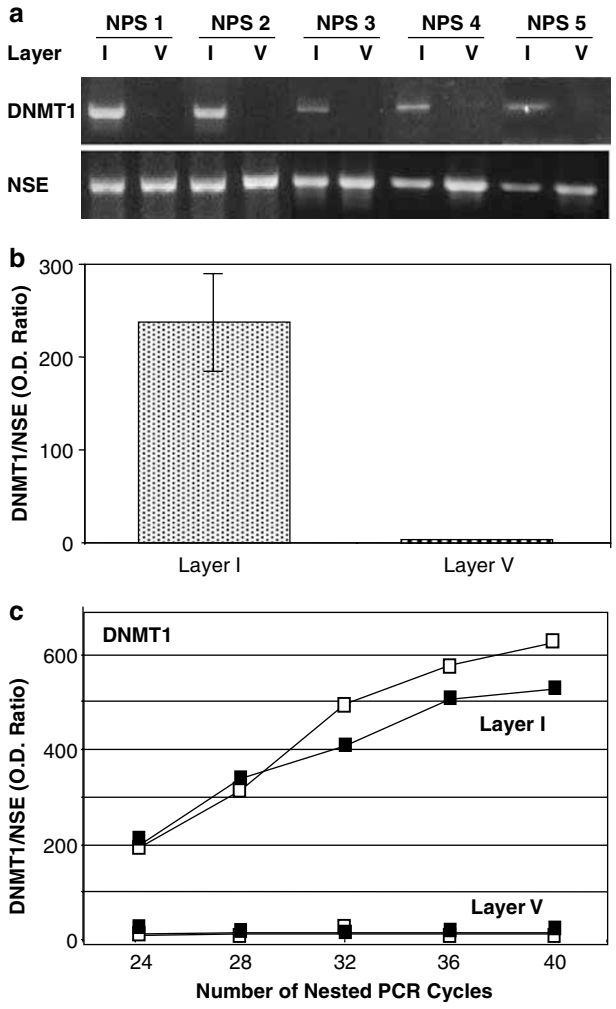

Figure 4 DNMT1 mRNA is expressed at higher levels in BA9 layer I GABAergic interneurons (layer I) than in BA9 layer V pyramidal neurons (layer V) of NPS. Samples ranging from 100 to 250 neurons were microdissected from layer I or V of five NPS BA9 brain samples. RNA was extracted and assayed for DNMT1 and NSE mRNAs using nested RT-PCR. (**a**) Whereas similar amounts of NSE mRNA are present in layer I and layer V samples, DNMT1 is detectable only in the layer I samples. The above PCRs were performed for 30 cycles each (first and nested) and at an annealing temperature of 65°C. (**b**) The average of five NPS samples per group is shown. (**c**) Samples NPS1 (□) and NPS2 (■) were used for nested RT-PCR for the DNMT1 and NSE mRNAs. PCRs consisted of 30 cycles of first PCR and between 24 and 40 cycles of nested PCR to demonstrate that after normalization to the NSE mRNA content of the sample, the difference in expression of DNMT1 mRNA between pyramidal neurons and GABAergic interneurons is robust and independent of the PCR conditions.

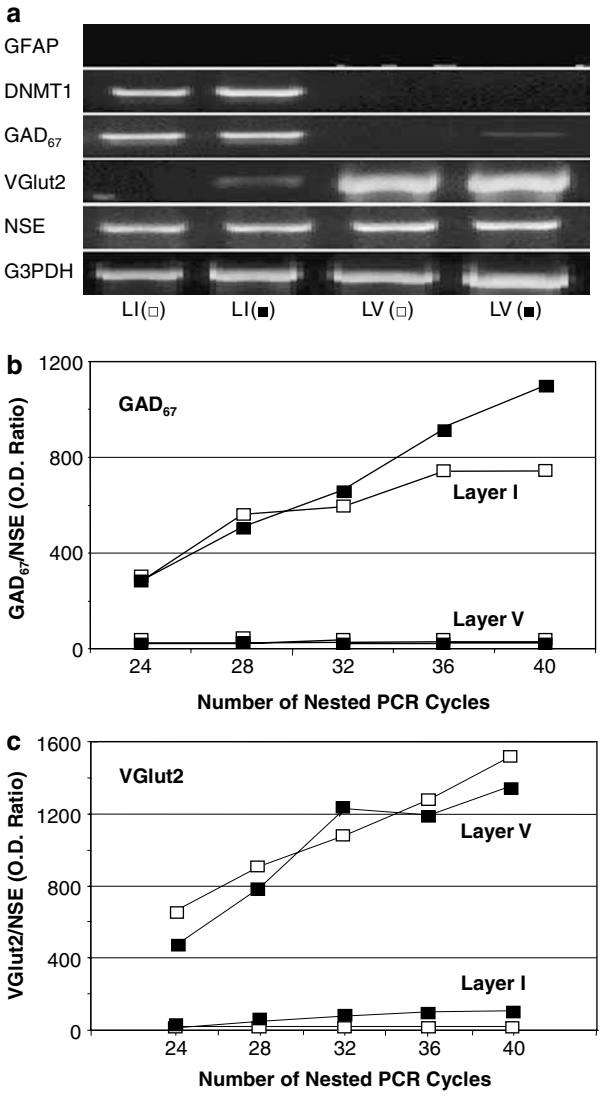

Figure 5 Characterization of cells microdissected from BA9 layer I (LI) or layer V (LV) of two NPS brains. The RNA extracted from samples NPS1 (□) and NPS2 (■) described in Figure 4 was assayed for GFAP, DNMT1, GAD_{67}, VGlut2, NSE and G3PDH mRNA content using nested RT-PCR. (**a**) Gels showing the PCR products for each mRNA after 30 and 32 cycles (first and nested) of nested RT-PCR. In all samples GFAP mRNA content was below the limit of detection. The DNMT1 and GAD_{67} mRNAs are coexpressed at high levels in layer I and are barely detectable in layer V neurons. VGlut2 mRNA is highly expressed in layer V and at very low levels in layer I. NSE and G3PDH mRNAs are easily detectable and expressed at similar levels in all samples. Variable numbers of nested PCR cycles were run for the GAD_{67} (**b**) and VGlut2 (**c**) mRNAs to demonstrate that the difference in GAD_{67} and VGlut2 mRNAs between layer I GABAergic interneurons and layer V pyramidal neurons is both robust across a wide range of amplification and present during the linear range of amplification.

GABAergic neurons by collecting microdissected sections of layer I and layer V tissue (Figure 1) from slices of eight NPS and eight SZP BA9 post-mortem tissue samples from the above-mentioned cohort (for demographic characteristics, see Table 1). DNMT1, GAD_{67}, reelin, NSE, and G3PDH mRNAs were quantitated with appropriate ISs using nested competitive RT-PCR. Figure 6a shows that after correcting for the NSE mRNA content, DNMT1 mRNA expres-

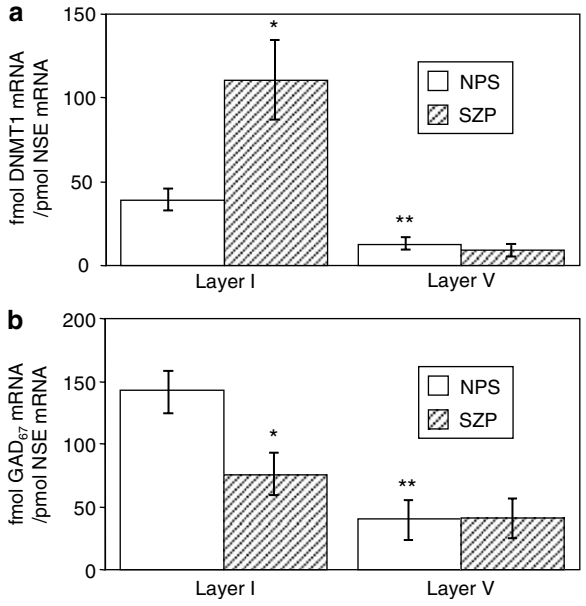

Figure 6 DNMT1 mRNA is overexpressed and GAD_{67} mRNA is underexpressed specifically in layer I of the SZP PFC (BA9). RNA was extracted from tissue sections microdissected from layers I and V of BA9 slices. DNMT1, GAD_{67} and NSE mRNAs were quantitated with nested competitive RT-PCR with ISs. The data represent mean $\pm$ s.e. of samples microdissected from the brains of eight subjects per group. As shown in panel **a**, the DNMT1 mRNA content of layer I of SZP was significantly different from that of NPS ($t = 3.4$; df = 14; $^*P = 0.004$). There was also a statistically significant difference when the DNMT1 mRNA content of NPS layer I was compared with that of NPS layer V ($t = 8.4$; df = 14; $^{**}P < 0.001$). Panel **b** demonstrates a statistically significant difference in the content of layer I GAD_{67} mRNA when SZP was compared with NPS ($t = -2.6$; df = 14; $^*P = 0.023$). In addition, when the content of GAD_{67} mRNA in layers I and V of NPS was compared, there was a significant difference ($t = 4.9$; df = 14; $^{**}P < 0.001$).

sion is approximately threefold higher in samples microdissected from layer I of SZP relative to samples microdissected from NPS. In the same samples, GAD_{67} mRNA is underexpressed by about twofold in samples obtained from layer I of SZP as compared with samples microdissected from layer I of NPS (Figure 6b). Interestingly, the overexpression of DNMT1 and the underexpression of GAD_{67} mRNAs are specific to layer I GABAergic interneurons, as we found no difference in the DNMT1 or GAD_{67} mRNA content of tissue microdissected from layer V (Figure 6a and b). In layer I, the difference in DNMT1 and GAD_{67} mRNA expression between NPS and SZP is similar in extent when the expression of DNMT1 or GAD_{67} mRNAs in the samples is related to the amount of the housekeeping gene G3PDH mRNA (Table 2). In a small group of samples (three SZP and three NPS), we also compared reelin mRNA expression normalized to G3PDH mRNA levels. Reelin mRNA is downregulated approximately twofold in layer I of SZP as compared with layer I of NPS (Table 2), and this difference is again specific to layer I, as it is not present in layer V of the same samples.

Table 2 DNMT1, GAD_{67} and RELN mRNAs normalized to G3PDH mRNA content in three selected patients per group

Group	*fmol DNMT1 mRNA/pmol G3PDH mRNA*	*fmol GAD_{67} mRNA/pmol G3PDH mRNA*	*fmol reelin mRNA/pmol G3PDH mRNA*
NPS layer I	10 ± 1.6	45 ± 10.8	3.3 ± 0.7
SZP layer I	24 ± 4.3[a]	14 ± 4.7[a]	1.2 ± 0.2[a]
NPS layer V	7.0 ± 0.9	47 ± 14.8	$0.14 \pm .03$
SZP layer V	8.6 ± 2.4	48 ± 9.5	$0.15 \pm .03$
P-value	0.046	0.040	0.037

[a]The level of gene expression in SZP layer I was significantly different from that in NPS layer I, with *P*-values listed in the last row of the table. Gene expression was not significantly different between layer V of SZP and NPS for any of the genes. For all data listed, $n = 3$.

Tissue microdissected from layer I of the cortex is greatly enriched in GABAergic interneurons when compared with tissue microdissected from layer V, where only about 20–30% of the neurons are GABAergic,[23,40] and which is instead enriched in pyramidal neurons. In fact, when normalized to NSE content, GAD_{67} mRNA content is four times more abundant in layer I samples than in layer V samples collected from the same NPS brains (Figure 6b). As for samples collected cell by cell, tissue collected from layer I does not contain VGlut2 mRNA, whereas tissue collected from layer V is greatly enriched for VGlut2 mRNA after correction for NSE mRNA content (Figure 7).

As contamination of the samples with glial cells remains a potential issue, GFAP mRNA content was assayed by nested RT-PCR in layer I and layer V tissue samples microdissected from the same three NPS BA9 tissue samples. We found no significant correlation (Pearson's correlation test) between GFAP and NSE mRNAs ($R^2 = 0.105$, $P = 0.847$), or GFAP and DNMT1 mRNAs ($R^2 = 0.525$, $P = 0.285$), whereas the correlation between NSE and DNMT1 mRNA content in the same samples was significant ($R^2 = 0.905$, $P = 0.034$). As the GFAP mRNA content of the samples does not correlate with that of the neuronal mRNAs we are interested in, and *in situ* hybridization studies show that GFAP-positive cells do not express appreciable amounts of DNMT1, GAD_{67} or reelin,[10] we are confident that glial contamination will not confound our conclusions on the expression levels of these neuron-specific mRNAs.

Demographic characteristics

As shown in Table 1, variables such as PMI, age of subjects, pH and freezer storage duration were not matched perfectly. However, none of these variables, with the exception of freezer storage duration, were

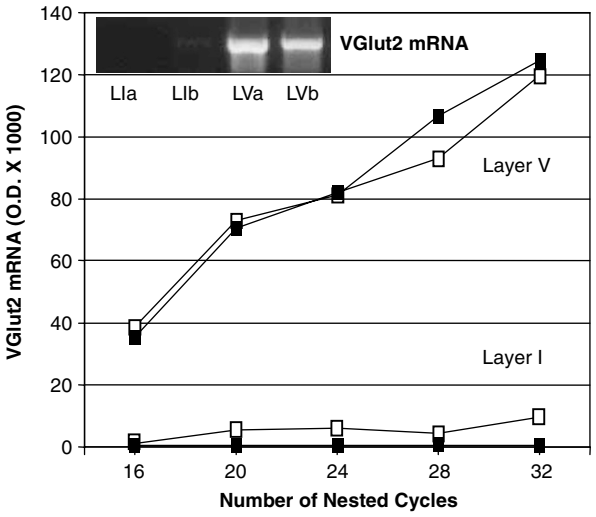

Figure 7 Layer V tissue sections are highly enriched in VGlut2 mRNA with respect to layer I samples. RNA was extracted from tissue sections microdissected from layer I or layer V of two NPS BA9 samples (NPSa – ■ and NPSb – □). NSE mRNA was measured by nested competitive RT-PCR. The samples were diluted to contain equal concentrations of NSE mRNA and then used for nested RT-PCR with 30 cycles of first PCR and various nested cycle numbers for Vglut2 mRNA. VGlut2 mRNA amplicon OD units represent the OD value of the amplicon*1000. The inset shows the VGlut2 mRNA amplicon from each sample after 30 and 28 cycles of nested RT-PCR.

significantly different between NPS and SZP groups when compared according to the Student's *t*-test. Neither age, brain hemisphere, pH, duration of storage, PMI, nor disease onset had any effect on DNMT1 or GAD_{67} expression. Furthermore, cumulative lifetime dose of fluphenazine was not correlated with the expression of DNMT1 or GAD_{67}. The only variable that was significantly (Student's *t*-test) associated with the increase of DNMT1 mRNA expression or with the decrease of GAD_{67} mRNA expression in layer I was the diagnosis of schizophrenia. In addition, when PMI, pH, freezer storage duration and other demographic variables listed above were included as covariates, ANCOVA produced results similar to those obtained by Student's *t*-test. For example, the comparison of layer I DNMT1 mRNA content yielded a *P*-value of 0.004 by Student's *t*-test and a *P*-value of 0.002 by ANCOVA, and the comparison of layer I GAD_{67} mRNA content yielded a *P*-value of 0.023 by Student's *t*-test and a *P*-value of 0.038 by ANCOVA.

Discussion

Cortical GABAergic neurons, but not pyramidal neurons or glial cells, express detectable levels of DNMT1

Non-terminally differentiated progenitor neurons and astroglial cells express high levels of DNMT1, which by catalyzing DNA (cytosine-5) methylation at CpG dinucleotide sequences influences the epigenetic processes of chromatin remodeling (DNA methylation, histone code modifications) and transcriptional regulation.[28,41–44] It has been proposed that in progenitor neurons, aberrant epigenetic events may stem from alterations in chromatin remodeling in response to abnormal intrinsic or extrinsic cues. These modifications may contribute to inherited intra-individual phenotypic variations in candidate gene expression relevant to psychiatric disorders in the absence of DNA sequence mutations.[45,46]

In most cells, including neurons, DNA methylation drops dramatically following terminal differentiation.[4,43,47] Thus, in terminally differentiated neurons in adult human brain that no longer face the challenges of differentiation and heritability of levels of gene activity, one would expect DNMT1 to be expressed at very low levels. To test this hypothesis experimentally, we studied the cellular localization of DNMT1 in the PFC of NPS with immunohistochemical techniques and developed quantitative nested competitive RT-PCR technologies using appropriate ISs to measure DNMT1 mRNA levels in populations of neurons laser-microdissected from layers I and V. The cells collected from layers I and V are nearly homogenous populations of GABAergic interneurons and glutamatergic pyramidal neurons, respectively (Figures 1 and 5). We found that DNMT1 mRNA is virtually absent in pyramidal neurons and glial cells, but in contrast, is highly expressed in a population of layer I GABAergic interneurons (Figure 4).

Cajal[48] termed the cortical GABAergic interneurons 'the butterflies of the soul', owing to the large number of actions they play in the regulation of cortical functions. These interneurons are involved in complex tasks such as shaping the receptive fields of pyramidal cells, controlling pyramidal cell excitability, setting up the synchronous activity of cortical neurons associated with various cognitive states and even controlling the types of information processed within the PFC.[49–53] To achieve these functions, GABAergic interneurons express a unique pattern of electrical activity and can be easily recognized electrophysiologically from other types of cortical neurons because of their fast-spiking discharge rate.[52] Hence, the fast-spiking cortical GABAergic interneurons must have a prompt and sensitive adaptive mechanism operative in the formation, storage and transfer of information in response to sudden changes in synaptic activity. A possible role for the high levels of DNMT1 found in cortical GABAergic interneurons may be enactment of this fast adaptive mechanism. By facilitating DNA methylation processes in response to transient intrinsic or extrinsic cues, DNMT1 may function to control, via this epigenetic mechanism, transcription of specific genes, such as *GAD1* and *RELN*, which are important in the control of cortical excitability and maintenance of cortical plasticity.[20,22,23,26,29]

DNMT1 mRNA overexpression in PFC of SZP is restricted to a subset of GABAergic interneurons

The human cortex contains a highly heterogeneous population of GABAergic neurons that express different specialized functions. In primate neocortex, the density of GABAergic neurons varies across layers – from 20 to 30% in the deep cortical layers to nearly 100% in layer I.[23,40,54,55] In human PFC (BA9, BA10 and BA46), every GABAergic neuron expresses GAD_{65} and/or GAD_{67}, but very likely not every GABAergic neuron synthesizes and secretes reelin. For example, in layers I and II of primate PFC, the number of GAD_{67}- and reelin-expressing neurons is virtually identical, whereas in layers V and VI, only 33–50% of GAD_{67}-containing neurons express reelin.[40]

One subset of GABAergic interneurons is the burst-spiking cells, which includes horizontal and bitufted cells that are abundant in layers I and II of the cortex.[52,53] Horizontal cells, which are considered the mature form of the embryonic Cajal–Retzius cells, express high levels of reelin[15] and are predominantly located in layer I, where they represent the majority of neurons. Along with bitufted cells, horizontal cells form inhibitory synapses with the apical dendrites of pyramidal neurons extending from their soma in layers II, III, V and VI. In contrast, fast-spiking GABAergic interneurons, such as chandelier and basket neurons, are mostly located in layer III, but are also present in layers V and VI, and innervate the axon initial segments and somata of pyramidal neurons, respectively.[23,52,56]

To investigate the involvement of these distinct cell types in gene expression changes associated with schizophrenia, we measured changes in DNMT1 and GAD_{67} expression in selected populations of GABAergic neurons microdissected from layer I or layer V post-mortem PFC tissue samples obtained from NPS and SZP. In these microdissected cortical layer samples, we made three important observations: (1) DNMT1 mRNA expression is increased approximately threefold in PFC layer I GABAergic neurons of SZP as compared with the same cells of NPS; (2) this increase of DNMT1 is accompanied by a twofold decrease in GAD_{67} mRNA expression; and (3) DNMT1 is not increased and GAD_{67} mRNA levels are not decreased in layer V GABAergic neurons of these patients. In the few samples analyzed, reelin mRNA is also decreased in layer I but not in layer V of SZPs. The increase in DNMT1 and the decrease in GAD_{67} or reelin mRNA expression in BA9 of SZPs are independent of changes in the levels of the neuronal marker NSE mRNA and the housekeeping gene G3PDH mRNA. These data agree with previous studies,[10,11,15] suggesting that in schizophrenia, alterations of DNMT1, GAD_{67} and reelin levels are not owing to changes in neuronal density, but rather reflect bona fide changes in gene expression associated with the psychopathology of the disease.

Previous studies have demonstrated that in schizophrenia, reelin mRNA expression is decreased not only in layer I and II GABAergic neurons of the PFC, but also in other neuronal populations. For example, in the cortex, it has been shown that reelin mRNA expression is downregulated also in interstitial white matter neurons.[17] It remains to be established whether these neurons also express DNMT.[10] Additionally, in the cerebellum, reelin mRNA expression occurs in glutamatergic granule neurons and these cells express decreased levels of reelin mRNA in SZP.[15] It is important to note that in the cerebellum, glutamatergic granule neurons express DNMT.[10] This observation suggests that reelin promoter hypermethylation may be operative in multiple subtypes of reelin-producing neurons, causing a downregulation of reelin expression independent of the neuronal phenotype.

What are the underlying mechanisms responsible for DNMT1 overexpression in a specific subset of cortical GABAergic interneurons in SZP?

The human DNMT1 gene maps to chromosome 19p13.2[7] and linkage studies only weakly implicate markers spanning this chromosome region as susceptibility factors in schizophrenia vulnerability.[57] This, and the fact that in the cortex the overexpression of DNMT1 mRNA is restricted to only certain subsets of GABAergic neurons, makes a DNMT1 DNA sequence mutation as the cause of an alteration of DNMT1 transcription an unlikely possibility. The findings presented here raise the question of why the observed changes in DNMT1 expression in SZP are specific to the GABAergic interneurons of layer I and are not found in the GABAergic interneurons of layer V. One way of conceptualizing how such layer differences in DNMT1 expression in GABAergic cortical neurons may occur is to consider that DNMT1 expression levels may be established epigenetically in embryonic neural progenitor cells, and then inherited by specific subsets of postmitotically differentiated GABAergic neurons in the adult brain. Alternatively, in adult differentiated cortical GABAergic interneurons, overexpression of DNMT1 may be induced after terminal differentiation by the dysfunction of afferent cortico-limbic excitatory inputs to telencephalic GABAergic neurons. A reasonable question to ask is whether the superficial layers of PFC receive excitatory projections that are different from the deeper cortical layers.

The activity of the fast-spiking basket and chandelier cells in layers III and V is regulated by distinct excitatory synaptic inputs to their dendritic shafts by axon terminals projecting to the PFC from neurons in the medio-dorsal nucleus of the thalamus (MD). In contrast, the burst-spiking horizontal and bitufted cells of layers I and II receive on their dendrites both excitatory axon terminal projections from thalamus and cortico-cortical pyramidal neurons[53] and massive excitatory axon terminal projections from the basolateral nucleus of the amygdala.[58–61] It has been suggested that amygdalo-cortical or cortico-cortical excitatory inputs to layer I and II cortical GABAergic interneurons may help to suppress information arriving at distal pyramidal neuron dendrites and den-

dritic spines directly from MD afferent synapses.[52,53] Therefore, as proposed by Benes,[58] and based on the data reported in this study, one can hypothesize that the selective decrease in GABAergic inhibitory function observed in layer I of SZP PFC may be the consequence of a specific disorganization of the complex neuronal afferent network reaching GABAergic interneurons of layers I and II from the amygdala or other cortico-limbic regions.[58–61] In schizophrenia, the disorganization of the MD afferent network reaching the GABAergic interneurons of the deeper cortical layers may be less pronounced or localized to only a small subset of neurons such as the chandelier neurons, which have been shown to be dysfunctional in SZP.[23]

Is DNMT1 overexpression in layer I cortical GABAergic neurons causally related to the decrease of GAD_{67} and reelin?

Although the increase in DNMT1 expression in layer I GABAergic neurons of SZP is associated with the decrease of GAD_{67} and reelin, the results of the present post-mortem brain study cannot directly prove that the increase in the DNMT1 content of layer I GABAergic interneurons is the cause of GAD_{67} and reelin downregulation in these cells. However, this concept is upheld by previous studies of an epigenetic mouse model of schizophrenia[19,62,63] and studies of primary and clonal neuronal cell lines *in vitro*.[64–66] These studies show that in GABAergic neurons, when the level or function of DNMT1 is increased, *GAD1* and *RELN* promoter CpG islands are hypermethylated and the *GAD1* and *RELN* genes are transcriptionally repressed. In contrast, when the action of DNMT1 is abated, for example, by administration of DNMT1 antisense RNA, *GAD1* and *RELN* CpG islands are hypomethylated and the *GAD1* and *RELN* genes are transcriptionally upregulated.[18] Furthermore, studies in SZP suggest that *RELN* promoter hypermethylation is restricted to a subset of cytosine residues in a region of the promoter important in transcription factor binding and RNA polymerase II complex assembly.[12,18] Thus, it is likely that the increase of DNMT1 expression in telencephalic GABAergic neurons of SZPs provides the molecular mechanism for *GAD1* and *RELN* promoter hypermethylation and transcriptional downregulation.

Implications for schizophrenia treatment

Our findings implicate epigenetic alteration of the GABAergic neurons in layer I of the PFC in the pathophysiology of schizophrenia. GABAergic interneurons of layer I are in a unique position in the cortex, which allows them to synapse with apical dendrites extending from the cell bodies of pyramidal neurons in all other layers. GABAergic inhibition of the apical dendrites of pyramidal neurons influences the amplitude of glutamate-mediated dendritic voltage-gated currents and the generation of Ca^{2+}-dependent dendritic action potentials distal from the cell body.[67] These mechanisms are active in modulating the amplitude of excitatory input delivered by thalamic or cortico-cortical fibers impinging upon pyramidal neuron apical dendritic spines.

Dysfunction of GABAergic neurotransmission in layer I, brought about by the hypermethylation of GABA-related promoters by overexpressed DNMT1, suggests two pharmacological routes that may prove beneficial in the treatment of schizophrenia. The first route is to attempt to correct the downregulation of the *RELN* and *GAD1* genes by targeting aberrant epigenetic mechanisms within the nucleus. Drugs currently being evaluated for efficacy in cancer treatment, such as procainamide and zebularine, inhibit the action of DNMT1. Alternatively, histone deacetylase (HDAC) inhibitors may be effective in alleviating the downstream effects of hypermethylation of GABA-related promoters. The long-used drug valproate has recently been shown to be active in the brain as an HDAC inhibitor[62] and has been shown to be beneficial in the treatment of SZP when coadministered with neuroleptic medications.[68,69]

HDACs exist in many distinct isoforms showing tissue-specific expression patterns. Administration of elevated doses of valproate to SZP inhibits multiple HDAC isoforms, increasing with similar efficacy the acetylated histone content of many different brain regions. Other HDAC inhibitors act more selectively, such as the benzamide MS-275. This drug is active as an HDAC inhibitor in the cortex, where its action is especially potent in layer I GABAergic neurons, but it is a very weak HDAC inhibitor in the GABAergic medium spiny neurons of the striatum.[70] Although the concept of differences in the HDAC isoforms expressed by distinct neuronal subtypes within the different layers of the cortex requires further study, previous work supports the possibility of targeting HDAC isoforms specifically expressed by layer I GABAergic neurons with selective HDAC inhibitors.

A second possibility is to correct the layer I-specific deficit in GABAergic neurotransmission resulting from reduced GAD_{67} expression by targeting postsynaptic α_2 and α_5 subunit-containing $GABA_A$ receptors with benzodiazepines that do not act on $GABA_A$ receptors containing α_1 subunits. Several lines of evidence support the suggestion that benzodiazepines acting specifically at α_2 and α_5 subunit containing $GABA_A$ receptors may be better suited for treatment of schizophrenia than clinically used benzodiazepines such as diazepam. By acting on $GABA_A$ receptors containing α_1 subunits, drugs such as diazepam induce side effects including tolerance, dependence, amnesia and sedation.[22,71–73] Drugs that specifically interact with α_2 and α_5 subunit-containing $GABA_A$ receptors and fail to act on $GABA_A$ receptors containing α_1 subunits, such as imidazenil, do not cause tolerance, dependence, sedation, or amnesia, and have been shown to be effective in correcting several schizophrenia-like phenotypes in an epigenetic mouse model of the disease.[19,71] Furthermore, α_5 subunit-containing $GABA_A$ receptors are concen-

trated in the apical dendrites of pyramidal neurons.[74] Thus, selective modulation of α_5 subunit-containing $GABA_A$ receptors with drugs such as imidazenil appears to be ideally suited to rectify the layer I-specific deficit of GABA transmission implicated by our findings in the pathogenesis of schizophrenia.

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