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Epigenetic mechanisms expressed in basal ganglia GABAergic neurons differentiate schizophrenia from bipolar disorder

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Abstract

In the cerebral prefrontal cortex (PFC), DNA-methyltransferase 1 (DNMT1), the enzyme that catalyzes the methylation of cytosine at carbon atoms in position 5 in CpG dinucleotides, is expressed selectively in GABAergic neurons and is upregulated in layers I and II of schizophrenia (SZ) and bipolar disorder patients with psychosis (BDP).

To replicate these earlier findings and to verify whether overexpression of DNMT1 and the consequent epigenetic decrease of reelin and glutamic acid decarboxylase (GAD) 67 mRNA expression also occur in GABAergic medium spiny neurons of the caudate nucleus (CN) and putamen (PT) of SZ and BDP, we studied the entire McLean 66 Cohort (Harvard Brain Tissue Resource Center, McLean Hospital, Belmont, MA) including SZ and BDP, which were matched with nonpsychiatric subjects.

The data demonstrate that in GABAergic medium spiny neurons of CN and PT, unlike in GABAergic neurons of layer I and II PFC, the increased expression of DNMT1 and the decrease of reelin and GAD67 occur in SZ but not in BDP.

This suggests that different epigenetic mechanisms must exist in the pathogenesis underlying SZ and BDP and implies that these disorders might involve two separate entities that are characterized by a well-defined neuropathology.
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1. Introduction

Current studies have consistently shown that the prefrontal cortex (PFC) GABAergic interneurons of psychotic patients [schizophrenia (SZ) and bipolar disorder with psychosis (BDP)] express a downregulation of glutamic acid decarboxylase $(GAD)_{67}$ (one of the two decarboxylases that synthesize GABA) and reelin (an extracellular matrix protein that is preferentially synthesized and secreted by GABAergic interneurons) (Benes et al., 1992; Akbarian et al., 1995; Impagnatiello et al., 1998; Guidotti et al., 2000; Fatemi et al., 2000; Eastwood and Harrison, 2003; Woo et al., 2004; Lewis et al., 2005). Upon secretion into the extracellular matrix, reelin adheres to the dendritic shafts and surrounds dendritic spines of cortical pyramidal neurons. This protein, perhaps by impinging on synaptically located

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integrin receptors, modulates event-related protein synthesis and may influence dendritic spine expression density (Costa et al., 2001; Liu et al., 2001; Dong et al., 2003), markedly changing LTP and cognitive function expression (Larson et al., 2003; Carboni et al., 2004; Beffert et al., 2005; Qiu et al., 2006).

Reelin and GAD_{67} promoters are embedded in large CpG islands and express methylation consensuses (Grayson et al., 2005). In the PFC of SZ patients, a decrease of reelin expression was associated with cytosine hypermethylation in the promoter region of the gene encoding for this protein (Grayson et al., 2005; Abdolmaleky et al., 2005). The regulatory role played by promoter CpG island methylation in the expression level of reelin can be inferred by an increase by up to 80 fold occuring the human reelin promoter following hypomethylation (Chen et al., 2002).

In the PFC of SZ and BDP patients, we have also quantified the expression of DNA methyltransferase 1 (DNMT1), which catalyzes the methylation of the carbon atom in position 5 of cytosines in CpG dinucleotides of various gene promoter regions of GABAergic neurons. We found that in these PFC neurons, DNMT1 is highly expressed whereas this enzyme cannot be detected in pyramidal neurons (Veldic et al., 2004; Ruzicka et al., in press). Moreover, DNMT1 expression is increased in a subset of cortical GABAergic interneurons in SZ and BDP patients. For example, it is increased in cortical layers I, II, and IV GABAergic neurons but not in GABAergic neurons of layers III, V and VI (Veldic et al., 2005; Ruzicka et al., in press). In PFC GABAergic neurons of SZ and BDP, the extent of the DNMT1 increase was accompanied by a related decrease of reelin and GAD_{67} expression (Veldic et al., 2004, 2005). Hence, these studies suggest that the downregulation of GAD_{67} and reelin, or that of other genes expressed in cortical GABAergic neurons of psychotic patients, may be mediated by a 5-cytosine hypermethylation of the promoter CpG dinucleotides elicited by the increased expression of DNMT1 (Grayson et al., 2006).

Postmortem studies of human brain suggest that SZ may be associated with a GABAergic neuron downregulation detected not only in the cortex but also in the striatum (Impagnatiello et al., 1998). High affinity binding studies with [^{3}H]muscimol show an increase in the number of $GABA_A$ recognition sites in the caudate nucleus (CN) of SZ patients (Hanada et al., 1987). In addition the expression levels of reelin mRNA are decreased by more than 70% in the CN of SZ patients compared to matched nonpsychiatric subjects (NPS) (Impagnatiello et al., 1998). In previous studies, we have shown that CN GABAergic neurons of SZ patients overexpress DNMT1 whereas reelin expression is downregulated (Veldic et al., 2004).

The goal of the present study is to replicate in the McLean 66 Cohort (Harvard Brain Tissue Resource Center, McLean Hospital, Belmont, MA) earlier findings of a DNMT1 mRNA increase in GABAergic neurons in Brodmann's area (BA) 9 and BA10 (Veldic et al., 2004, 2005; Ruzicka et al., in press) and to verify whether there is an overexpression of DNMT1 and a parallel downregulation of reelin and GAD_{67} in CN and putamen (PT) medium spiny GABAergic neurons, in SZ and BDP patients.

2. Experimental/materials and methods

2.1. Tissue collection

Tissue samples isolated from CN, PT, and BA9 of NPS, SZ or BDP patients were obtained from the Harvard Brain Tissue Resource Center, McLean Hospital, Belmont, MA (Table 1). All specimens were fixed in 4% formaldehyde. RNA quality was established by the

Table 1
Demographic characteristics of brain samples[a, b] obtained from the Harvard Brain Tissue Resource Center, McLean Hospital, Belmont, MA (The McLean 66)

	PATIENT COHORT		
	NPS (n=27)	SZP (n=20)	BP (n=14)
Male/female	19/8	13/7	7/7
Hemisphere (L/R)	15/12	12/8	9/5
Age/years	58±18	56±18	64±16
Postmortem interval/h	21±5.8	21±5.5	21±9.5
Fixation days × 10^3	1.8±0.6	2.2±0.4	2.0±0.6
Brain pH	6.4±0.3	6.4±0.3	6.4±0.2
3′/5′ G3PDH ratio	1.5±0.4	1.6±0.6	1.6±0.8
3′/5′ β-actin ratio	2.3±0.9	2.4±0.8	2.7±1.3
RNA 28S/18S ratio	1.1±0.3	1.1±0.5	1.0±0.3
% present call	46±4.0	46±4.1	44±7.1
Age of illness onset/years	–	23±9.9	38±17
Duration of illness/years	–	34±18	28±18
% Suicide	0	16	21
% Abuse or dependence	0	47	43
% CNS medications			
Atypical antipsychotics	0	32	43
Typical antipsychotics	0	37	29
Typical and atypical	0	5	0
Valproate	0	16	71

[a]Formaldehyde fixed BA9; The values are mean±SD; L = left; R = right; NPS = nonpsychiatric subjects; SZP = schizophrenia patients; BP = bipolar disorder. [b]The psychiatric diagnoses were established by two senior psychiatrists based on clinical and family histories and according to Feighner et al. (1972) for SZ and The American Psychiatric Association (1994) *Diagnostic and Statistical Manual of Mental Disorders IV* criteria for BDP.

Table 2
Detailed demographic characteristics of the Harvard Brain Tissue Resource Center Study Group

a	b	c	d	e	f	g	h	i	j	k	l
Nonpsychiatric subjects											
M	66	19	6.8	1.1	1.4	1.5	44	–	1	–	–
M	40	28	6.5	1.7	3.4	0.5	46	–	3	–	–
M	69	15	6.9	1.3	2.8	1.5	44	–	1	–	–
F	78	14	6.2	1.6	2.5	1.3	42	–	1	–	–
M	36	24	6.3	1.5	2.3	1.1	44	–	1	–	–
M	37	19	6.7	1.2	1.4	1.3	49	–	3	–	–
M	49	25	6.8	1.0	1.5	1.4	45	–	1	–	–
M	29	18	7.1	1.2	1.8	1.3	44	–	5	–	–
F	53	24	5.8	2.3	3.6	0.6	44	–	2	–	–
M	40	17	6.2	1.2	2.0	1.2	48	–	1	–	–
F	74	12	6.3	1.5	2.3	0.8	45	–	5	–	–
M	54	24	6.6	1.0	1.5	1.3	53	–	1	–	–
M	36	20	6.0	1.6	2.3	0.9	48	–	1	–	–
F	70	22	6.3	2.3	3.7	0.4	37	–	2	–	–
M	67	22	6.4	1.1	1.7	1.4	48	–	1	–	–
M	42	18	6.8	1.0	1.4	1.1	54	–	1	–	–
M	79	21	6.7	1.5	2.8	0.6	49	–	2	–	–
F	78	24	6.7	1.4	2.3	0.9	48	–	2	–	–
M	38	29	6.5	1.1	1.5	1.5	49	–	1	–	–
F	65	24	6.4	1.1	1.8	1.3	52	–	5	–	–
F	66	7	6.0	1.5	2.5	0.6	47	–	2	–	–
M	89	7	6.4	1.5	2.4	1	47	–	2	–	–
M	35	26	6.3	1.2	1.4	1.4	40	–	5	–	–
M	50	24	6.0	2.1	3.1	1.1	42	–	5	–	–
M	84	29	6.4	2.2	5.0	0.5	44	–	1	–	–
M	73	20	6.0	1.5	2.5	1	40	–	5	–	–
Schizophrenia patients											
M	66	22	6.4	1.0	1.4	1.8	48	20	1	H	Y
F	83	23	5.9	2.0	3.5	0.2	42	55	3	H	Y
M	44	19	6.0	3.0	4.0	0.9	34	20	1	Cz	N
M	35	28	6.2	1.6	2.1	1	46	31	1	UN	N
M	46	18	6.3	3.2	2.6	1	45	19	3	V, O	Y
M	26	16	6.7	1.3	2.6	1.6	50	23	4	F	Y
M	42	27	6.6	1.0	2.0	1.2	45	16	2	NT	Y
M	47	19	6.6	2.0	4.0	1.9	47	17	2	NT	N
M	31	15	6.5	1.2	2.0	1.1	49	19	5	R, O, Bp	Y
M	80	11	6.4	1.4	1.8	0.5	41	17	1	Th, Mr	N
M	49	19	6.6	1.0	1.2	1.8	50	19	4	H	Y
M	61	20	6.7	1.5	2.2	1.5	46	25	3	H	N
F	73	24	6.1	1.6	3.0	0.5	47	23	2	R, Fx	N
M	63	22	6.5	1.1	2.8	1.3	50	7	1	Cz, Ta, H	N
F	72	22	6.6	1.2	2.0	0.7	50	24	2	R, Pa	N
F	84	26	6.1	1.7	2.8	0.5	40	34	1	V	N
M	42	18	6.3	1.3	2.1	1	46	27	4	Ta	Y
F	78	13	6.8	1.5	2.3	1.2	46	16	3	L, H	N
F	48	34	6.6	1.2	1.5	1.3	47	17	1	V, R	Y
Bipolar patients with psychosis											
F	64	11	6.7	1.0	1.7	1.3	51	20	1	V, Cr, T, D	N
M	38	41	6.5	1.0	1.2	1.4	43	37	4	Se, L, V	N
F	80	12	6.5	3.0	4	1	27	38	3	Pe, V	N
F	42	16	6.3	1.1	1.7	1.3	52	20	4	V, L, Pe	Y
M	78	30	6.3	1.9	4.2	0.7	42	49	1	Ch, F, V, L	N
F	76	23	6.6	1.9	3.8	0.5	42	67	1	L	Y
Bipolar patients with psychosis											
M	74	25	6.5	1.4	3.4	0.8	47	50	1	V, Q	Y
M	74	7	6.7	2.9	4.1	1.3	33	72	1	O	N
F	55	18	6.5	–	–	0.7	47	40	2	V	Y
F	73	21	6.3	1	1.6	1.2	51	25	3	R	N
M	74	14	6.3	1.9	2.7	0.5	38	18	1	O, L, V	N
F	73	17	6.4	2.0	3.5	0.5	42	25	3	V, R, Se	N
M	40	31	6.0	1.3	2.4	0.6	48	25	4	R, Nf, Z	Y
M	83	17	6.6	1.6	3	1.3	47	43	1	V, Pa	Y

a = Gender; b = Age (years); c = Postmortem interval — interval between death and brain fixation in formaldehyde (h); d = Brain pH; e = 3′/5′ G3PDH ratio; f = 3’/5’ -actin ratio; g = 28 s/18 s RNA ratio; h = percent probe sets present; i = Age of onset of illness (years); j = Cause of death: 1 — cardiopulmonary failure; 2 — cancer; 3 — other; 4 — suicide; 5 — unknown. k = Medication: Bp — buproprion; Cr — carbamazepine; Ch — chlorpromazine; Cz — clozapine; D — doxepin; Fx — fluoxetine; F — fluphenazine; H — haloperidol; L — lithium; Mr — mirtazapine; Nf — nefazodone; O — olanzapine; Q — quetiapine; Pa — paroxetine; Pe — perphenazine; P — phenelzine; R — risperidone; Se — sertraline; Th — thioridazine; Ta — trazodone; T — trifluoperazine; V — valproic acid; Z — ziprasidone. UN = never treated; NT = not treated six months or more before death; UK = unknown; l = Abuse or dependency history: Y — yes; N — no.

Harvard Brain Tissue Resource Center, McLean Hospital, Belmont, MA (http://www.brainbank.mclean.org/) (Table 2). The brain was cut rostro-caudally in 0.5-cm-thick slices along the coronal axis, starting from the frontal pole. We received Section 6 for the CN and PT and Section 3 for BA9.

2.2. In situ hybridization and immunocytochemistry

Free-floating 40-μm sections were incubated for 48 h with a mixture of 50 pmol/ml of antisense oligonucleotides (Rodriguez et al., 2002; Veldic et al., 2004) using probes complementary to bases a) 1627–1650 and 4801–4824 of the human DNMT1 cDNA (GenBank accession no. NM_001379); b) 1729–1752, 5505–5528, and 10102–10125 of the human reelin cDNA (GenBank accession no. NM_005045); c) 885–908 and 1661–1684 of the human GAD_{65} cDNA (GenBank accession no. NM_000818); and d) 1063–1086 and 2674–2697 of the human GAD_{67} cDNA (GenBank accession no. NM_000817).

These nucleotides failed to match either DNMT3a or DNMT3b sequences or any other known mRNA sequence and were selected according to the criteria reported by Veldic et al. (2004).

Neuron-specific nuclear protein (NeuN) immunolabeling was studied in 40 μm floating sections incubated with a mouse anti-NeuN monoclonal antiserum (Chemicon,

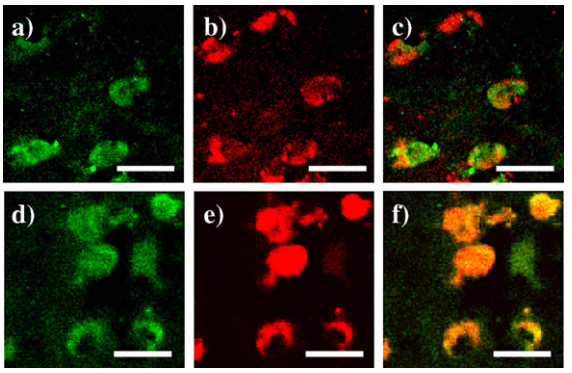


Fig. 1. Colocalization of DNMT1mRNA and $GAD_{65/67}$ immunoreactivity in CN of NPS and SZ patients. (a) DNMT1 mRNA-positive neurons in CN of NPS, color-coded in green. (b) $GAD_{65/67}$ immunoreactivity in CN of NPS, color-coded in red. (c) Merge of A and B. (d) DNMT1 mRNA-positive neurons in CN of SZ patient, color-coded in green (e) $GAD_{65/67}$ immunoreactivity in CN of SZ patient, color-coded in red. (f) Merge of A and B (scale bar, 20 μm).

Temecula, CA) diluted 1:500 as previously described (Veldic et al., 2004).

2.3. Confocal fluorescence microscopy

After DNMT1 mRNA in situ hybridization was completed, sections were processed for immunohistochemistry with antibodies directed against $GAD_{65/67}$ (Chemicon, Temecula, CA, 1:2000) (Veldic et al., 2004).

Cy-5-conjugated goat anti-rabbit IgG (Amersham Biosciences, Piscataway, NJ, 1:1000) were used to label the antibodies that reacted with $GAD_{65/67}$. Cy2-conjugated streptavidin (1:1000) was used to label DNMT1 mRNA. After washing, the sections were incubated in 10 mM $CuSO_4$ and 50 mM CH_3COONH_4 for 30 min to eliminate interference by lipofuscin-mediated autofluorescence (Veldic et al., 2004).

2.4. Three-dimensional counts

The cell counts were performed as described by Veldic et al. (2004). The reproducibility and reliability of our in situ histochemical procedures was established as reported by Veldic et al. (2005).

In BA9, the definition of the border separating layer III from layer IV may not always be distinct because this layer contains large pyramidal cells intermixed with small granular cells (Rajkowska and Goldman-Rakic, 1995). To avoid confusion regarding layer III boundaries and to optimize our counting reproducibility, we adopted the procedure described by Volk et al. (2000) in which the borders of the lower part of layers III and IV were designated as layers III–IV.

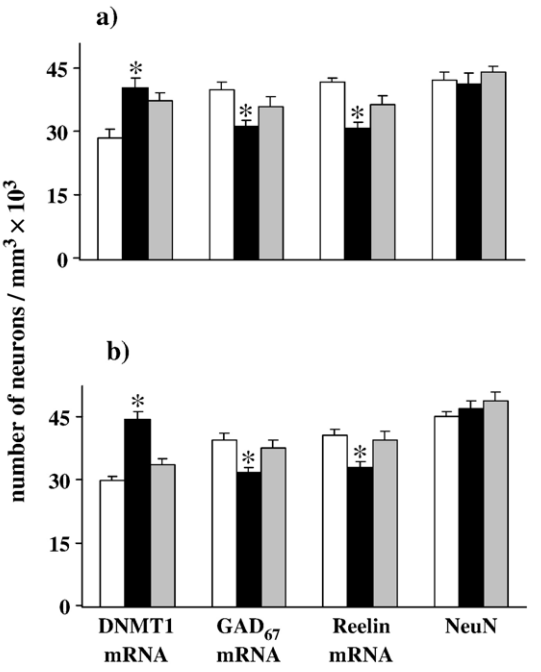
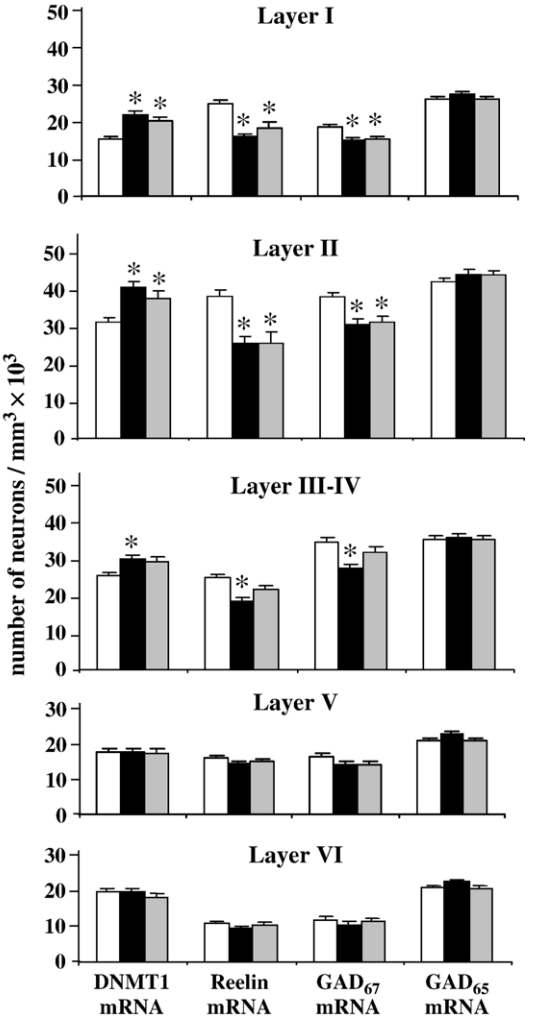


Fig. 2. Density of DNMT1, reelin, GAD_{67} mRNAs, and NeuN protein immunoreactive neurons in CN and PT from NPS and psychiatric patients. □ NPS; ■ SZ; ▩ BDP. **a)** *Caudate nucleus (CN)*. Each bar represents the mean±SE of 27† NPS, 20 SZ and 14† BDP patients. *, asterisk indicates a statistically significant difference at $P \leq 0.013$ vs. NPS. ***DNMT1 mRNA positive neurons**: ANOVA ($F_{2,56}=9.1$, $P<0.001$); Bonferroni post hoc analysis ($P<0.001$ of NPS vs. SZP and $P=NS$ of NPS vs. BP);**reelin mRNA positive neurons**: ANOVA ($F_{2,54}=20.1$, $P<0.001$); Bonferroni post hoc analysis ($P<0.001$ of NPS vs. SZP and $P=NS$ of NPS vs. BP);**GAD_{67} mRNA positive neurons**: ANOVA ($F_{2,55}=4.8$, $P=0.012$); Bonferroni post hoc analysis ($P=0.009$ of NPS vs. SZP and $P=NS$ of NPS vs. BP); NeuN: $P=NS$.* †On closer inspection of the data, it was noticed that the density of the reelin mRNA-expressing neurons in one NPS and one BDP patient was 3 SD above the mean density, GAD_{67} mRNA-expressing cells in one NPS was also 3 SD above the mean density. These cases were therefore excluded from the numerical density comparisons reported herein. **b)** *Putamen (PT)*. Each bar represents the mean±SE of 27 NPS, 20 SZ and 14 BDP‡ patients. The difference between SZP and BDP patients with psychosis versus NPS was calculated by ANOVA and P values were evaluated by Bonferroni multiple comparisons (t test). *, asterisk indicates a statistically significant difference at $P \leq 0.013$ vs. NPS. ***DNMT1 mRNA positive neurons**: ANOVA ($F_{2,55}=34.0$, $P<0.001$); Bonferroni post hoc analysis ($P<0.001$ of NPS vs. SZP and $P=NS$ of NPS vs. BP);**reelin mRNA positive neurons**: ANOVA ($F_{2,56}=7.4$, $P=0.001$; Bonferroni post hoc analysis ($P=0.002$ of NPS vs. SZP and $P=NS$ of NPS vs. BP);**GAD_{67} mRNA positive neurons**: ANOVA ($F_{2,56}=7.1$, $P=0.002$); Bonferroni post hoc analysis ($P=0.001$ of NPS vs. SZP and $P=NS$ of NPS vs. BP); NeuN: $P=NS$.* ‡On closer inspection of the data, it was noticed that the numerical density of the DNMT1 mRNA-expressing cells in one BP patient was 3 SD above the mean density. This case was therefore excluded from the average numerical density comparisons reported herein.

To count strongly stained cells and exclude weakly stained cells or non-specifically-labeled cells from the analysis, the threshold intensity of staining was established at 3× the background (measured by Leica Confocal Software).

2.5. Statistical analyses

Statistical analyses were performed using univariate or repeated measures analysis of variance (ANOVA) or covariance (ANCOVA) with Bonferroni corrections and the identification of diagnostic groups (controls versus patients, schizophrenics versus bipolars) using logistic regression on the SPSS and SAS statistical packages. Since we were examining DNMT1, GAD_{67}, and reelin mRNAs we used a Bonferroni procedure to correct for type I error and therefore for all *t* tests we accepted as significance $P<0.013$ (i.e., $.05 \div 3$).



3. Results

3.1. Medium spiny neurons of CN and PT

3.1.1. CN

Fig. 1 (a,b,c) shows that in the CN of the NPS group several but not all $GAD_{65/67}$-containing neurons express detectable amounts of DNMT1 mRNA. In contrast, in the CN of SZ patients (Fig 1d,e,f), almost every $GAD_{65/67}$-positive neuron is DNMT1 mRNA-positive.

Three-dimensional counting established that the average number of DNMT1 mRNA-positive neurons is increased in the SZ group by ~40% compared to the corresponding number of DNMT1-positive neurons in NPS (Fig. 2a). In contrast in subjects with BDP, statistical analyses fail to demonstrate a significant increase in the number of DNMT1 mRNA positive neurons (Fig. 2a). The number of NeuN positive neurons is virtually identical in SZ, BDP, and NPS (Fig. 2a).

In the CN of NPS, more than 90% of the NeuN-positive neurons express reelin and GAD_{67} mRNAs

Fig. 3. DNMT1 upregulation is associated with reelin and GAD_{67} downregulation in BA9 neurons of SZ and BDP patients. □ NPS; ■ SZ; ▣ BDP. *, asterisk indicates a statistically significant difference at $P \leq 0.013$ vs. NPS. Each bar represents the mean±SE of three-dimensional counts of DNMT1, reelin, GAD_{67}, and GAD_{65} mRNA-positive neurons in various layers of BA9 from NPS ($N=26$), SZ ($N=19$), and bipolar BDP ($N=14$) patients. *Denotes a statistically significant difference when SZ or BDP disorder groups are compared with NPS. ***DNMT1 mRNA positive neurons***: *Layer I [ANOVA ($F_{2,56}=17.4$; $P<0.001$); Bonferroni post hoc analysis ($P<0.001$ of NPS vs. SZP and $P=0.001$ of NPS vs. BP)]; Layer II [ANOVA ($F_{2,56}=12.2$; $P<0.001$)); Bonferroni post hoc analysis ($P<0.001$ of NPS vs. SZP and $P=0.010$ of NPS vs. BP)]; Layer III–IV [ANOVA ($F_{2,56}=6.4$; $P=0.003$); Bonferroni post hoc analysis ($P=0.004$ of NPS vs. SZP and P=NS of NPS vs. BP)]; Layer V (NS); Layer VI (NS). None of the conclusions derived from ANOVA were affected by including confounding variables in the analysis.* ***Reelin mRNA positive neurons***: *Layer I [ANOVA ($F_{2,56}=24.1$; $P<0.001$); Bonferroni post hoc analysis ($P<0.001$ of NPS vs. SZP and $P<0.001$ of NPS vs. BP)]; Layer II [ANOVA ($F_{2,56}=15.0$; $P<0.001$)); Bonferroni post hoc analysis ($P<0.001$ of NPS vs. SZP and $P<0.001$ of NPS vs. BP)]; Layer III–IV [ANOVA ($F_{2,56}=12.5$; $P<0.001$); Bonferroni post hoc analysis ($P<0.001$ of NPS vs. SZP and P=NS of NPS vs. BP)]; Layer V (NS); Layer VI (NS).* ***GAD_{67} mRNA positive neurons***: *Layer I [ANOVA ($F_{2,56}=10.4$; $P<0.001$); Bonferroni post hoc analysis ($P<0.001$ of NPS vs. SZP and $P=0.003$ of NPS vs. BP)]; Layer II [ANOVA ($F_{2,56}=16.3$; $P<0.001$)); Bonferroni post hoc analysis ($P<0.001$ of NPS vs. SZP and $P<0.001$ of NPS vs. BP)]; Layer III–IV [ANOVA ($F_{2,56}=10.6$; $P<0.001$)); Bonferroni post hoc analysis ($P<0.001$ of NPS vs. SZP and P=NS of NPS vs. BP)]; Layer V [ANOVA ($F_{2,56}=3.7$; $P=0.031$); NS when confounding variables were taken into account]; Layer VI (NS).* ***GAD_{67} mRNA positive neurons***: *Layer I (NS); Layer II (NS); Layer IIIs (NS); Layer III–IV (NS); Layer V (NS); Layer VI (NS).*

(Fig. 2a). The number of reelin mRNA positive neurons decreases by 26% in SZP but virtually does not change when BDP counts are compared to NPS (Fig. 2a). The number of GAD_{67} mRNA-positive neurons is also significantly decreased in SZ (21%), but not in BDP (Fig. 2a).

3.1.2. PT

Fig. 2b shows that in the PT, the number of DNMT1 mRNA-positive neurons is increased (~49%) and the number of neurons expressing reelin and GAD_{67} mRNAs is decreased in the SZ group. In contrast, the number of DNMT1, reelin and GAD_{67} mRNA positive neurons fails to change in the BDP patient group (Fig. 2b).

In SZ and BDP patients, there is no difference between groups in the number of NeuN positive neurons (Fig. 2b). We also compared the number of DNMT1, GAD_{67}, and reelin mRNA-positive neurons in SZ versus the BDP group. The increase in DNMT1 and the decrease of reelin and GAD_{67} mRNA comparing SZP and BDP were statistically different; DNMT1 ($P<0.001$), GAD_{67} ($P=0.01$), and reelin ($P=0.01$) (Student's *t*-test).

3.2. Prefrontal cortex BA9

As shown in Fig. 3, changes in DNMT1, GAD_{67}, and reelin ***mRNA*** expression in BA9 are cortical layer-and disease-specific: a) the number of DNMT1 mRNA-positive neurons is increased in layers I and II of SZ and BDP patients compared to NPS, b) in layer III–IV, the increase in DNMT1 mRNA-positive neurons is smaller than in layers I and II and occurs in SZ but not in BDP, c) a difference in the number of DNMT1 mRNA-positive neurons failed to be detected in the layers V and VI of both SZ or BDP compared to NPS.

Fig. 3 also shows that the counts of reelin and GAD_{67} mRNA-positive neurons are lower in layers I and II of SZ or BDP compared to NPS.

The number of reelin and GAD_{67} mRNA positive neurons is decreased in layers III–IV of SZ but not in BDP compared to NPS. Moreover in layers III–IV, the decrease of reelin and GAD_{67} mRNAs in SZ patients was significantly different from BDP using the Student's t-test (reelin mRNA: $P=0.047$; GAD_{67} mRNA: $P=0.01$).

Importantly, in cortical layer I, which virtually express only GABAergic neurons (Gabbott and Somogyi, 1986) and where the changes of DNMT1, reelin and GAD_{67} expression are more pronounced than in other layers, there is a negative correlation between the densities of DNMT1 and that of GAD_{67} mRNA-positive neurons or reelin mRNA-positive neurons. (Fig. 4). In contrast, there is a positive correlation between the densities of reelin and GAD_{67} mRNAs positive neurons (Fig. 5).

No differences between the patient and NPS groups were detected in the number of GAD_{65} mRNA-positive neurons (Fig. 3) or in the number of NeuN-immunopositive neurons in every cortical layer of BA9 (not shown).

3.3. Confounding variables

We tested whether background variables or other possible confounding variables may have influenced the results: 1) Average values for the confounding variables listed in Table 1 were similar in all three groups. 2) Repeated measure ANCOVAs of various cortical layers, CN, and PT with confounding variables as covariates or factors (gender, hemisphere) failed to show any significant direct differences between the three groups of the four outcome variables. However there was an exception, the counts of GAD_{67} mRNA-expressing neurons were weakly associated with PMI in most areas, when examined individually by linear regression, none of these correlations were significant. Most likely this occurred by chance alone due to the large number of

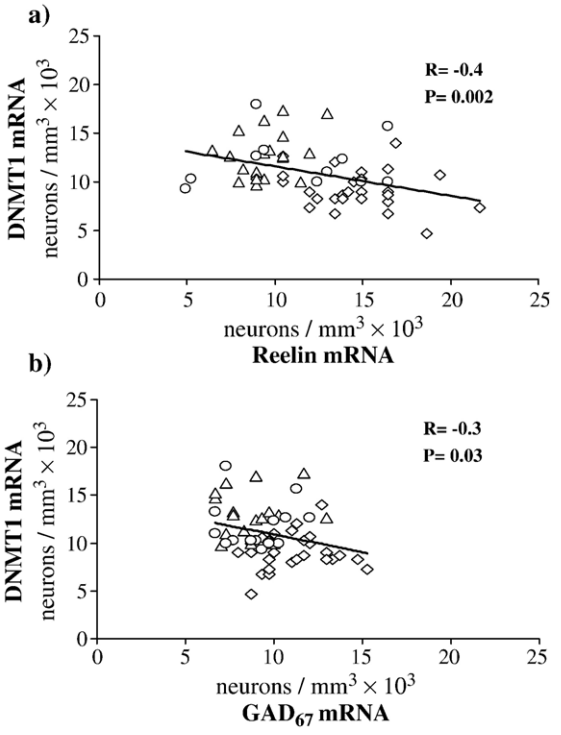


Fig. 4. Pearson correlation between counts of DNMT1 mRNA and reelin mRNA ($R=-0.4$; $P=0.002$) (a) or GAD_{67} mRNA ($R=-0.3$; $P=0.03$) (b) positive neurons in layer I BA9 of ◇ NPS; △ SZ; and ○ BDP.

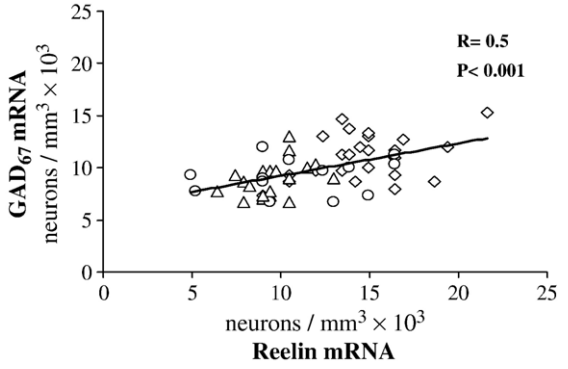


Fig. 5. Pearson correlation between counts of reelin mRNA and GAD_{67} mRNA positive neurons in layer I BA9 of SZ, BDP, and NPS. ◇ NPS; △ SZ; and ○ BDP ($R=0.5$; $P<0.001$).

confounding variables considered. Furthermore, the measures of RNA quality did not alter the results. 3) More importantly, we failed to find that any of the confounding variables differentially affected the difference between the diagnostic groups on any of tested variables.

In our cohort, several patients had a current history of alcohol or other substance abuse. The neuronal counts of DNMT1, reelin, GAD_{65}, GAD_{67} mRNAs, or NeuN were virtually identical whether the subjects with a history of substances of abuse were included or excluded from the analysis, neither did our ANCOVA find any significant change for this variable.

Increases in DNMT1 and decreases in reelin and GAD_{67} mRNA-positive neurons in SZ and BDP patients also failed to correlate with antipsychotic drug dosage or type of antipsychotic treatment.

4. Discussion

4.1. DNMT1 is overexpressed in GABAergic neurons of caudate/putamen from SZ but not from BDP patients

From the study of DNMT1 mRNA expression in the CN, PT, and BA9 of the entire McLean 66 cohort, it was found that in the telencephalon: a) DNMT1 was exclusively expressed in GABAergic neurons, b) in SZ patients, the number of GABAergic neurons expressing DNMT1 mRNA was increased not only in BA9 layers I, II, III–IV, but also in CN and PT, and that c) in BDP, the number of DNMT1 mRNA-positive neurons was increased in layers I and II of BA9, but not in GABAergic neurons of CN, PT, or layers III–IV in BA9.

Thus although in PFC (BA9), both SZ and BDP may have an epigenetically-mediated neuropathology of GABAergic neurons as a common denominator, in the basal ganglia, GABAergic neurons appear to overexpress DNMT1 and underexpress reelin and GAD_{67} only in SZ patients.

These differences between SZ and BDP are consistent with previous reports suggesting that GABAergic neurons located in the upper layers of the PFC may exhibit a similar neuropathology in SZ and BDP, whereas in the GABAergic neurons of the lower layers, this neuropathology is specific to SZ (Volk et al., 2000; Lewis et al., 2005) but not to BDP (Woo et al., 2004).

Because of the findings presented here, one must question why the changes in DNMT1 expression in SZ and BDP are selective in specific subsets of GABAergic neurons.

To relate neurochemical changes occurring in selected populations of GABAergic neurons to the psychopathology in SZ and in BDP, it is important to consider the characteristic network in which different populations of GABAergic neurons operate. In the PFC, cortical GABAergic neurons of layers I and II receive excitatory afferent inputs primarily from amygdala and in part from the mediodorsal nucleus of the thalamus (Barbas and De Olmos, 1990; Woo et al., 2004) whereas GABAergic interneurons of layers III–IV, V, and VI receive their excitatory inputs mostly from the mediodorsal thalamic cortical afferents (Rotaru et al., 2005). Several lines of evidence suggest that neuropathologies in the thalamus (Popken et al., 2000) and amygdala (Aleman and Kahn, 2005) contribute to SZ morbidity. Hence, abnormal function of the amygdala afferents in the cortex could be responsible for the overexpression of DNMT1 and the consequent downregulation in the expression of GAD_{67} and reelin in layer I and II GABAergic neurons in SZ and BDP.

GABAergic medium spiny neurons represent at least 95% of the total neuronal population in the CN and PT (Tisch et al., 2004). In primates, the CN receives cortical afferents primarily from PFC association areas (BA9, BA10, BA46), whereas somatosensory cortical areas encompassing BA 1–3 and motor cortical areas (BA 4–6) send projections primarily to the PT (Kemp and Powell, 1970; Kunzle, 1975; Selemon and Goldman-Rakic, 1985; Parent and Hazrati, 1995).

Cortical afferents in the CN and PT may play a critical role in integrating diverse types of information derived from prefrontal associative or sensory motor cortices. Hence, disturbances in information processing at cortico-CN and cortico-PT neuronal circuits might contribute to the differences in the expression of DNMT1 observed in the basal ganglia of SZ versus BDP.

A recent study (Benes et al., 2006) reported that the expression of preapoptotic genes is increased in bipolar disorder but not in SZ postmortem brains. These and

other related data support the view that fundamental differences must exist in the pathogenetic alterations underlying SZ and BDP. Thus, the differences between SZ and BDP in DNMT1, reelin, and GAD_{67} expression in the GABAergic neurons of various cortical layers and basal ganglia might not be the consequence of a continuum of disease severity but rather a characteristic feature of the differential pathogenetic mechanisms associated with the neuropathology and symptomatology of BDP as distinguished from SZ.

4.2. Is DNMT1 overexpression in telencephalic GABAergic neurons of SZ and BDP causing an epigenetic downregulation of GAD_{67} and reelin expression?

Our study shows that in GABAergic neurons of the CN, PT, and BA9, when DNMT1 mRNA expression is increased there is a corresponding decrease of reelin and GAD_{67} expression (Figs. 2–4). However, in the CN and PT of BDP, the number of GABAergic neurons expressing DNMT1 mRNA failed to change significantly and the expression of reelin and GAD_{67} also failed to change.

The hypothesis that the relationship between DNMT1 overexpression and GAD_{67} and reelin downregulation in GABAergic neurons is cause-related is suggested by the existence of a statistically significant inverse correlation between the increase of DNMT1 and the decrease of reelin or GAD_{67} mRNA positive neurons demonstrated in layer I of SZ, BDP, and NPS (Fig. 4). This hypothesis is further supported by studies of Tremolizzo et al. (2002, 2005) and Dong et al. (2005) in an epigenetic mouse model of SZ, and by studies of Chen et al. (2002), Mitchell et al. (2005), and Noh et al. (2005), in primary and clonal neuronal cell lines in vitro. These studies show that when the level of DNMT1 is increased and presumably hypermethylation occurs in GAD_{67} and reelin promoter CpG islands, GAD_{67} and reelin genes are transcriptionally repressed. In contrast, when the action of DNMT1 is abated, for example by administration of DNMT1 antisense RNA, GAD_{67} and reelin promoter CpG islands are hypomethylated and GAD_{67} and reelin are transcriptionally upregulated (for a review Grayson et al. (2006)). Furthermore, studies in SZ patients suggest that reelin promoter hypermethylation is restricted to a subset of cytosine residues in a specific promoter region important for the binding of the complex assembly of transcription factors and the RNA polymerase II (Grayson et al., 2005). Thus, it is likely that the increase of DNMT1 expression in telencephalic GABAergic neurons of SZ and BDP patients represents the molecular mechanism for GAD_{67} and reelin promoter hypermethylation and the consequent GAD_{67} and reelin transcription downregulation.

4.3. The overexpression of DNMT1 in BA 9 and basal ganglia GABAergic neurons of SZ or BDP does not bring about an indiscriminate downregulation of all the genes expressed in these neurons

Although in addition to GAD_{67} and reelin, other genes such as NR2A (Woo et al., 2004) and GAT1 (Lewis et al., 2005), are downregulated in GABAergic neurons of SZ patients, GAD_{65} mRNA and protein levels are not altered in the BA 9, CN, and PT of SZ or BDP (Fig. 3). Interestingly, the GAD_{65} promoter is embedded in CpG islands (Costa et al., 2002) and therefore, an increased expression of DNMT1 in GABAergic neurons would predict GAD_{65} promoter hypermethylation and GAD_{65} mRNA and protein expression downregulation. Based on this possibility, we can speculate that the unexpected lack of GAD_{65} downregulation in the same neurons in which epigenetic promoter hypermethylation likely induces reelin and GAD_{67} expression downregulation: a) the GAD_{65} promoter, unlike *RELN* and GAD_{67} promoters, is already hypermethylated in NPS and therefore in SZ and BDP patients its expression cannot be further downregulated by DNMT1 overexpression; and b) DNMT1 fails to target GAD_{65} promoters due to the inhibitory role of specific/local chromatin factors, such as histone modifying enzymes, chromatin remodeling proteins, and the protein complexes, including the repressor MeCP2 proteins, which alter the affinity of DNMT1 for *S*-adenosylmethionine and CpG island promoters (Jenuwein and Allis, 2001; Robertson et al., 2004; Hong et al., 2005). This consideration suggests study of whether the expression of other gene promoters rich in CpG islands is also downregulated in GABAergic neurons showing elevated levels of DNMT1.

4.4. Conclusions

It is well established that telencephalic (PFC, CN and PT) GABAergic neurotransmission is involved in various aspects of normal psychomotor behavior and if altered, the change in GABAergic transmission may contribute to the severity of the clinical morbidity in SZ and BDP (Hanada et al., 1987; Impagnatiello et al., 1998; Benes and Berretta, 2001; Tekin and Cummings, 2002; Guidotti et al., 2005; Lewis et al., 2005). The data presented here support the view that the dysfunction of GABAergic neurotransmission (including the downregulation of reelin and GAD_{67}) in BA9, CN, and PT of SZ is brought

about by the hypermethylation of gene promoters in GABAergic neurons elicited by the overexpression of DNMT1. Since it is evident that the extent of the DNMT1 increase and of the reelin or GAD_{67} expression decrease in SZ and BDP is cortical layer and brain region-selective, it is suggested that a study of the alterations of the afferent inputs (glutamatergic, cholinergic, or dopaminergic) to telencephalic GABAergic neurons may help to clarify the pathogenetic mechanisms that differentiate the epigenetic nature of the dysfunction operative in SZ and BDP and perhaps lead to specific models in which drug efficacy in SZ and BDP could be tested.

Epigenetic mechanisms involve marking of DNA (promoter hypermethylation) and of its associated chromatin remodeling processes (i.e., covalent histone tail acetylation or methylation). Drugs currently being evaluated for their ability to reduce DNMT1 activity in cancer therapy, such as procainamide or zebularine (Brueckner and Lyko, 2004), should be also tested in appropriate protocols to evaluate SZ and BDP treatment efficacy. Such drugs may be associated with valproate or other histone deacetylase inhibitors that may induce the activity of a brain demethylase and thereby decrease the untoward effects caused by DNMT1 induction (Costa et al., 2003a,b; Detich et al., 2003; Tremolizzo et al., 2005; Mitchell et al., 2005; Simonini et al., 2006).

Induction of a brain DNA demethylase may be facilitated by valproate (Detich et al., 2003), hence an issue that requires attention is the understanding of the pharmacological protocols capable of inducing brain demethylase to detect whether the efficacy of the presently unsatisfactory armamentarium for the treatment of psychosis can be improved.

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