For several decades the biochemical targets to monitor the brain molecular changes associated with psychosis were the monoamines dopamine, serotonin and norepinephrine [1]. Recently, an emerging new target appears to be the GABAergic neurotransmission [2]. Hence, the overarching hypothesis guiding current research in schizophrenia (SZ) has been focused on a dysfunction of the telencephalic GABAergic system, which is considered a key component in the pathophysiology of this psychiatric disorder [2]. Consistent with this new strategy, we and others have shown that the expression of glutamic acid decarboxylase (GAD67), reelin [2–6] and other genes [4,7–10] is downregulated by aberrant epigenetic events in cortical, hippocampal and basal ganglia GABAergic neurons of SZ postmortem brains [9–15]. This downregulation is among the most robust and consistent findings reported by various groups in studies of postmortem brains of SZ patients. Owing to the prominent role of GABAergic neurotransmission in the maintenance of the normal function of telencephalic pyramidal circuits, we have inferred that, very likely, reelin and GAD67 downregulation reflects a brain molecular mechanism underlying SZ and bipolar (BP) disorder symptomatology.

Reelin, an extracellular matrix protein, is preferentially synthesized and secreted by GABAergic neurons in the cortex, hippocampus and caudate putamen. Upon secretion into the extracellular matrix, reelin adheres to the dendritic shafts and surrounds the dendritic spines of cortical pyramidal neurons [2,16]. This protein, perhaps by impinging on postsynaptically located apolipoprotein E2, very large-density lipoprotein or integrin receptors [17,18], modulates event-related protein synthesis, including that of the immediate-early gene activity regulated cytoskeletal protein (Arc) [18], and induces tyrosine phosphorylation of NMDA-selective glutamate receptor subunit-modulating NMDA receptor activity, markedly enhancing long-term potentiation associated with cognitive function [17]. Additionally, a reelin deficiency, such as that observed in the brain of the heterozygous reeler mouse, results in a net decrease of pyramidal neuron dendritic spine expression in the frontal cortex and hippocampus [2,19].

Reelin and GAD67 promoter regions are embedded in large CpG islands and express methylation that regulates the transcriptional activity of these genes [13,14]. The efficiency of this regulation can be underscored by stating...
that, when the human reelin promoter is hypomethylated in vitro, reelin expression levels can increase by up to 80-fold; on the contrary, when the reelin promoter is hypermethylated, for example, by the addition of methionine to the culture medium, the reelin expression level is decreased significantly [14]. Noh et al. demonstrated that, in primary cortical cultures of GABAergic neurons, a reduction of DNA methyltransferase (DNMT; cytosine-5) expression facilitated by antisense technology blocks methionine-induced reelin and GAD67 promoter hypermethylation and reelin mRNA downregulation [20].

Grayson et al. reported that, in the frontal cortex of SZ patients, the reelin promoter regions containing PAX6 binding sites are hypermethylated [13]. Abdolmaleky et al. found a similar hypermethylation further upstream proximal to the CREB regulatory element [19]. These data are in keeping with the hypothesis that the decrease of reelin and GAD67 or other genes expressed in cortical GABAergic neurons of psychotic patients may be mediated by cytosine-5 hypermethylation in the respective promoters elicited by the increased expression of DNMTs (Table 1) [21].

Table 1. DNA methyltransferase 1 mRNA is overexpressed but glutamic acid decarboxylase and reelin mRNAs are underexpressed in layer I cortical GABAergic interneurons of schizophrenic patients’ brains.

<table>
<thead>
<tr>
<th>mRNAs</th>
<th>NPS</th>
<th>SZ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LI</td>
<td>LV</td>
</tr>
<tr>
<td>DNMT1</td>
<td>10 ± 1.6</td>
<td>7 ± 0.9</td>
</tr>
<tr>
<td>GAD67</td>
<td>45 ± 11</td>
<td>47 ± 15</td>
</tr>
<tr>
<td>Reelin</td>
<td>3.3 ± 0.07</td>
<td>0.14 ± 0.03</td>
</tr>
</tbody>
</table>

Values are expressed as the ratio of fmol of DNA methyltransferase 1, glutamic acid decarboxylase or reelin mRNA/pmol G3PDH mRNA. Each value is the mean ± standard deviation of three to six samples. *p < 0.05 compared with the respective samples of the NPS group.

LI: Layer; NPS: Nonpsychotic patients; SZ: Schizophrenia. Reprinted with permission from [12].

There is substantial evidence suggesting that the downregulation of reelin, GAD67 and other promoters expressed in GABAergic neurons of SZ and BP disorder patients is not caused by:

- A genetic defect. The reelin and GAD67 genes map to chromosome 7q22 [23] and 2q31 [24], respectively; neither region has been clearly implicated in SZ pathogenesis;
- A highly conserved single nucleotide polymorphism within the regulatory regions of the genes. In fact, only in few cases has this polymorphism been associated with an increased risk of psychotic symptoms [25–27] but, in a number of other studies [28,29], no association has been reported. Although the polymorphism detected in the reelin and GAD67 regulatory regions may be insufficient per se to alter gene expression, it may sensitize these genes to the influence of an epigenetic regulation by environmental factors, toxins, drugs or viruses [9,25];
- The loss of GABAergic neurons. In fact, studies describing the downregulation of reelin and GAD67 have found no changes in the expression of other specific genes of GABAergic neurons, for example GAD65 [3,4].

On the contrary, accumulating evidence suggests that the downregulation of reelin, GAD67 and other genes in SZ and BP patients is very likely mediated by epigenetic hypermethylation of the related promoters. In fact, it has been shown in SZ and BP patients that there is:

Genetic versus epigenetic mechanisms in the GABAergic dysfunction expressed by SZ & BP disorder patients

Although a downregulation of reelin, GAD67 and other genes expressed in GABAergic neurons of psychotic patients could reflect a genetic linkage, it has been suggested that the complex inheritance and selective pathology in GABAergic neurons may be better explained by the action of epigenetic factors on susceptible candidate genes [10,21,22].

Figure 1. Density of DNMT1 and DNMT3a mRNA-positive neurons in Brodmann’s area 10 from NPS and SZPs. Each bar represents the mean ± standard error of eight NPS (filled bars) or seven SZPs (checkered bars). The difference between SZPs and NPS was calculated by t-test.

*Statistically significant difference when SZPs are compared with NPS. DNMT1 mRNA-positive neurons:
L I: p = 0.02; LII: p < 0.01; LIII: p = 0.04; LIV: p = 0.03; LV: p = not significant;
LVII: p = not significant.
DNMT3a mRNA-positive neurons:
LI: p = 0.01; LII: p < 0.01; LIII: p = not significant; LIV: p < 0.01; LV: p = not significant;
LVI: p = not significant.
DNMT: DNA methyltransferase; L: Layer; NPS: Nonpsychotic patients; SZP: Schizophrenic patients.
For experimental details see [11].
• An increased number of cortical (Figure 1) or caudate–putamen GABAergic neurons staining positive for DNMT1 and DNMT3a mRNA and proteins [11,12], the enzymes that catalyze the methylation of promoter cytosine (Figure 2);

• An increased level of S-adenosyl-methionine (SAM) in the prefrontal cortex (PFC) [30]. SAM is the universal methyl donor utilized by the catalytic activity of DNMTs;

• The presence of reelin and other GABAergic gene promoters that are hypermethylated in the brain of SZ and BP patients [10,13–15].

**Neuronal molecular mechanisms in the epigenetic regulation of gene expression**

In multicellular organisms, the cells of various tissues are specialized to perform distinct functions. They are differentiated for this purpose and this process is accompanied by distinct patterns of gene expression. Thus, despite the fact that, with few exceptions, every cell in an organism has identical DNA, a huge number of distinct transcriptional profiles are created and maintained. Among the most important in controlling these processes are the epigenetic factors that establish and maintain the epigenetic regulation of gene expression.

In psychiatric patients, the importance of epigenetic factors in non-Mendelian inheritance of complex disorders such as SZ, BP disorder and autism is exemplified by the 50% morbidity discordance observed in monozygotic twins, despite their identical DNA sequences [22]. This phenomenon has been explained as an effect of epigenetic (environmental) factors that likely induce an aberrant epigenetic chromatin remodeling, causing morbidity in one of the two genetically predisposed monozygotic twins [22].

Fundamental to the concept of epigenetic modulation of gene expression is the fact that, in the nucleus, DNA is packed around a histone octamer known as a nucleosome that forms a highly regulated and complex DNA–protein structure known as chromatin. Along a single chromosome, various regions of DNA can be packed in two different extreme configurations of chromatin:

• A highly condensed tightly coiled chromatin referred to as heterochromatin. In this structural conformation, DNA is inaccessible to transcription factor regulation, therefore can be considered transcriptionally inactive;

• A relaxed, loosely coiled chromatin, referred to as euchromatin. The DNA in euchromatin is readily accessible to transcription factors and is transcriptionally active.

**DNA promoter methylation**

In the mammalian genome, DNA methylation specifically occurs on carbon in position 5 of the cytosine residues embedded in CpG dinucleotides. This methylation is a process catalyzed by a family of brain DNMT enzymes including DNMT1, 3a and 3b. These enzymes transfer a methyl group from SAM to the carbon 5 of cytosine. Hypermethylation of cytosines embedded in CpG dinucleotides of reelin, GAD67, and other promoters is associated with gene expression downregulation (Figure 3), whereas cytosine promoter demethylation is associated with gene expression upregulation (Figure 3).

It was thought that, in neurons, DNA methylation patterns were finalized during development and remained stable thereafter [31]. However, there is now increasing evidence that, in adult neurons, DNA methylation patterns change rapidly and, thus, DNA methylation provides a platform on which the environment can sculpt the genome and affect neuronal phenotype profiles throughout life without altering the genotype [32,33]. Recent data suggest that, in addition to DNMTs, neuronal promoter methylation can be regulated by the activity of a putative DNA demethylase (see later) that can remove a methyl group from carbon 5 of cytosine (Figure 3). Hence, this evidence suggests that, in neurons, promoter methylation is a dynamic process that can be altered in response to environmental factors such as stress, drugs and various psychopathologies.

**Histone tail covalent modifications**

In addition to DNMT and DNA demethylase, DNA methylation can be regulated indirectly by changes in the covalent modifications of nucleosomal histone tails. For example, covalent histone-3 lysine 9,14 acetylation or histone-3 lysine 4 (tri) methylation is associated
with a downregulation of DNA promoter methylation and contributes in changing the rate of gene expression in neurons by two mechanisms:

- Reducing DNA affinity for histones and making the DNA accessible to DNA demethylases and transcription factors;
- Reducing the recruitment of chromatin repressor complexes including histone deacetylases (HDACs), methyl-binding protein (MeCP)-2, DNMTs and other corepressors [32–34].

Based on the original observation that there is a recruitment of methyl DNA-binding proteins to methylated gene promoters, it was inferred that, in neurons, DNA methylation regulates chromatin conformation. However, it is becoming clear that the relationship between chromatin remodeling and DNA methylation is bidirectional. For example, the targeting of DNA methylating/demethylating enzymes to gene promoters (Figure 3) may be regulated by chromatin-modifying enzymes. Hence, signaling pathways that activate chromatin-modifying enzymes could potentially result in alterations of DNA methylation patterns. As we will discuss later, this concept has significant important functional implications for the use of HDAC inhibitors (i.e., valproate [VPA]) or histone methyl transferase activators (one recently discovered activator is clozapine [CLZ]) [9] as drugs that may affect methylation of GABAergic neuron promoters and cognate protein expression [9,35–37].

**Aberrant epigenetic regulation of GABAergic neurons in SZ & BP disorder patients**

*In the adult human brain, the expression of DNMTs is restricted to GABAergic neurons*

Central to the general hypothesis that a pathology of epigenetic mechanisms is operative in the transcriptional downregulation of reelin and GAD67 expression in SZ and BP patients is the evidence that DNMTs (DNMT1 and DNMT3a) in the cortex and basal ganglia are highly expressed in GABAergic neurons (Figure 1 & Table 1) [11,12]. DNMT1 is the most frequently expressed protein and it functions as a maintenance methylating enzyme, while DNMT3a, which is also abundant, is a protein with de novo methylating activity. In contrast to DNMT1 and DNMT3a, DNMT3b is expressed at very low levels in adult human PFC (Veldic M, Pers. Comm.).

Since the expression and the activity of DNMTs in somatic cells generally decreases after cell division and differentiation, it is surprising that, in the adult human brain, DNMT1 and DNMT3a are highly expressed in a subset of terminally differentiated GABAergic neurons. In a recent histochemical and biochemical laser-assisted microdissection study, we have provided evidence that DNMT1 and DNMT3a are highly expressed in cortical layer I, II and IV GABAergic interneurons. The expression of both proteins is also found in the caudate–putamen GABAergic medium spiny neurons but is virtually absent in pyramidal neurons [11,12]. Since cortical GABAergic neurons are terminally differentiated cells, it is likely that, in these cells, DNMTs play a role in important functions beyond the well-known DNA methylation function at replication foci in dividing cells.

**In SZ & BP disorder patients, DNMT overexpression is restricted to a subset of GABAergic neurons**

DNA methyltransferase has been shown to be increased in GABAergic caudate–putamen medium spiny neurons, and in GABAergic interneurons of the PFC layers I, II and IV in SZ patients with psychosis (Figure 1). This upregulation (an increase of two-to-threefold over control) occurs in the same neurons expressing a decrease of reelin and GAD67 (Table 1). In addition, in the PFC of the same patients, the increase of DNMTs is associated with an elevated (60–80%) level of SAM [30]. Such an increase was not detected in the PFC of depressed patients. Since our studies show that the concentrations (low µmol range) of SAM in the PFC of control subjects are below the concentrations required to saturate DNMT catalytic sites, an increase in SAM levels as observed in...
SZ and BP patients may reflect an increase of DNMT1 activity. Hence, the available data indicate that the brains of SZ or BP patients may express reelin and GAD 67 promoter hypermethylation and downregulation of the cognate proteins for two reasons:

• The increased expression of DNMTs that selectively occurs in GABAergic neurons in the upper layer of the cortex that are contiguous to apical dendrites

• The increased availability of SAM that is not present in saturating concentrations in proximity to DNMTs

**Methionine regulation of epigenetic mechanisms**

In addition to environmental factors including stress and maternal behavior, the administration of methyl donors has also been identified as an important epigenetic factor contributing to the aberrant regulation of reelin, GAD 67, and also to other promoters. For example, folic acid intake affects agouti gene expression in Avy/a mice and unbalance global methylation levels of the genome. Maternal methyl supplementation in mice induces epigenetic variations, including DNA methylation in offspring.

The concept that reelin and GAD 67 expression deficits in SZ are mediated via epigenetic promoter hypermethylation can be related to clinical observations in the late 1960s, which demonstrated that daily administration of high methionine doses could exacerbate or even trigger a psychotic episode in 40–50% of SZ patients but not in nonpsychiatric patients. In the mammalian brain, methionine functions as the precursor of SAM that is not present in saturating concentrations in proximity to DNMTs to maintain the methylation patterns of GABAergic promoters. This hypothesis was tested in a methionine-induced murine endophenotype model of SZ.

Mice treated for a protracted period with repeated doses of methionine (5.2 mmol/kg subcutaneously twice a day for 15 days) model some of the behavioral patterns (prepulse inhibition and social interaction deficits) and molecular neuropathologies that are similar to SZ symptomatology, including:

• Elevated PFC levels of SAM;

• Reelin and GAD 67 promoter hypermethylation;

• Downregulation of reelin and GAD 67 expression;

• Recruitment of methyl-CpG-binding domain proteins (MeCP2 and MDB2) on hypermethylated reelin and GAD 67 promoters, which suggests that this recruitment may be required to elicit reelin and GAD 67 downregulation.

In these mice, the HDAC inhibitor VPA, which increases brain levels of acetylated histones in cortical GABAergic neurons, also prevents methionine-induced reelin and GAD 67 promoter methylation, and downregulates the increase of MeCP2 binding to reelin and GAD 67 promoters. VPA also either prevents or reverses the decrease of reelin and GAD 67 expression elicited by methionine. These data suggest that treatment with methionine may produce a mouse model of SZ because of the casual relationship among elevated brain SAM levels, increased DNMT1 activity, reelin and GAD 67 promoter hypermethylation, and induction of behavioral responses reminiscent of SZ symptomatology. Furthermore, these findings suggest that DNA hypermethylation and the associated chromatin remodeling may be critically important in mediating the epigenetic downregulation of reelin and GAD 67 expression detected in cortical and other GABAergic interneurons of SZ patients.

Collectively, these data challenge the classical concept that DNA (cytosine-5) methylation patterns remain stable in postmitotic cells. Our findings with promoter hypermethylation of reelin and GAD 67 in GABAergic neurons of adult mice receiving methionine, and the recent report by Weaver et al. on the epigenetic reprogramming of glucocorticoid receptors in hippocampal pyramidal neurons of methionine-treated rodents, suggest that promoter methylation is a dynamic process mediated by DNMT1 or DNMT3a.

Evidence that the methionine-induced reelin, GAD 67, and exon1 glucocorticoid receptor promoter hypermethylation can be prevented or effectively reversed by VPA and other HDAC inhibitors, perhaps inducing a DNA demethylation process, supports the concept that, in addition to DNMT1 and DNMT3a, a putative DNA (cytosine-5) demethylase may also play a pivotal role in regulating the appropriate dynamic balance of DNA cytosine-5 methylation patterns in animals receiving methionine.

**Pharmacological strategies to reduce reelin & GAD 67 promoter hypermethylation in SZ patients**

Based on the evidence that DNMT1 and DNMT3a expression is increased in cortical GABAergic neurons of SZ patients, the logical and theoretical considerations depicted in Figure 3 suggest that...
a new approach for the treatment of SZ morbidity should address the hypermethylation of reelin, GAD$_{67}$, and other gene promoters expressed in GABAergic neurons. This approach should include the study of:

- Inhibitors of DNMT1 catalytic activity;
- Drugs that downregulate the expression of DNMT1 and DNMT3a;
- Drugs (VPA or sulpiride [SULP], alone or associated with antipsychotics) that induce DNA demethylation (Table 2).

**DNMT inhibitors**

Most of the inhibitors of DNMT1 catalytic activity available today are molecules that can be incorporated into the DNA of proliferating cells (Table 2) [45]. The most-studied compounds are the cytosine analogs 5-azacytidine and zebularine. Both nucleoside analogs are converted in vivo into deoxynucleotide triphosphate derivatives and are incorporated into replicating DNA. DNMTs bind to the nucleoside analogs and remain irreversibly trapped in the DNA, leading to depletion of DNMT stores [45]. These two inhibitors have been used with some success to downregulate cell division in cancer cells in vitro and in vivo, but it is expected that they would fail to act on postmitotic cells and therefore are expected to be devoid of action in differentiated neurons. However, recent studies in rats show that zebularin and 5-aza-cytidine injected locally into the CA1 region of the hippocampus prevent the DNMT1-induced promoter hypermethylation of the ‘memory suppressor gene’ PPI [48]. Unfortunately, 5-azacytidine and zebularine do not readily cross the BBB and, at the high doses needed to reach the brain, they elicit significant toxicity.

Another potent inhibitor of DNMT activity is doxorubicin. This antibiotic derivative of antracycline intercalates into the DNA, thus preventing the action of DNMT and reducing its expression [46]. Doxorubicin has been tested in the treatment of peripheral tumors, but it causes considerable toxicity because it reduces mitochondrial oxidative phosphorylation and causes severe cardiotoxicity [46]. In addition, this compound does not cross the BBB readily and therefore cannot be considered a candidate to pharmacologically reduce DNMT activity in the brain.

A prospective DNA methylation inhibitor active in differentiated neurons is procainamide, a drug used to treat cardiac arrhythmias that also inhibits DNA methylation. This compound is a non-nucleoside competitive inhibitor of DNA methylation [47] and is expected to be an effective inhibitor of DNA hypermethylation in neurons. In initial studies, we demonstrated that, given to mice in doses of 0.29 mmol/kg subcutaneously four-times a day, procainamide prevents the hypermethylation of reelin and GAD$_{67}$ promoters induced by protracted methionine treatment (5.2 mmol/kg subcutaneously twice daily) [33].

**Drugs that downregulate DNMT1 expression**

The expression of several transcription factor binding sites on DNMT promoters [48,49] suggests that their expression in neurons can be regulated by both environmental stimuli and neuroactive drugs. Therefore, to downregulate DNMT expression upregulation with drugs, one should also consider non-nucleoside compounds that either decrease DNMT1 expression or reduce its catalytic activity. As reported in Table 2, a non-nucleoside drug proven to be an effective indirect DNA demethylase inhibitor in cancer chemotherapy is hydralazine. This drug is used to treat hypertension and, in nontoxic doses, downregulates DNA methylation in T cells by inhibiting the ERK pathway and decreasing DNMT expression [50]. This drug should be tested in psychopathologies once its ability to cross the BBB has been established.

Another small non-nucleoside molecule that should be considered is nicotine. We have recently found that nicotine downregulates cortical DNMT1 mRNA and protein expression, suggesting that nicotine abuse, which is very frequent in SZ patients, may be motivated by an attempt to reduce the SZ symptoms via a downregulation of the increase of brain DNMT1. In fact, our results suggest that nicotine and selective agonists of $\alpha_4/\beta_2$ nicotinic cholinergic receptors downregulate DNMT1 expression and increase GAD$_{67}$ expression in cortical and hippocampal GABAergic neurons [51].

**HDAC inhibitors**

A possible strategy to elicit a pharmacological normalization of the reduced reelin or GAD$_{67}$ expression in cortical GABAergic neurons of SZ patients is to administer HDAC inhibitors to reduce DNMT1 accessibility to cytosine embedded in the promoter CpG islands of these genes. The possible success of such a strategy is supported by a report showing VPA administered in therapeutic doses to human subjects increases acetylation of chromatin histones [52] and, at least in mice and rats, this drug prevents the reelin and GAD$_{67}$ promoter hypermethylation induced by methionine [33–37].

Four major classes of HDAC inhibitors have been described (Table 2): hydroxamic acids, short-chain fatty acids, cyclic tetrapeptides and benzamides.

The hydroxamic acid derivative trichostatin A is the most potent HDAC inhibitor available and acts in vitro in the nanomolar concentration range. However, this compound does not readily cross the BBB and, if given parentally, cannot inhibit CNS HDACs. Suberoylanilide hydroxamic acid is another hydroxamic acid derivative active in micromolar concentrations in vitro but it appears to be a weak inhibitor of brain HDACs in vivo [53], presumably because of its short half-life and its low BBB penetrability. Better results were obtained with the benzamide derivatives MS-275 [33,54] and SULP [57]. When injected in mice, both compounds are at least ten-times more potent than VPA in increasing brain acetyl histone-3 content and they are well tolerated when administered repeatedly during the day for several days.

Interestingly, SULP is a dopamine D2 and D3 receptor antagonist with a low extrapyramidal side-effect liability. Sulpiride is used in Europe as an effective antipsychotic to treat psychotic exacerbation episodes in chronic SZ patients [55]. Because of its HDAC inhibitory activity, its efficacy as an antipsychotic cannot be ascribed exclusively to its D2, D3 receptor function inhibition but can also involve the inhibition of HDACs.
It was previously assumed that the HDACs expressed in the brain were approximately equally sensitive to different inhibitors of these enzymes. While this may be the case for trichostatin A and its derivatives, recent indirect evidence suggests that this might not be the case for VPA and MS-275. For example, it has been reported that class II HDACs are five-times less susceptible to VPA inhibition than class I HDACs [53]. Furthermore, it has been reported that, among the class I HDACs, HDAC1 is most sensitive to MS-275, whereas HDAC3 and HDAC8 have a significantly lower sensitivity [53,54]. This finding suggests that the development of new HDAC inhibitors targeted to specific HDACs combined with pertinent information on selective tissue expression of the various molecular forms of HDACs may allow the preparation of tailored HDAC inhibitor cocktails to be tested as pharmacological interventions to treat psychiatric episodes in SZ and BP disorders.

**HDAC inhibitors & DNA methylation**

Recent studies suggest that GABAergic neurons, in addition to DNMTs and SAM, also express an active DNA-demethylating enzyme that may play an important role in determining the level of gene-specific cytosine methylation [33,37,38]. Thus, to study whether hypermethylated reelin and GAD67 promoters in the brain can be demethylated by the action of DNA demethylase expressed in the mouse brain, we tested whether reelin and GAD67 promoter hypermethylation induced by protracted methionine administration is reversed after methionine withdrawal, and whether the demethylation characteristics can be modified by the association of VPA or MS-275 with antipsychotics.

In mice receiving methionine (5.2 mmol/kg subcutaneously twice daily for 7 days), reelin and GAD67 promoters isolated from the frontal cortex were hypermethylated compared with promoters extracted from glycine-treated controls [33,34]. This hypermethylation declined by 50% after 6 days of methionine withdrawal [33]. By contrast, when VPA (2 mmol/kg) was given after termination of methionine treatment, it dramatically accelerated the reelin and GAD67 promoter demethylation that occurred in the following 48–72 h [33]. At the doses used (0.25–2.2 mmol), VPA increased the content of nuclear acetyl-histone 3 bound to reelin and GAD67 promoters by 40–50% [34], suggesting that a histone code involving covalent histone tail modification may facilitate the efficacy of DNA demethylation by this drug.

The HDAC inhibitors (i.e., VPA and MS-275) ability to demethylate reelin and GAD67 promoters could be facilitated by an inhibition of DNMT activity, or the activation of a DNA (cytosine-5) demethylase.

There are two sets of data that suggest that the accelerated reelin and GAD67 promoter demethylation elicited by VPA or MS-275 treatment could be directly or indirectly related to a DNA demethylase induction but not to reduced DNMT1 function:

- Reduction of DNMT1 activity with procainamide fails to activate demethylation of the hypermethylated reelin and GAD67 promoters [33];
- VPA fails to inhibit SAM biosynthesis and in fact may even facilitate its biosynthesis [33], suggesting that an indirect reduction of DNMT activity due to a reduced availability of SAM cannot be related to VPA-induced promoter demethylation.

Hence, we believe that, in mice, the mechanism by which the recovery of reelin and GAD67 downregulation is accelerated by VPA or MS-275 after methionine withdrawal could be related to the induction of a putative DNA demethylase activity. These data agree with a previous report by Detich et al. demonstrating that VPA triggers a replication-independent DNA demethylation [44].

Since the biochemical identification of a putative DNA demethylase in mammalian cells is still unclear [32], one cannot infer whether the accelerated reelin and GAD67 promoter demethylation elicited by HDAC inhibitors in the mouse frontal cortex is the result of DNA demethylase activation or an induction of DNA demethylase biosynthesis. The characterization of an inducible demethylase activity in the mammalian brain is an attractive possibility for discovering a new epigenetic mechanism that includes a putative DNA demethylase. Obviously, the identification of a DNA demethylase would be essential to develop a new line of pharmacological intervention to treat psychosis in SZ or BP disorder patients.

**GABAergic gene promoter demethylation as a new epigenetic target for antipsychotics**

Published clinical studies infer that drugs such as VPA are efficacious in the treatment of SZ and BP disorder when used in combination with typical or atypical antipsychotics [56–58]. However, no study has suggested the mechanisms underlying this interaction.

The symptomatic benefits elicited by a combination of VPA and antipsychotics in the treatment of SZ prompted us to study whether the targets of this drug combination could lead to changes of specific chromatin remodeling events mediated by the activation of a DNA demethylase operative at selected hypermethylated GABAergic promoters (i.e., reelin and GAD67).

Our experiments were carried out with various typical and atypical antipsychotics, including haloperidol (HAL), CLZ, SULP and olanzapine (OLZ). HAL (a butyrophenone derivative) is a typical antipsychotic that relieves the positive but not the negative symptoms of SZ. In addition, it is a potent D2 receptor blocker causing a high liability for extrapyramidal side effect induction. CLZ (pyperazinyl-dibenzo-[1-4]-diazepine) and OLZ (pyperazinyl-[1-5]-benzodiazepine) are two atypical antipsychotics with low efficacy on negative symptoms. At the doses used as antipsychotics, they have weak antagonistic action on D2 receptors and therefore express low extrapyramidal side-effect liability. SULP (aminosulfonyl-methoxybenzamide) is an atypical antipsychotic that also ameliorates the severity of negative symptoms and, only at high doses, may act as a D3/D2 receptor antagonist that may include extrapyramidal side effects. In a recent study, we report that frontocortical and striatal reelin and GAD67 promoter hypermethylation induced in mice by a 7-day methionine administration can be reversed by atypical antipsychotics (i.e., CLZ or SULP) given either alone or coadministered with VPA, but not by HAL or OLZ given either alone or with VPA [37].
As shown in Figure 4, CLZ (3.8–15 µmol/kg subcutaneously) administered twice a day for 3 days elicits a dose-related increase of frontocortical reelin promoter demethylation. Furthermore, at every dose studied, CLZ or SULP (12.5–50 µmol/kg subcutaneously) synergistically enhance reelin promoter demethylation elicited by a dose of VPA that, per se, only partially (30–35%) increases promoter demethylation (Figure 4).

As opposed to CLZ and SULP, HAL (1.5–4 µmol/kg subcutaneously) (Figure 4) and OLZ (4–16 µmol/kg subcutaneously) [37] failed to induce statistically significant changes even when administered in combination with VPA.

To elicit a marked reelin promoter demethylation, CLZ or the combination of CLZ and VPA must be given repeatedly (multiple injections for 3 days); in fact, reelin promoter demethylation was not increased 2 h after a single injection of CLZ (15 µmol/kg subcutaneously) or a single coadministration of VPA (0.5 mmol/kg subcutaneously) plus CLZ (15 µmol/kg subcutaneously). For example, after a single injection, the percentage of reelin promoter methylation is 62 ± 5% in vehicle, 59 ± 8% in CLZ alone and 47 ± 13% in VPA plus CLZ-treated mice (n = 3) [37].

The association of VPA (0.5 mmol/kg subcutaneously) with CLZ (15 µmol/kg subcutaneously twice daily) at a dose schedule that induces reelin and GAD67 promoter demethylation in the frontal cortex also exerts a similar effect in the striatum [37]. We used this brain area because its GABAergic neurons are a possible target for the beneficial action of typical neuroleptics and cannot be generalized to other tissues or cell types.

Promoter demethylation induced by coadministration of VPA and CLZ is brain selective. In the liver, the reelin promoter is constitutively hypermethylated (more than 80% of the cytosine sites are methylated) and in fact its degree of methylation is not significantly increased by methionine administration [37]. Moreover, in the liver, the methylation of reelin promoter cytosines failed to be lowered by the coadministration of VPA (0.5 mmol/kg twice a day for 3 days) or CLZ (15 µmol/kg twice a day for 3 days) at doses that dramatically decrease reelin promoter methylation in the frontal cortex and striatum of the same mice [37].

Taken together, these data suggest that coadministration to mice of well-tolerated, clinically relevant doses of VPA and CLZ or VPA and SULP induces brain reelin and GAD67 promoter demethylation, presumably by activating a DNA demethylase that must be expressed in selected populations of cortical or striatal GABAergic neurons. Nonetheless, such an activity cannot be expected when VPA is associated with HAL or OLZ.

The similarities between the frontal cortex and striatum and the difference of both tissues from the liver further suggest that DNA demethylation induced by coadministration of clinically relevant doses of VPA and CLZ, or VPA or SULP, is a process restricted to selected populations of brain GABAergic neurons and cannot be generalized to other tissues or cell types.

**Expert commentary**

Typical and atypical antipsychotics targeted to dopamine, norepinephrine or serotonin neurotransmission have represented a fundamental breakthrough in the treatment of positive symptoms in SZ and BP disorder patients. However, these drugs are ineffective for treatment of negative symptoms and cognitive dysfunction. Indeed, the advances made during the last 50 years, since the introduction of chlorpromazine in the treatment of negative symptoms or cognitive deficits in SZ and BP disorder, have been minimal [1]. Thus, there is a need to identify novel molecular targets with a well-defined role in the pathophysiology of these illnesses.

Recent breakthroughs in the study of aberrant epigenetic mechanisms operative in SZ and BP disorder, including an increased expression of DNMT1, suggest that promoter hypermethylation in CNS GABAergic neurons could be the pathogenetic mechanism underlying specific symptoms, such as lack of motivation, anhedonia and working memory and executive function deficits.
It is clear that presently available treatments for SZ and BP disorder are not directly targeted to the molecular abnormalities in the GABAergic dysfunction operative in these disorders. To correct GABAergic neuron deficits, we propose the following two principal strategies:

- **Enhancement of defective GABAergic transmission by drugs acting as selective positive allosteric modulators of GABA action at pertinent GABA₆ receptor subtypes** [59];

- **Drugs acting to correct chromatin remodeling abnormalities due to epigenetic dysregulation mechanisms (as described in this review).**

Elaborating on the first strategy, one may consider that benzodiazepines, which are devoid of intrinsic activity at GABA₆ receptors, including α1 subunits, and act exclusively at GABA₆ receptors expressing α2, α3, α5 subunit combinations, should counteract the GABAergic signal-transduction deficit without eliciting sedation, amnesia, tolerance or dependence liabilities. Benzodiazepines acting at α1-expressing GABA₆ receptor subtypes are prescribed to psychotic patients but one has to deal with problems related to their sedative action. One benzodiazepine devoid of intrinsic activity at GABA₆ receptors, including α1 subunits, but acting as a full allosteric modulator at α5, and perhaps α2 and α3 subunits, is imidazenil. This drug is anxiolytic and anticonvulsant and, moreover, it fails to produce sedation or amnesia [59]. Hence, we suggest that a combination of imidazenil with antipsychotics should be considered and eventually tested in the treatment of the GABAergic dysfunction operative in SZ and BP disorders.

An alternative strategy to correct the GABAergic neuron deficit in SZ and BP disorder patients may be to use drugs that diminish the DNMT1 overexpression typical of these illnesses. One can speculate that a protocol to treat SZ and BP disorders may include procainamide (a competitive inhibitor of DNMTs) or nicotine acetylcholine receptor agonists such as A-85380 or varenicline to downregulate the expression of DNMT1 [51]. These agonists may be administered with VPA used to inhibit HDACs and to activate chromatin transcriptional activity of selective GABAergic genes including, for example, GAD₆₇, which is downregulated in SZ and BP disorders [51]. This is probably the reason why VPA is presently prescribed with antipsychotics as an augmentation strategy to treat multiple symptoms of the SZ syndrome.

The site of action of antipsychotics is not clear. However, recent studies suggest that they may act on nuclear chromatin remodeling in GABAergic neurons [37]. Hence, antipsychotic drugs and their coadjuvants (imidazenil and HDAC inhibitors) should be studied in animal experiments to evaluate their putative action on chromatin remodeling, and their ability to correct the GABAergic downregulation typical in SZ and BP disorder with psychosis.

**Five-year view**

For over a decade, VPA has been associated with atypical antipsychotics in the treatment of the cognitive deficits and anxiety, which are two symptoms commonly expressed by SZ and BP disorder patients [56–58]. It has been suggested that when VPA is used in combination with atypical antipsychotics, this cotreatment is not only well tolerated, but in fact leads to an earlier and more pronounced improvement of psychotic symptoms compared with that following antipsychotic monotherapy [56–58]. The data presented here suggest that the coadministration of VPA and atypical antipsychotics may correct the altered nuclear epigenetic function operative in selected populations of cortical GABAergic neurons of SZ and BP disorder patients. In fact, both VPA and CLZ, by covalently modifying histone tail lysines (via acetylation or methylation), activate DNA demethylation and increase GAD₆₇ mRNA and protein expression in the frontal cortex of rodents [9,34–36]. To prove the concept that ‘histone code’ remodeling is at the base of VPA augmenting antipsychotic action, a reasonable working hypothesis is to associate antipsychotics with other more-potent, more-specific and chemically unrelated HDAC inhibitors [33].

Although HDAC inhibitors, such as benzamide MS-275, elicit brain histone code modifications and activate DNA demethylation in a manner similar to that caused by VPA [33,34], there are no clinical studies as yet reporting on the action of MS-275 administered alone or in association with antipsychotics in the treatment of psychosis. However, we can predict that, in the future, increasing our knowledge of the cellular brain distribution of various HDAC subtypes or of DNA demethylases will be possible and would help to develop specific nontoxic epigenetically active drugs to target neuronal populations dysfunctional in SZ or BP disorder brains. However, as a general rule, we must avoid the use of drugs that produce a generalized and indiscriminate increase of DNA demethylation, because such an activation may induce serious neuropathologies (i.e., loss of myelin stability in the brains of multiple sclerosis patients [60]).

Our working hypothesis is that the epigenetic processes operative in the pathophysiology of SZ and BP disorder may include a dysfunction of DNA demethylase activity presumably expressed in brain GABAergic neurons of these patients. However, the histochemical localization and the biochemical properties of such an enzyme require further investigation. Moreover, the induction of a DNA demethylase by a specific class of antipsychotics may mediate the promoter hypomethylation of vulnerable GABAergic genes that are downregulated in psychosis by promoter hypermethylation. A drug that upregulates DNA demethylase by a specific class of antipsychotics may mediate the promoter hypomethylation of vulnerable GABAergic genes that are downregulated in psychosis by promoter hypermethylation. A drug that upregulates DNA demethylase by a specific class of antipsychotics may mediate the promoter hypomethylation of vulnerable GABAergic genes that are downregulated in psychosis by promoter hypermethylation. A drug that upregulates DNA demethylase by a specific class of antipsychotics may mediate the promoter hypomethylation of vulnerable GABAergic genes that are downregulated in psychosis by promoter hypermethylation.
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Key issues

- A deficit of GABAergic neurotransmission emerges as the pathophysiological core mechanism operative in schizophrenia (SZ) and bipolar (BP) disorders.
- In telencephalic GABAergic neurons of SZ and BP disorder patients, the expression of glutamic acid decarboxylase (GAD$_{67}$), the enzyme that synthesizes the inhibitory neurotransmitter GABA, and reelin, an extracellular matrix protein with a trophic synaptic function, are consistently downregulated.
- The downregulation of GAD$_{67}$ and reelin is most likely caused by their promoter hypermethylation catalyzed by an increased expression of DNA methyltransferase 1 (DNMT1).
- An approach to correct the defective GABAergic transmission of psychotic patients should be to administer drugs acting as positive allosteric modulators of GABA action at selective GABA$_A$ receptor subtypes. Here, we propose that a benzodiazepine such as imidazenil – which fails to induce sedation because it is devoid of action on α1-containing GABA$_A$ receptor subtypes – might be an ideal compound to upregulate GABAergic transmission deficits in psychotic patients.
- A pharmacological strategy to reverse the GAD$_{67}$ and reelin promoter hypermethylation is to use drugs that reduce DNA methylation by acting on chromatin remodeling.
- We have established that clozapine, when coadministered with valproate (a histone deacetylase inhibitor), facilitates chromatin remodeling and induces reelin and GAD$_{67}$ promoter demethylation in a manner that may lead to normalization of the downregulation of GABAergic gene expression in SZ and BP patients.

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**Affiliations**

- Erminio Costa, MD
  Professor and Director, Psychiatric Institute, Department of Psychiatry, University of Illinois at Chicago, 1601 Taylor, Chicago, IL 60612, USA
  Tel.: +1 312 413 4591
  Fax: +1 312 413 4569
costa@psych.uic.edu

- Ying Chen, PhD
  Department of Psychiatry, University of Illinois at Chicago, 1601 Taylor, Chicago, IL 60612, USA
  Tel.: +1 312 413 4591
  Fax: +1 312 413 4569
ychen@psych.uic.edu

- Erbo Dong, PhD
  Department of Psychiatry, University of Illinois at Chicago, 1601 Taylor, Chicago, IL 60612, USA
  Tel.: +1 312 413 4591
  Fax: +1 312 413 4569
edong@psych.uic.edu

- Dennis R Grayson, PhD
  Department of Psychiatry, University of Illinois at Chicago, 1601 Taylor, Chicago, IL 60612, USA
  Tel.: +1 312 413 4591
  Fax: +1 312 413 4569
dgrayson@psych.uic.edu

- Marija Kundakovic, PhD
  Department of Psychiatry, University of Illinois at Chicago, 1601 Taylor, Chicago, IL 60612, USA
  Tel.: +1 312 413 4591
  Fax: +1 312 413 4569
mkundakovic@psych.uic.edu

- Ekrem Maloku, MD
  Department of Psychiatry, University of Illinois at Chicago, 1601 Taylor, Chicago, IL 60612, USA
  Tel.: +1 312 413 4591
  Fax: +1 312 413 4569
emaloku@psych.uic.edu

- William (Brad) Ruzicka, PhD
  Department of Psychiatry, University of Illinois at Chicago, 1601 Taylor, Chicago, IL 60612, USA
  Tel.: +1 312 413 4591
  Fax: +1 312 413 4569
wruzicka@psych.uic.edu

- Rosalba Satta, PhD
  Department of Psychiatry, University of Illinois at Chicago, 1601 Taylor, Chicago, IL 60612, USA
  Tel.: +1 312 413 4591
  Fax: +1 312 413 4569
rsatta@psych.uic.edu

- Marin Veldic, MD
  Department of Psychiatry, University of Illinois at Chicago, 1601 Taylor, Chicago, IL 60612, USA
  Tel.: +1 312 413 4591
  Fax: +1 312 413 4569
mveldic@psych.uic.edu

- Adrian Zhubi, MD
  Department of Psychiatry, University of Illinois at Chicago, 1601 Taylor, Chicago, IL 60612, USA
  Tel.: +1 312 413 4591
  Fax: +1 312 413 4569
azhubi@psych.uic.edu

- Alessandro Guidotti, MD
  Scientific Director, Psychiatric Institute, Department of Psychiatry, University of Illinois at Chicago, 1601 Taylor, Chicago, IL 60612, USA
  Tel.: +1 312 413 4594
  Fax: +1 312 413 4569
aguidotti@psych.uic.edu