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Epigenetic GABAergic Targets in Schizophrenia and Bipolar Disorder

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Abstract

It is becoming increasingly clear that a dysfunction of the GABAergic/glutamatergic network in telencephalic brain structures may be the pathogenetic mechanism underlying psychotic symptoms in schizophrenia (SZ) and bipolar (BP) disorder patients. Data obtained in Costa's laboratory (1996–2009) suggest that this dysfunction may be mediated primarily by a downregulation in the expression of GABAergic genes (e.g., glutamic acid decarboxylase$_{67}$ [GAD$_{67}$] and reelin) associated with DNA-methyltransferase (DNMT)-dependent hypermethylation of their promoters.

A pharmacological strategy to reduce the hypermethylation of GABAergic promoters is to administer drugs, such as the histone deacetylase (HDAC) inhibitor valproate (VPA), that induce DNA-demethylation when administered at doses that facilitate chromatin remodeling. The benefits elicited by combining VPA with antipsychotics in the treatment of BP disorder suggest that an investigation of the epigenetic interaction of these drugs is warranted.

Our studies in mice suggest that when associated with VPA, clinically relevant doses of clozapine elicit a synergistic potentiation of VPA-induced GABAergic promoter demethylation. Olanzapine and quetiapine (two clozapine congeners) also facilitate chromatin remodeling but at doses higher than used clinically, whereas haloperidol and risperidone are inactive. Hence, the synergistic potentiation of VPA's action on chromatin remodeling by clozapine appears to be a unique property of the dibenzepines and is independent of their action on catecholamine or serotonin receptors.

By activating DNA-demethylation, the association of clozapine or its derivatives with VPA or other more potent and selective HDAC inhibitors may be considered a promising treatment strategy for normalizing GABAergic promoter hypermethylation and the GABAergic gene expression downregulation detected in the postmortem brain of SZ and BP disorder patients.

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Introduction

This review summarizes our present understanding of the topic of neuroepigenetics in major psychotic disorders. To elucidate the molecular mechanisms whereby nurture (biological or environmental epigenetic factors) and nature (genetic factors) interact to cause major psychiatric disorders such as schizophrenia (SZ) and bipolar (BP) disorder was at the center of Dr. Costa's mission for the last 15 years of his research at the Psychiatric Institute at the University of Illinois at Chicago (Costa et al., 2002).

Existing drugs used to treat major psychiatric disorders have limited efficacy and substantial side effects. Hence, the challenge for Dr. Costa and his colleagues has been to find new ways to prevent and treat psychiatric disorders with pharmacological agents that fail to have major unwanted side effects.

The challenge to identify the core pathophysiological mechanisms underlying schizophrenia (SZ) and bipolar (BP) disorder that are targeted by antipsychotics

Unfortunately, there are few new leads for future drug development for the treatment of major psychiatric disorders (Miller, 2010a). A fundamental barrier to the identification of more efficacious, less toxic, and faster acting drugs than those presently available to treat BP disorder and SZ is the incomplete understanding of the etiopathogenetic mechanisms underlying the symptomatology of these diseases.

Population, family, and twin studies indicate that SZ and BP disorders are highly heritable but a single allele conferring increased risk has been identified in only a small proportion of the observed phenotypic variants (Li, 2010, Sullivan et al., 2008). Hence, the hypothesis that complex psychiatric disorders are attributable to a relatively few common genetic variants has been questioned. Rather, it appears that these disorders are the consequence of synergistic interactions of multiple susceptibility genes with environmental *neuroepigenetic* factors (Ptak and Petronis, 2008).

"Neuroepigenetics" refers to the reversible regulation of various genomic functions mediated principally through changes in DNA methylation and chromatin structure in neurons.

The findings reviewed in this article marshaled by the pioneering work of Costa and his collaborators (1996–2010) suggest that an epigenetic downregulation of telencephalic GABAergic genes may represent a contributing factor to the behavioral and cognitive impairments experienced by SZ and BP disorder patients.

Despite the large number of pharmacological studies performed in recent years to delineate the receptor affinity profile of different antipsychotics (Table 1) (Gary and Roth 2007, Jarskog et al. 2007, Roth et al. 2004), little is known about the action of antipsychotics on specific epigenetic mechanisms in GABAergic or glutamatergic neurons. In fact, the

antipsychotic medications presently used clinically were not designed to target altered GABAergic or glutamatergic neurotransmission.

A primary objective of our studies has been to understand more about the specific intracellular signal transduction processes and nuclear events involved in the pharmacological action of antipsychotics by testing whether *antipsychotic drugs elicit functionally relevant neuroepigenetic changes at GABAergic and glutamatergic gene promoters*.

Understanding more about the action of antipsychotic drugs on neuroepigenetic mechanisms will not only accelerate the development of novel pharmacological agents to treat SZ and BP disorder but should also provide insights into the underlying neurobiological causes of these disorders.

The reciprocal interaction between GABAergic interneurons and glutamatergic pyramidal principal neurons is altered in the cortex or hippocampus of SZ or BP disorder patients

When the postmortem brain of SZ and BP disorder patients is compared to that of non-psychiatric subjects, GABAergic neuropathology is detected in the hippocampus and cortex (Akbarian et al., 1995; Benes et al., 1992; Benes and Berretta, 2001, Fatemi et al., 2000, Guidotti et al., 2000; 2005, Impagnatiello et al., 1998; Lewis et al., 2005; Veldic et al., 2007). The GABAergic neuropathology found in the brain of SZ and BP disorder patients is characterized by a decrease in the expression of glutamic acid decarboxylase$_{67}$ (GAD$_{67}$). GABAergic pathology is also characterized by decreased expression of nicotine acetylcholine receptor (nAChR) α4 and α7 subunits (Breese et al., 2000) and by a decrease in other proteins abundantly expressed in GABAergic neurons, such as the NMDA receptor subunit NR2A and the kainate receptor subunit GluR5 (Bitanihirwe et al., 2009; Woo et al., 2004, 2008). Further, there are decreases in somatostatin, tyrosine kinase B (TrkB) receptors, cholecystokinin, GABA transporter-1, parvalbumin (Lewis et al., 2005), and reelin (for a review see Guidotti et al., 2005).

Reelin is a large (400 kDa) extracellular matrix protein that is most abundantly synthesized in GABAergic neurons of cortical layers I and II, and in the hippocampus, caudate, and putamen (Guidotti et al. 2000,Costa et al., 2001, Veldic et al. 2007, Guidotti et al., 2009, Levenson et al., 2008). In the cortex, upon secretion reelin adheres to postsynaptic densities located on dendritic spines and shafts of pyramidal neurons (Costa et al., 2001). This protein binds to specific receptors [apolipoprotein E 2 (ApoE2), very low density lipoprotein (VLDL), and integrin], harmonizing local dendritic protein synthesis rates necessary for: 1) dendritic spine formation, 2) spine maturation, and 3) the regulation of glutamate receptor structure and function (Costa et al., 2001; Guidotti et al., 2009, Levenson et al., 2008, Pujadas et al., 2010; Tueting et al., 1999, 2010). It is important to note that a decrease of GAD$_{67}$ and reelin expression in brain of SZ or BP disorder patients has not been always confirmed. For example in one study an increase instead of a decrease of GAD$_{67}$ was reported (Dracheva et al. 2004) and in another study lack of a significant decrease of reelin

mRNA has been reported (Lipska et al. 2006). Presumably differences in the demographic characteristics, drug treatment, methodology, and composition of brain tissue samples including the ratio of white vs gray matter may actually account for these apparent discrepancies. The later point is highlighted by our more recent studies that identified GAD_{67} and reelin downregulation occurring specifically in GABAergic interneurons of layer I and II (Ruzika et al. 2007, Veldic et al. 2007) suggesting that different cell populations within the same brain areas may have a quite distinct GABAergic deficit profile.

At the molecular level, in addition to reelin, brain-derived neurotrophic factor (BDNF) could be a potential mediator of the reduction of the neuropil expression detected in the prefrontal cortex (PFC) of SZ and BP disorder patients (Lewis et al., 2005). BDNF stimulates the growth of dendrites and increases spine density on dendrites of cortical pyramidal neurons (McAllister et al., 1996). The expression of BDNF mRNA and protein is lower in the PFC of subjects with SZ (for a review see Roth et al. 2009) and BDNF levels and spine density on the basilar dendrites of deep layer 3 pyramidal neurons are positively correlated in SZ patients (Hill et al., 2005). It is important to mention that in SZ patients, reduced BDNF expression in pyramidal neurons is accompanied by a significant decrease in TrkB mRNA levels in GABAergic interneurons (Lewis et al., 2005). Therefore, reduced levels of BDNF and TrkB as well as reelin may be required to produce the decrease in spine density observed in the brain of SZ patients.

An important new finding is that the selective partial elimination of the essential NR1 subunit of the NMDA receptor in GABAergic cortical and hippocampal neurons of mice during early adolescence generates a phenotype characteristic of the GABAergic pathology in SZ (decreased GAD_{67}, decreased parvalbumin, disinhibition of cortical excitatory neurons, reduced neuronal synchrony, and SZ-like behaviors) (Belforte et al., 2010).

GABAergic neuropathologies could explain the disturbance of the reciprocal interaction between GABAergic interneurons and principal glutamatergic pyramidal neurons that likely underlies the appearance of the positive and negative symptoms and cognitive dysfunctions seen in psychotic patients (Lewis and Gonzales-Burgos, 2008, Lisman et al., 2008). We and others have suggested that SZ and BP disorder are diseases characterized by a deficit of GABAergic transmission with consequent glutamatergic and monoaminergic network dysfunction (Benes et al., 2007, Guidotti et al., 2005, 2009, Lewis and Gonzalez-Burgos, 2008, Lisman et al., 2008, Moghaddam, 2003, Woo et al., 2004).

Is an altered epigenetic regulation of gene expression the molecular mechanism mediating the GABAergic and glutamatergic dysfunction in SZ and BP disorder?

In SZ and BP disorder patients, the downregulation of GAD_{67}, reelin, and other genes expressed in GABAergic neurons could be a sign of a genetic abnormality. Although a highly conserved single nucleotide polymorphism (SNP) has been identified in the vicinity of the regulatory region of GAD_{67} (Straub et al., 2007) and of the reelin gene (Shifman et

al., 2008, Wedenoja et al., 2008), it is possible that these polymorphisms are associated with an increased risk of psychotic symptoms in a small number of cases.

However, there are several epidemiological, clinical, and molecular peculiarities associated with SZ or BP disorder that are difficult to reconcile with Mendelian genetic disorders and in contrast, correspond to features of an altered epigenetic homeostasis (Ptak and Petronis, 2008). Such features include: 1) incomplete phenotypic concordance between monozygotic twins, 2) fluctuating disease course with periods of remission and relapse, 3) peaks of susceptibility to disease coinciding with major hormonal changes, and 4) parent-of-origin effects. These observations have led to speculation about the importance of epigenetic factors in mediating psychosis susceptibility.

In support of a role for aberrant epigenetic mechanisms in the pathogenesis of SZ and BP disorder, we have recently reported that the downregulation of GAD_{67} or reelin expression in GABAergic neurons of SZ and BP patients is associated with an overexpression of DNA methyltransferase 1 (DNMT1) and DNA methyltransferase 3a (DNMT3a) in layers I and II of BA9, BA10, BA17, but not in layers V and VI (Costa et al., 2007, Ruzicka et al., 2007, Veldic et al., 2004, 2005, 2007, Zhubi et al., 2009) or in the striatum of BP disorder patients (Veldic et al., 2007). DNMTs belong to a family of enzymes that catalyze the transfer of a methyl group from the methyl donor S-adenosylmethionine (SAM) to the carbon 5’ of cytosines embedded in cytosine phosphodiester guanine (CpG) islands of many gene promoters (Van Emburg and Robertson, 2008). Although increased promoter methylation induced by the overexpression of DNMTs in SZ and BP disorder patients may be the cause of the downregulation of GABAergic genes, the inhibitory action of DNMTs on gene expression may also occur through the formation of repressor complexes. These chromatin complexes may contain other specific proteins (e.g., methyl CpG binding domain proteins, SIN3A, and histone deacetylases) that may repress transcription via a modification of chromatin structure, shifting chromatin from a permissive open conformation to a repressive closed conformation (Chen et al., 2007, Costa et al., 2007, Dong et al., 2005, Grayson et al., 2006, Kundakovic et al., 2009, Van Emburg and Robertson, 2008).

In addition to the increased expression of DNMT1 and DNMT3a, which is associated with a decrease of reelin and GAD_{67} expression in a selected population of GABAergic interneurons of the prefrontal cortex (PFC), the hypothesis that an epigenetic pathology of GABAergic promoters is operative in the transcriptional downregulation of several genes in SZ or BP disorder patients is supported by the following evidence: 1) increased S-adenosyl-methionine (SAM) in the PFC (Guidotti et al., 2007), 2) hypermethylation of cytosines in CpG islands of reelin (Abdolmaleky et al., 2005, Grayson et al., 2005, 2006) and other selected promoters (Mill et al., 2008) and the associated downregulation of cognate protein expression in the PFC of psychotic patients, although negative findings for reelin have also been reported (Mill et al., 2008)., 3) decreased histone methylation at GABAergic gene promoters (Huang et al., 2007), 4) increased histone deacetylase expression in the PFC (Benes et al., 2007; Sharma et al., 2008), 5) an inverse correlation between DNA methylation of the BDNF gene and the level of its expression in the PFC (Mill et al., 2008), and 6) evidence of epigenetic dysregulation of several other GABAergic and glutamatergic genes in major psychosis (Mill et al., 2008). For example, Vglut-1 is hypermethylated and

downregulated in SZ postmortem brain samples from females (Eastwood and Harrison, 2005, Mill et al., 2008).

These data are consistent with the epigenetic theory of major psychosis (Costa et al., 2002) and suggest that DNA-methylation or histone tail covalent modifications associated with GABAergic and glutamatergic gene promoters are important casual events in the pathogenesis of SZ and BP disorder. Furthermore, support for the hypothesis that an epigenetic chromatin remodeling pathology contributes to the downregulation of GABAergic or glutamatergic genes in psychotic patients is sustained by clinical studies conducted in the early 1970s (for review see Wyatt et al., 1971).

Methionine, the precursor of SAM (the universal methyl donor utilized by DNMTs to methylate cytosine in CpG islands), administered in large doses (10–20 g/day) for 3–4 weeks to SZ patients was reported to exacerbate psychotic symptomatology. In both mouse FC and neuronal cultures, the administration of large doses of methionine induces an increase in SAM and the hypermethylation of selective CpG-rich GABAergic promoters, including GAD_{67} and reelin, and facilitates the downregulation of their expression (Chen et al., 2007, Noh et al., 2005, Mitchell et al., 2005, Tremolizzo et al., 2002, 2005). Importantly, the brain levels of GAD_{65} and that of other housekeeping genes were not affected (Tremolizzo et al., 2002).

DNA promoter methylation patterns in neurons constitute a dynamic process

It was thought that in neurons, DNA methylation patterns were established during development and remained stable thereafter (Razin and Shemer, 1995). However, there is increasing evidence supporting the concept that in adult neurons, methylation patterns of specific cytosine/guanine (CpG) dinucleotide-rich promoters change rapidly. Thus, throughout life, DNA methylation provides a platform on which the environment can sculpt the genome and affect neuronal phenotype profiles without altering genotypes (Szyf, 2005). We tested this hypothesis in a behavioral, neuroanatomical, and biochemical epigenetic mouse model of SZ (Tremolizzo et al., 2002, 2005, Tueting, 2010), treating mice protractedly with methionine to induce reelin and GAD_{67} promoter hypermethylation (Fig. 1, **left side**). We quantified the ratio of 5 methyl cytosine (5mC) to the unmethylated cytosine (C) of the murine reelin CpG-enriched promoter region from −340 to + 160 bp (Fig. 2) or the murine GAD_{67} CpG-enriched promoter region from −760 to – 311 (Satta et al., 2008) by measuring the fraction of reelin or GAD_{67} promoter immunoprecipitated by specific anti-5mC or anti-methylcytosine binding protein-2 (MeCP2) antibodies with competitive RT-PCR and mutated internal standards (Dong et al., 2005). The results reported in Fig. 2 show that in the FC of mice treated for 14 days with methionine, the ratio of 5mC/C promoters measured with these two antibodies is virtually identical. These results were duplicated using methylation-specific PCR primers (MSP) (Dong et al., 2007). We found that the ratio of 5mC/C in the reelin promoter region −340 to + 160 bp was approximately proportional to the number of methylated CpG dinucleotides measured with sodium bisulfate mapping (Fig. 2), (Dong et al., 2007, 2008, Tremolizzo et al., 2005).

Methionine induces an increase of brain levels of 1) SAM (Table 2); 2) reelin and GAD_{67} promoter methylation (Fig. 2 and Dong et al., 2007, 2008), and 3) downregulation of reelin and GAD_{67} mRNA (Fig. 3) and cognate protein expression (Tremolizzo et al., 2002, 2005).

GAD_{67} and reelin are not the only gene promoters hypermethylated by methionine treatment. ChIP-on-chip assays show that in mice receiving methionine, about 5% of the genome promoters are hypermethylated in the FC (Dong et al., 2008). Interestingly, the GAD_{65}, NSE (Fig. 3), and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) promoters are not hypermethylated by the administration of methionine, suggesting that promoter hypermethylation is cell- and gene-specific.

As shown in Fig. 1, if methionine is withdrawn after seven days of treatment, reelin promoter hypermethylation decreases by ~50% after the seventh day and returns to control levels after 12–14 days of methionine withdrawal.

Methyl donors have been identified as important epigenetic factors contributing to the aberrant regulation of reelin and other gene promoters. For example, maternal methionine supplementation in rats induces epigenetic variations including DNA methylation alterations in offspring (Weaver et al., 2006). Further, there is an epigenomic reprogramming of reelin and glucocorticoid receptors in hippocampal pyramidal neurons after methionine administration (Weaver et al., 2006). Our studies in cultured cortical neurons (Kundakovic et al., 2009, Noh et al., 2005) not only show that the hypermethylation of promoters induced by methionine is blocked by siRNA-mediated DNMT-KO or by DNMT antagonists but also that this blockade induces the overexpression of reelin, GAD_{67}, or BDNF (Kundakovic et al., 2009).

Collectively, these data challenge the classic concept that DNA 5-mC patterns remain stable in postmitotic cells and strongly suggest that by increasing SAM brain content, methionine facilitates the promoter methylation mediated by DNMT1 or DNMT3a in the CNS. Unlike the DNA sequence of a cell, which is stable and strongly conserved, epigenetic processes are highly dynamic: they can be tissue-specific, developmentally-regulated, and modified by exposure to a range of drugs or environmental factors (Ptak and Petronis, 2008, Szyf, 2005).

Valproate (VPA) and other histone deacetylase (HDAC) inhibitors promote chromatin remodeling, induce DNA-demethylation, and regulate cognitive function

The dynamic nature of the epigenome means that, unlike pathogenic DNA sequence mutations, epigenetic disruptions are potentially reversible and thus a realistic target for pharmacological intervention.

In psychiatry, the use of VPA as a drug that enhances GABAergic transmission is based on the observation that protracted VPA treatment in rodents induces an increase of GAD_{67} expression (Fig. 3) (see also Loscher et al., 1999, Tremolizzo et al. 2002). Recent studies from our group and others suggest that GAD_{67} and reelin are increased by VPA because this drug not only inhibits HDACs and modifies the “histone code” but also decreases

methylated sites at reelin and GAD_{67} promoters, [(Fig. 1) and Dong et al., 2007], thus preventing the recruitment of a corepressor protein complex. This complex includes methyl-CpG-binding domain proteins (MeCP2, MDB2, MBD4), DNMTs, and other corepressor proteins (Dong et al., 2007). We have shown that if HDAC inhibitors such as VPA and the benzamide derivative MS-275 are given to mice after methionine withdrawal, they dramatically accelerate reelin and GAD_{67} promoter demethylation during the subsequent 24–48 hours (Fig. 1 and Dong et al., 2007).

The ability of VPA to facilitate the demethylation of reelin and GAD_{67} promoters was not caused by a direct inhibitory action of this drug on DNMT1 or DNMT3a expression (Table 3) or DNMT activity (DNMT activity in nuclear extract of NT2 cells is $5.0 \pm 0.1 \times 10^3$ cpm/μg protein in control and $5.5 \pm 0.6 \times 10^3$ cpm/μg protein in cells treated with 1 mM VPA for 12 hrs n=3) or on an inhibitory action of VPA on SAM biosynthesis (Dong et al., 2008). Moreover, these data are in line with a previous report by Detich et al. (2003) demonstrating that in human embryonic kidney cells (HEK-293), VPA triggers a replication-independent DNA demethylation that may be associated with an increase of histone acetylation. Since the biochemical identity of the catalytic process mediating DNA demethylation in mammalian cells remains unclear (Ma et al., 2009, Zhu, 2009), one cannot conclude that the accelerated reelin and GAD_{67} promoter demethylation elicited by HDAC inhibitors in the mouse FC (Fig. 1) is 1) the result of a direct induction of a DNA demethylase activity, or 2) the result of an indirect recruitment or activation of a preexisting demethylation mechanism related to “histone code” remodeling (Jenuwein and Allis, 2001).

Evidence has been obtained in rodents that methionine-induced reelin, GAD_{67}, and $exon1_7$ glucocorticoid receptor promoter hypermethylation can be prevented or effectively reversed by VPA and other HDAC inhibitors (Dong et al., 2007, Weaver et al., 2006) in the absence of an action on DNMT. This supports the concept that a putative DNA-demethylase activity may also play a pivotal role in regulating the appropriate dynamic balance of DNA cytosine-5 methylation patterns in the mammalian brain.

Recently, cortical DNA demethylation induced by chromatin modifications, especially histone tail acetylation, has been implicated in memory formation (Miller and Sweatt, 2007; Miller et al. 2010b). Increased histone tail acetylation induced by repeated administration of histone deacetylase inhibitors facilitates cognitive function in normal (Levenson et al., 2004) and aging mice (Peleg et al., 2010) and in a mouse model of neurodegeneration (Alarcon et al., 2004, Chuang et al., 2009). Taken together, these data suggest that pan-HDAC inhibitors such as VPA and other more selective chromatin remodeling agents may provide a potential adjuvant treatment for the cognitive deficits present in SZ and BP disorder patients or other neuropsychiatric disorders.

Epigenetic processes can be a target of antipsychotic drug action

Recent work has demonstrated that methylation of a promoter CpG island located ~30kb upstream of the gene encoding mitogen-activated protein kinase I (MEK1) is significantly correlated with lifetime antipsychotic use in *postmortem PFC* samples, with greater lifetime antipsychotic use associated with lower levels of DNA methylation (Mill et al., 2008). This

finding is interesting given the involvement of (MAPK1) signaling pathways in mediating intraneuronal signaling and the observation that clozapine, a widely used medication in the treatment of schizophrenia, selectively activates this pathway via an interaction with MEK1 (Browning et al., 2005).

Recently, Huang et al. (2007) reported that clozapine but not haloperidol increases histone3-lysine4 (H3K4) (tri) methylation at the GAD_{67} promoter. These effects were not mimicked by genetic ablation of D2 and D3 receptors, suggesting that dopamine receptor signaling is not required for clozapine-induced histone methylation.

To address the issue of whether antipsychotic drugs acting on epigenetic mechanisms alter DNA methylation of GABAergic gene promoters, experiments were carried out in which Swiss albino mice were treated with typical and atypical antipsychotics, including haloperidol, clozapine, olanzapine, quetiapine, and risperidone. We found that the reelin and GAD_{67} promoters were significantly demethylated in the FC of mice receiving three days of treatment with clinically relevant doses of clozapine and relatively high doses of quetiapine and olanzapine but not in mice receiving haloperidol or risperidone (Fig. 4, Table 4, and Dong et al., 2008).

Results similar to those in the FC were obtained in the striatum, which expresses an almost homogeneous population (~90%) of GABAergic medium spiny neurons that also synthesize reelin (Dong et al., 2008). Because in the same mice reelin promoter methylation in the liver fails to change, we infer that clozapine and congeners modify methylation in the CNS and specifically in GABAergic neurons. Importantly, the action of clozapine on GABAergic promoter demethylation appears to be independent of an inhibitory effect on DNMT (Satta, personal communication).

The administration of clozapine but not that of haloperidol, in parallel with the increase of DNA demethylation, induces increased nuclear H3 hyperacetylation at the reelin (Fig. 5) or GAD_{67} promoters in the FC (Dong et al., 2008). It is conceivable that antipsychotics that induce chromatin remodeling act by increasing histone acetylation, thereby recruiting putative DNA demethylating enzymes. This would convert chromatin at specific loci from an inactive/silenced state to an active/state positively modulating synaptic plasticity and regulating cognitive function (for a review see Guidotti et al., 2009). The precise mechanism whereby clozapine modulates the “histone code” and induces DNA-demethylation in GABAergic neurons remains to be elucidated. However, clozapine is marginally active by itself and we found it difficult to reconcile the structure of clozapine with that of any other currently known HDAC inhibitor.

Effect of VPA and clozapine on DNA-demethylation

Important for the translational implications, the DNA-demethylating actions of clozapine, olanzapine and quetiapine were synergistically potentiated by the co-administration of a threshold inhibitory dose of VPA (Fig 4, Table 4, Dong et al. 2008). Furthermore as shown in Table 5, the administration of clozapine (5 mg/kg s.c./three days/twice a day) in conjunction with VPA (70 mg/kg s.c./three days/twice a day) reverses the downregulation of GAD_{67} expression induced in mice by seven days of methionine administration, suggesting

an epigenetic action of this drug combination through histone acetylation and promoter demethylation.

In mammalian cells, active DNA-demethylation is achieved, at least in part, by a base-excision repair pathway that first requires the conversion of 5mC to thymine (T) through deamination (Ooi and Bestor 2008, Zhu, 2009). Recently, human breast cancer cell research suggested that DNMT3a and DNMT3b can convert 5mC to T through deamination; the resulting T is then removed by a G/T mismatch base-excision repair pathway (for a review see Zhu, 2009). Indeed, recent studies suggest that in mammalian cells including neurons, DNA-demethylation at promoter genes involved in memory and cognition or neurogenesis can be achieved by the coupled action of 5mC deamination [i.e., elicited by DNMT3a, 3b, AID (activation induced deaminase), or Apobec (apolipoprotein B RNA editing catalytic component)] and G/T mismatch DNA glycosylation presumably catalyzed by the methyl binding protein-4 (MBD4) (Ma et al. 2009, Kim et al., 2009).

It has been proposed that coupling between 5mC deaminase and G/T mismatch DNA glycosylase is favored by the presence of “growth arrest and DNA-damage-inducible protein 45 (Gadd45) α and β (Fig. 6). These are small active nuclear acidic proteins that are induced in neurons by stress or by drugs that increase neuronal activity (Gavin et al., 2010).

Recently, it has been reported that electroconvulsive treatment a) induces Gadd45 β expression, b) increases Gadd 45 β binding to cytosine deaminase or G/T mismatch glycosylase, and c) induces DNA-demethylation at specific gene promoters (i.e., BDNF, Fgf-1) that is abolished in Gadd45 KO mice (Ma et al., 2009). Hence, it is thought that Gadd45 proteins exert a putative regulatory role on DNA-methylation.

Given our data suggesting that subtypes of antipsychotic medications and VPA can synergistically interact to activate DNA-demethylation (Fig 3 and table 4), we examined the possibility that VPA and antipsychotic drugs elicit functionally-relevant DNA-demethylation changes altering the expression or activity of Gadd45 α or β. We found that in the FC of mice that had been given 70 mg/kg of VPA/three days/twice a day, Gadd45 β mRNA expression is increased compared to vehicle-treated controls (Fig. 7). Histochemical studies with specific Gadd45 β antibodies also show that cortical pyramidal neurons of mice treated with VPA (70 mg/kg/three days) exhibit increased nuclear Gadd45 β expression compared with vehicle-treated mice (Fig. 8). Moreover, clozapine in a dose that per se increases Gadd45 β potentiates the action of VPA (Fig. 7). Since these doses of clozapine and VPA induce promoter-demethylation (Fig. 4), taken together, the data suggest that in addition to DNMTs, neuronal promoter methylation can be regulated by the activity of a putative DNA demethylase, which can remove a methyl group from the carbon 5 of C. Hence, 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.

Concluding remarks

Recent breakthroughs in the study of aberrant molecular mechanisms operative in SZ and BP disorder point to a downregulation in the expression of several genes in GABAergic

interneurons, most likely caused by gene promoter hypermethylation mediated by overexpression of DNMT in these cells (Costa et al., 2007).

The epigenetic downregulation of telencephalic GABAergic function may be responsible for disinhibiting pyramidal neurons that in turn could provide an excitatory input to dopamine cells in the ventral tegmental area (VTA) or serotonin cells in the raphe nucleus and drive a hyper-dopaminergic or -serotoninergic state that further increases pyramidal neuron excitability and presumably induces psychotic symptoms in SZ patients (Lewis and Gonzales Burgos, 2008, Lisman et al., 2008).

Taken together, these data suggest that to produce a significant symptomatic improvement of SZ or BP disorder morbidity, it may be desirable to pharmacologically reverse the promoter hypermethylation in GABAergic neurons.

We have attempted to establish a preclinical strategy for evaluating drugs that facilitate DNA-demethylation either 1) by reducing DNMT activity (i.e., administering DNMT inhibitors), or 2) by promoting the nuclear recruitment of DNA demethylation machinery associated with changes in chromatin remodeling.

DNMT inhibitors that easily cross the blood-brain-barrier and are devoid of toxicity are presently not available. However, we have shown that the dibenzepine derivatives clozapine, quetiapine, and olanzapine but not the butyrophenone derivative haloperidol and the piperidyl-benzisoxazole derivative risperidone (Fig. 9) induce chromatin remodeling changes and activate DNA-demethylation of GABAergic gene promoters (Table 1), perhaps via this mechanism contributing to correction of the downregulation of GABAergic transmission present in the brain of BP and SZ patients.

An analysis of the data of Table 1 suggests that the action of clozapine and its derivatives on chromatin remodeling is independent of its action on catecholamine or serotonin receptors. To validate this concept, the effect of clozapine and congeners on chromatin remodeling should be studied in mice with a genetic ablation of dopamine or 5HT receptor subtypes.

Although double-blind studies with VPA in SZ patients offer contrasting results (Casey et al. 2008), this drug has been co-administered for over a decade with typical and atypical antipsychotics to medicate BP and SZ disorder patients (Kelly et al., 2006, Wassef et al., 2000). The data presented in this review strongly support the provocative concept that the co-administration of VPA with clozapine, by activating DNA demethylation, can reverse a repressed nuclear epigenetic function expressed in the postmitotic cortical GABAergic neurons of SZ or BP disorder patients.

To test the concept that chromatin remodeling modifications may be a possible mechanism operative in the VPA augmentation of antipsychotic efficacy, it seemed appropriate to associate antipsychotics with other more potent and chemically unrelated HDAC inhibitors, such as the benzamide MS-275, which is now in phase II clinical trials and elicits brain histone hyperacetylation and activates DNA-demethylation in a manner similar to that of VPA (Dong et al., 2007, Simonini et al., 2006; Chen et al., 2010).

Presently, the overarching goal of the studies inspired by the pioneer work of Dr. Costa is to develop a classification of antipsychotic drugs based on their action on chromatin remodeling and DNA demethylation in GABAergic neurons. However, the biochemical identity and function of the process mediating DNA demethylation in mammalian cells requires further clarification (Szyf, 2005, Zhu, 2009).

Recently, the search for active DNA demethylation activity in mammals has been characterized by the identification of several different mechanisms. Interestingly, various reports suggest that DNA demethylation is initiated by molecules that either stabilize (methyl binding domain 2 [MBD2]) or induce (DNMT3a) DNA-methylation marks (Ooi and Bestor 2008, Szyf, 2005). Although the complete characterization of this activity is in progress (Dong et al., 2010), the identification of the biochemical nature of brain DNA-demethylation and an understanding of how drugs induce DNA demethylase activity are crucial to the progress of a new line of pharmacological interventions to treat major psychiatric disorders.

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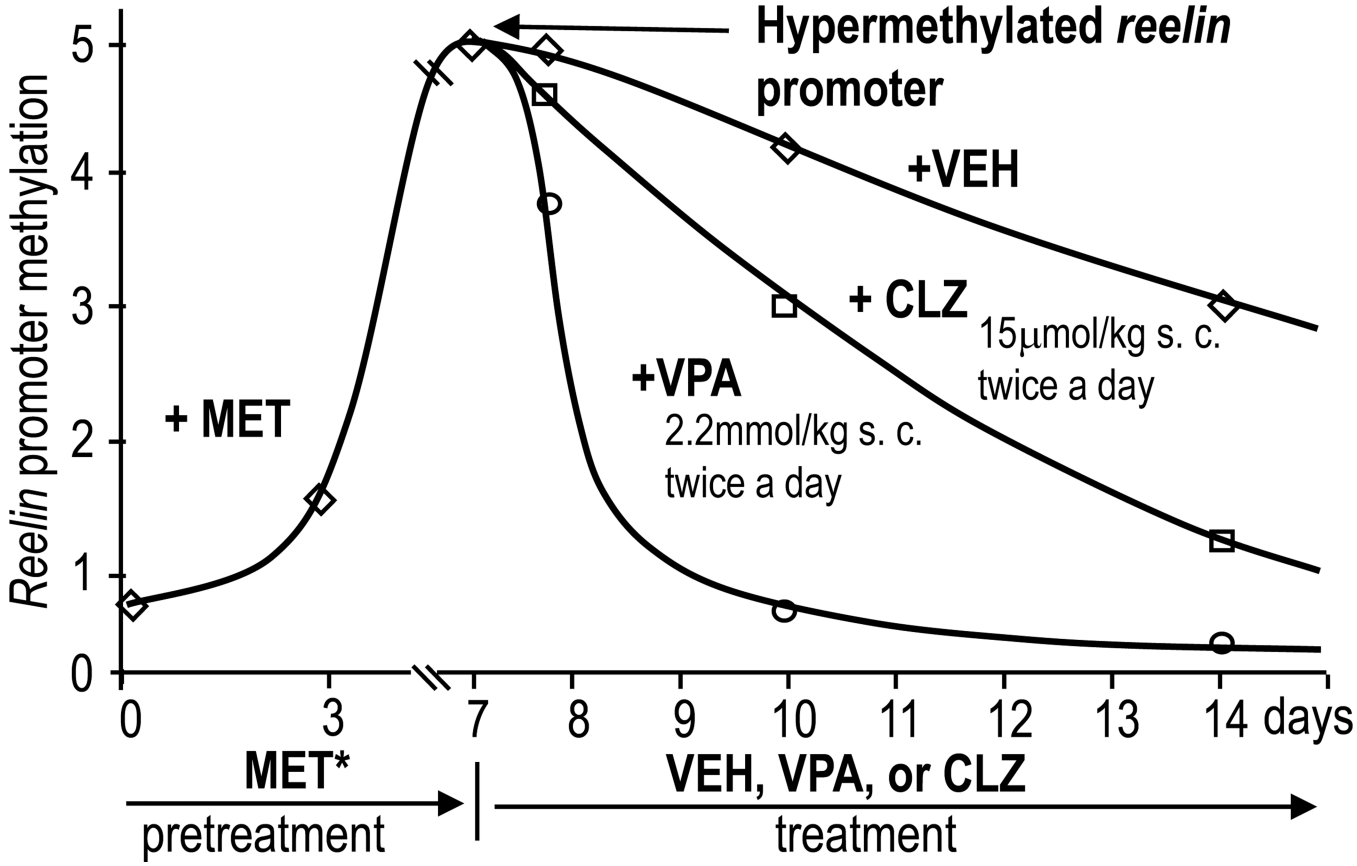
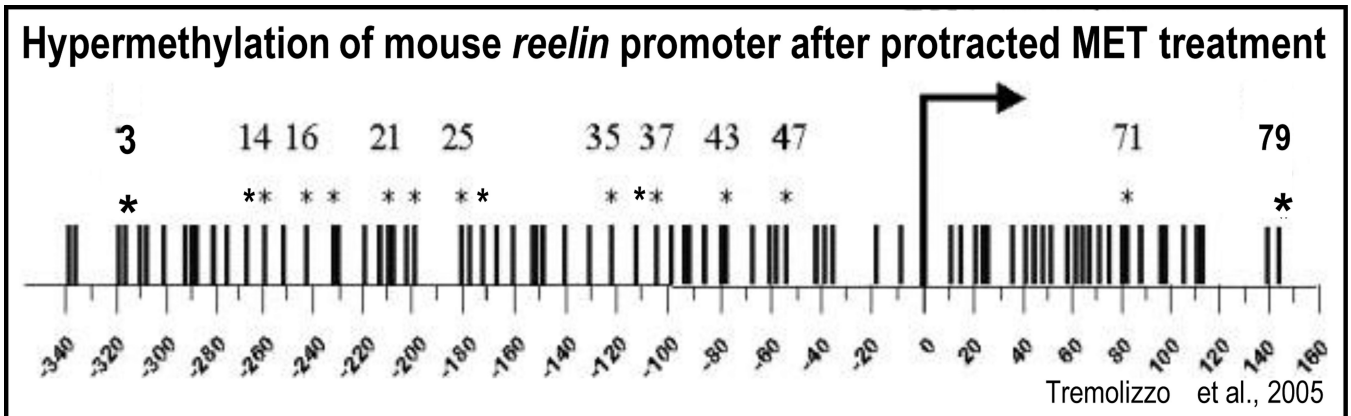


FIG 1. Valproate (VPA) and clozapine (CLZ) accelerate the demethylation of methionine (MET*)-induced hypermethylation of reelin promoter

Met*: mice were pretreated with L-methionine (5.2 mmol/kg s.c. twice a day) for 7 days to hypermethylate the reelin promoter. At 7 days MET was withdrawn and mice received vehicle (VEH) or VPA or CLZ as indicated. Reelin promoter methylation was measured using MeDIP and quantitative PCR assays. Similar results were obtained for the GAD_{67} promoter.

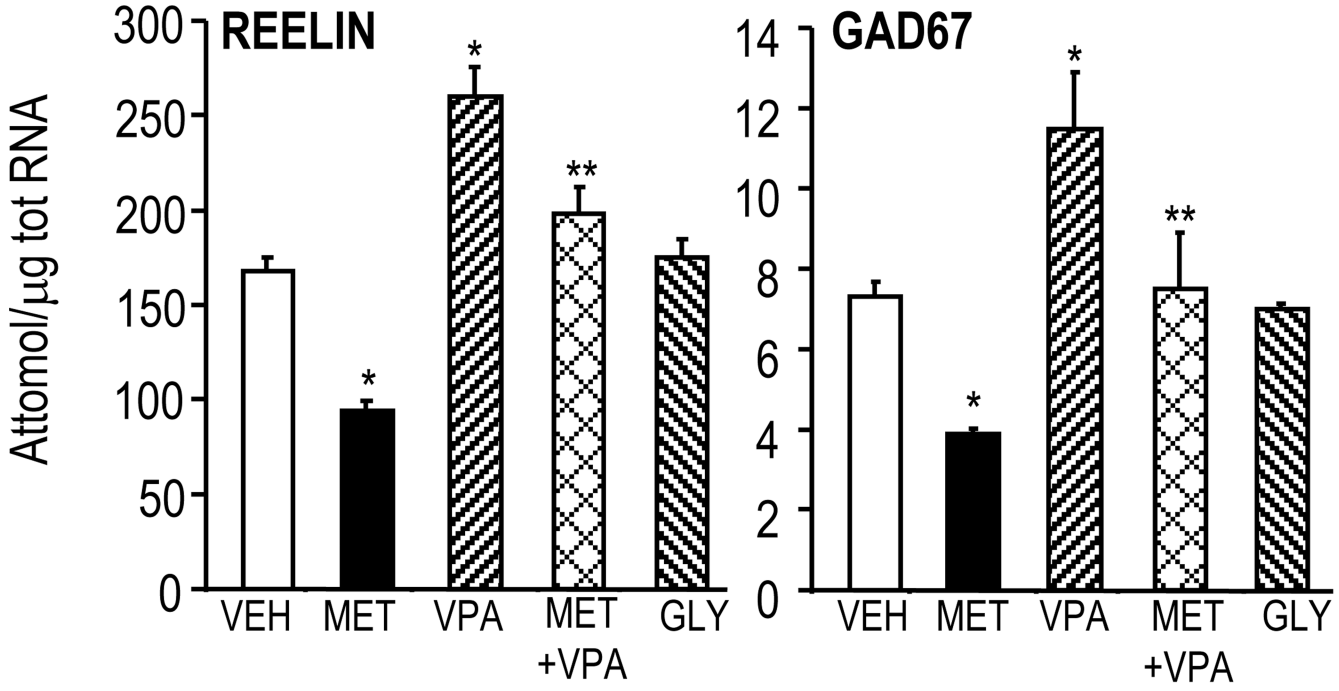
(Dong et al., 2007, 2008).



Treatment	Bisulfate sequencing	MeCP2	5mC	MSP
		Antibody		
	Methylated on 79 CpG sites	Cytosine Methylation (% of total)		
VEH	2	9.0 ± 0.9	11 ± 1.5	8.0 ± 1.2
VPA	0	2.6 ± 0.6[a]	3.0 ± 0.6[a]	-
MET	16	60 ± 4.3	58 ± 3.0	60 ± 4.3
VPA+MET	4	3.5 ± 0.4[a]	3.8 ± 0.3[a]	-

*denotes methylated cytosines; **MeCP2**: methyl CpG binding protein 2.
5mC: 5' methylated cytosine; **MSP**: methylation-specific PCR.
MET: methionine 5.2 mmol/kg twice a day for 14 days.
VPA: valproate 2.2 mmol/kg twice a day for 14 days.
a: P<0.05 compared to VEH group.

FIG 2. Cytosine methylation in a CpG island-enriched promoter region of reelin comparing various methods



	VEH	MET	VPA	MET+ VPA	GLY
	Attomol/ug RNA				
NSE	306±46	320±57	305±56	299±54	300±40
GAD65	44±4	45±4	45±5	43±3	44±5

FIG 3. Valproate (VPA) increases reelin and GAD_{67} mRNAs and reverses L-methionine (MET)-induced downregulation of these mRNAs

MET 5.2 mmol/kg; GLY (glycine) 5.2 mmol/kg; VPA 2.2 mmol/kg s.c. twice daily for 15 days.

*p< 0.05 vs VEH; **p< 0.05 vs MET.

Tremolizzo et al., 2005.

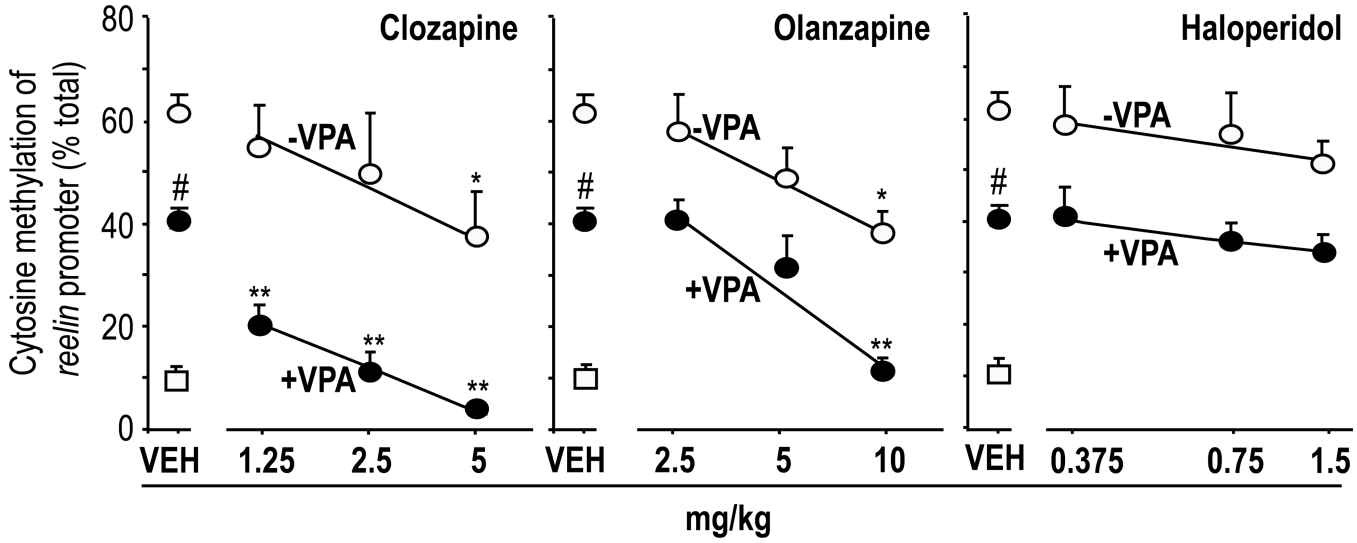


FIG 4. Clozapine or olanzapine alone or in combination with valproate (VPA) but not haloperidol induce reelin promoter demethylation in the mouse frontal cortex

VPA (70 mg/kg) and antipsychotics were given s.c. twice a day for three days after MET withdrawal. Open circles denote MET-pretreated mice that did not receive VPA. Filled circles denote MET-pretreated mice that received VPA. Open squares denote mice never treated with MET.

*p< 0.05 when clozapine or olanzapine in absence of VPA were compared with the respective VEH-treated mice.

**p<0.01 when clozapine + VPA or olanzapine + VPA-treated mice were compared with VEH + VPA-treated mice.

# p< 0.05 when VEH + VPA-treated mice were compared with the respective VEH-treated mice.

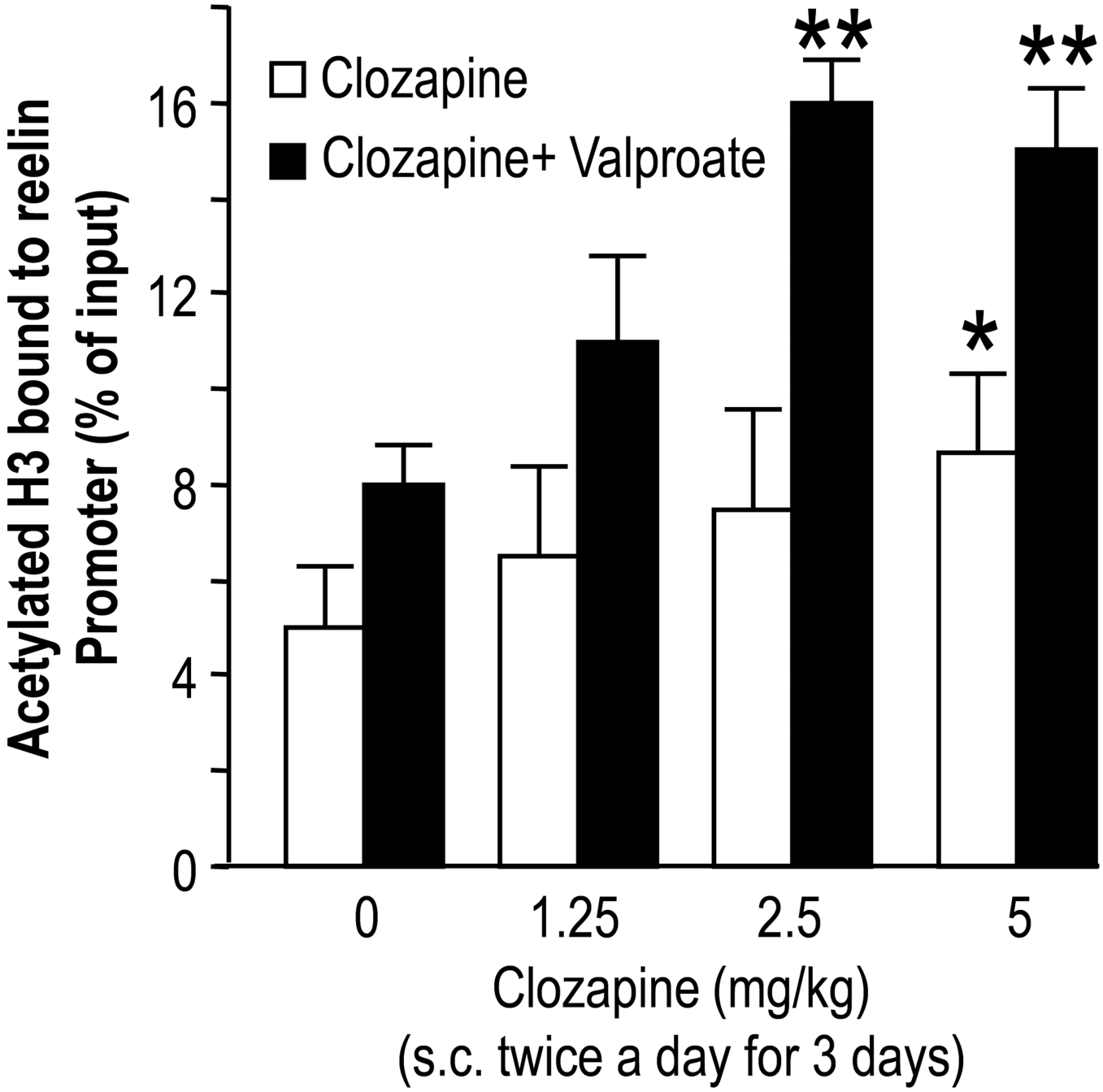


FIG 5. Clozapine increases acetylated H3-lysine9 frontal cortex levels at the reelin promoter

Open bars denote mice that did not receive valproate. Closed bars denote mice that received valproate (70 mg/kg s.c. twice a day for three days)

*p< 0.05; **p< 0.01 when compared with the respective controls.

Controls are mice that did not receive valproate or clozapine.

For details see Dong et al., 2008.

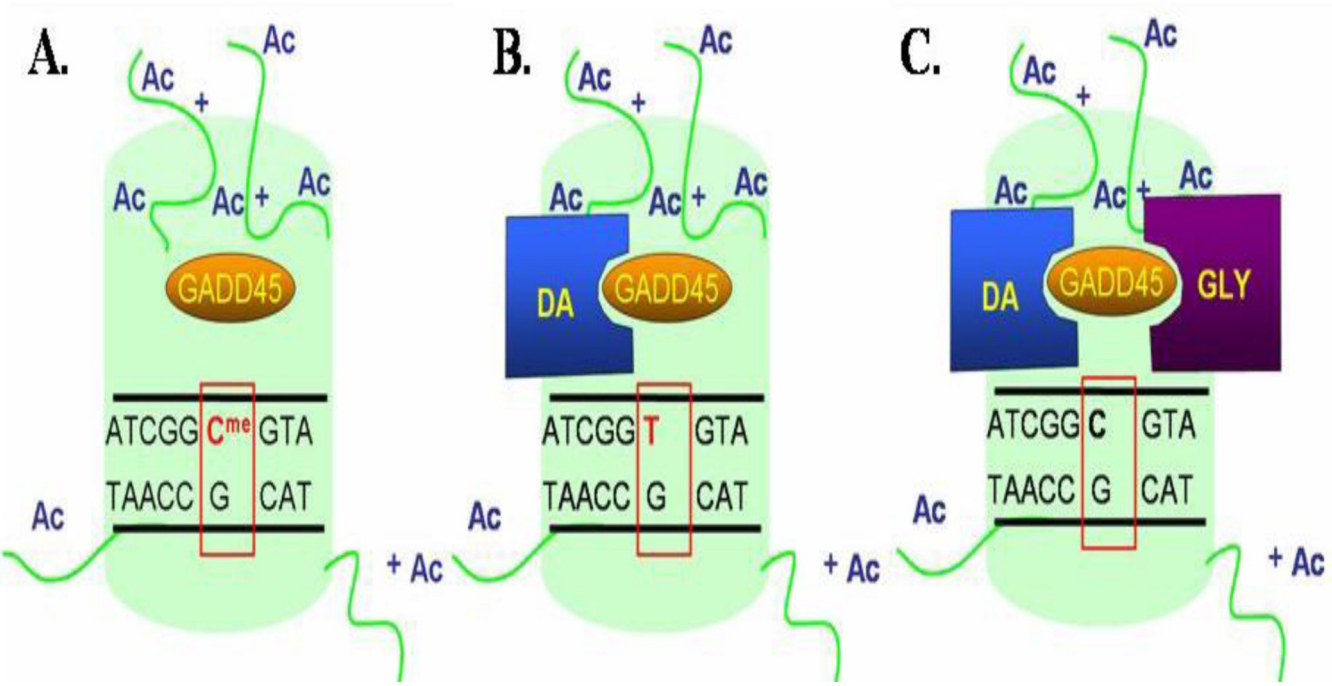


FIG 6. Proposed mechanism of activity-dependent CpG-rich promoter demethylation

A. Following depolarization GADD45 α, β protein levels increase and GADD45 α, β bind to a methylated promoter region proximal to an acetylated histone (green).

B. GADD45 recruits a deaminase (DA), which converts 5-methylcytosine to thymine leading to a T:G mismatch.

C. GADD45 recruits a DNA glycosylase (GLY), which removes thymine from the T:G mismatch. Thymine is later replaced with an unmethylated cytosine.

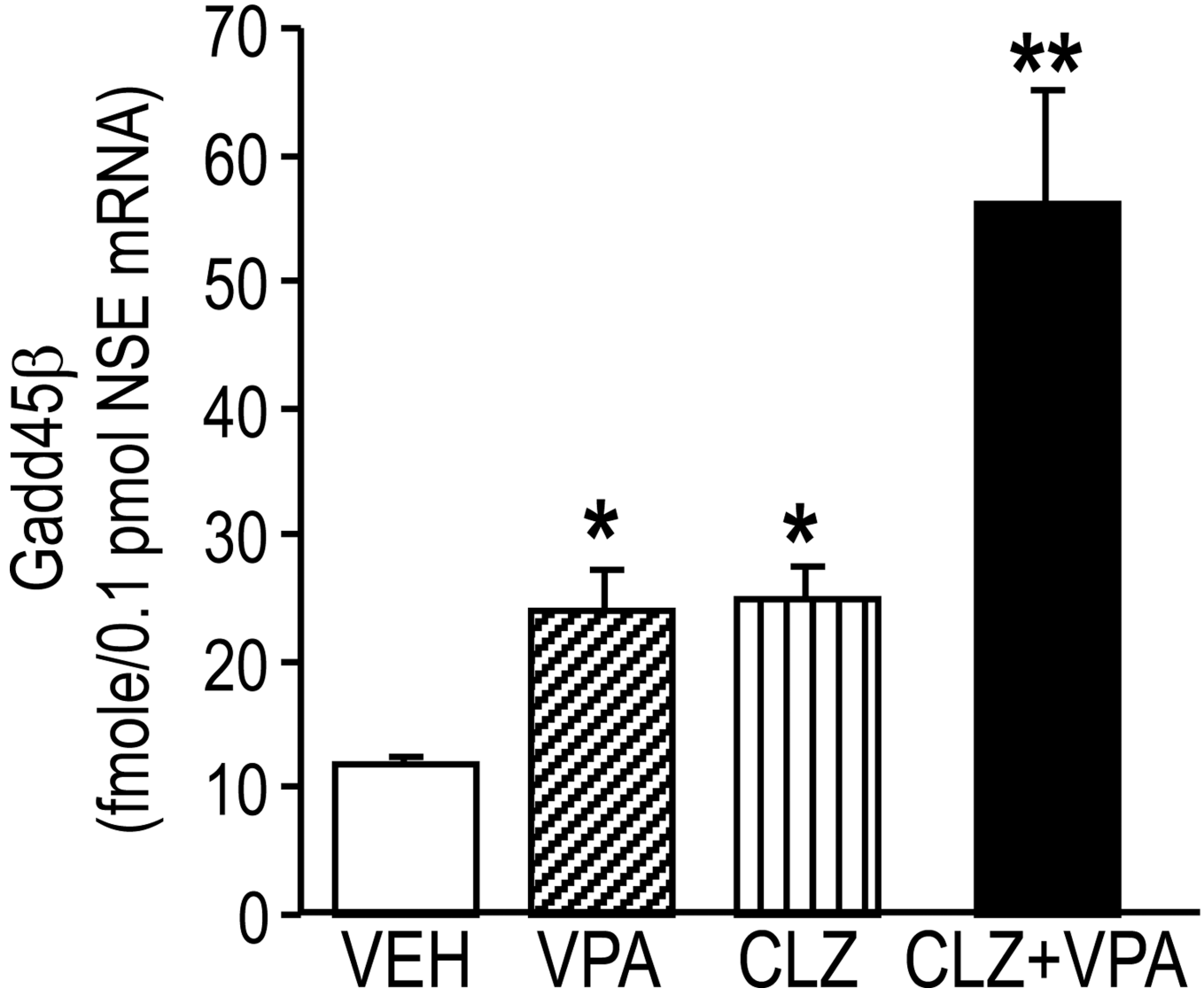


FIG 7. Clozapine (CLZ) alone or in combination with valproate (VPA) increases GADD45 β mRNA expression in FC of mice

CLZ (5 mg/kg s.c./3 days/twice a day); VPA (70 mg/kg s.c./3 days/twice a day); VEH, vehicle. GADD45 β mRNA expression was measured two hours after the last drug injection. *p< 0.05, **p< 0.01 vs VEH group. ANOVA followed by Bonferroni comparison; n=4–5 mice per group.

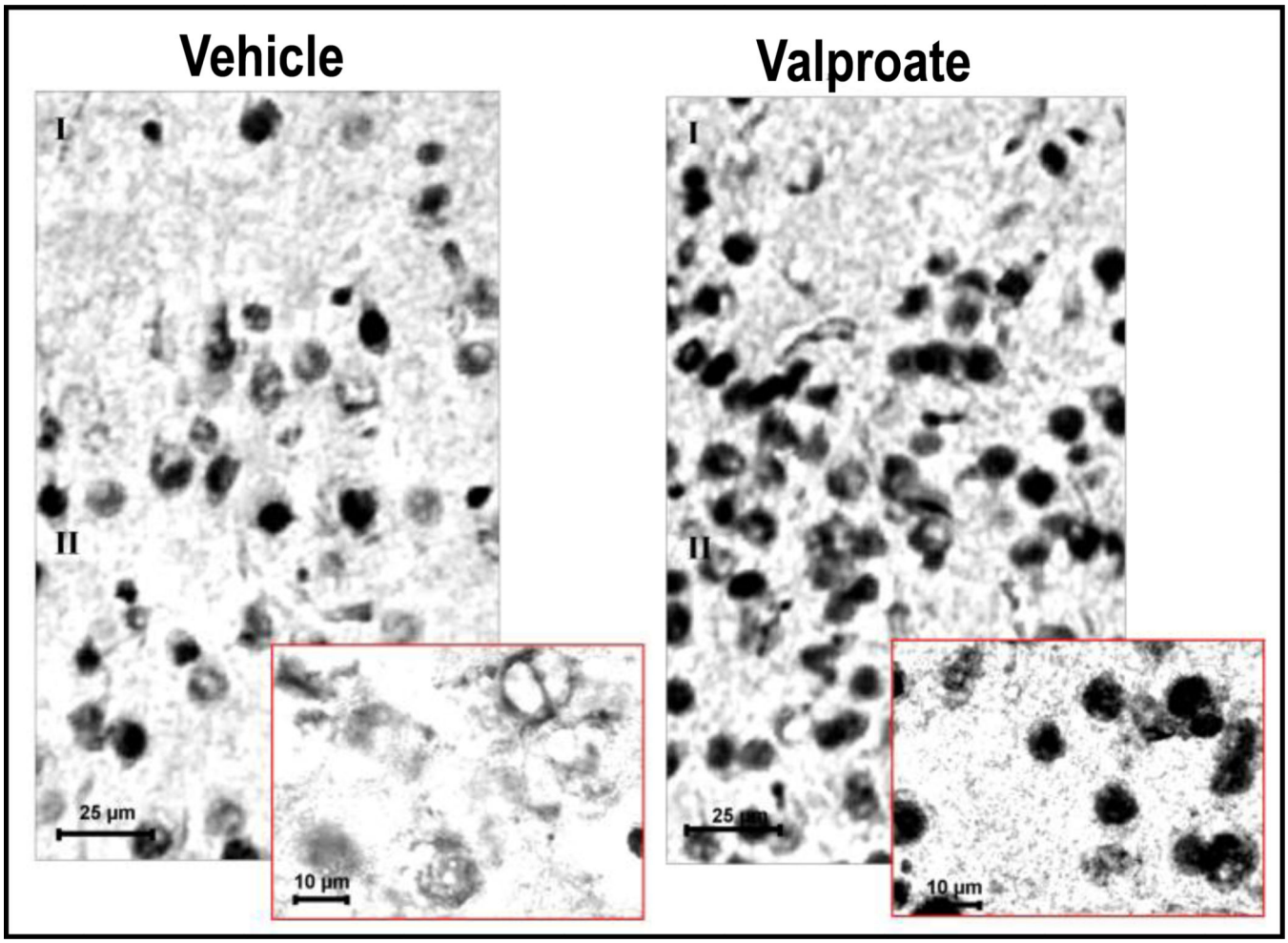


FIG 8. GADD45 β protein levels increase in FC neurons and coalesce in nuclei of mice treated with valproate

Mice were treated with either vehicle or 70 mg/kg s.c. valproate twice a day for three days. Samples were analyzed two hours after the last drug injection.

GADD45 β antibody (Santa Cruz) recognized a major band (~ 18 kDa) of immune-reactive material in western blot of FC extracts.I and II denote layers I and II of cortex.

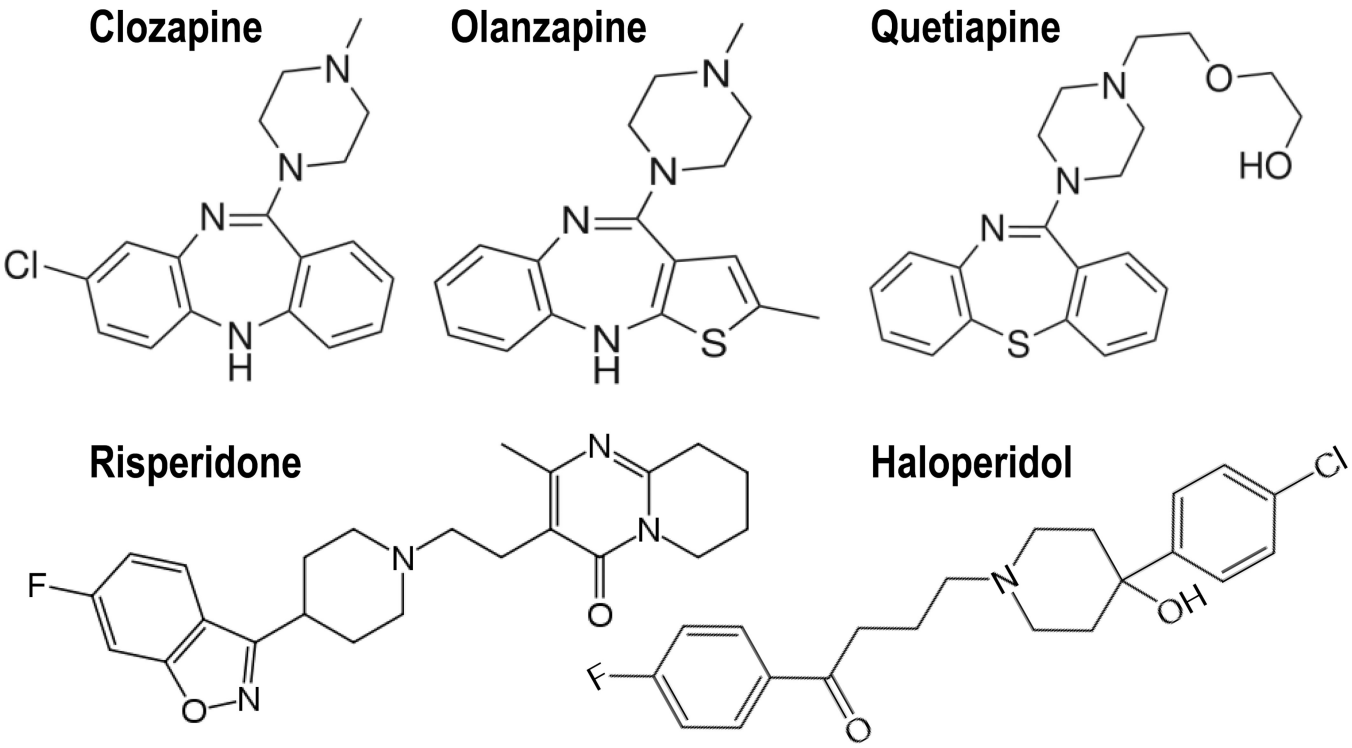


FIG 9. Structures of the antipsychotics used in the study

Table 1

Relative Neurotransmitter Receptor Affinity and GABAergic Promoters Demethylation for Antipsychotics and Valproate (VPA)

Drug	***Receptors***										
	D1	**D2**	**D4**	**5HT 2A**	**5HT 1A**	**5HT2 C**	**A1**	**H1**	**M1**	**mGlur 2/3**	**GABAergic* promoter demethylation**
Haloperidol	+	++++	+++	+	−	−	+++	−	−	−	−
Perphenazine	?	++++	+	+++	−	?	++	−	−	−	?
Olanzapine	++	++	++	+++	−	++	++	+++	++	−	+
Clozapine	+	++	++	+++	+	++	+++	++	++++	−	++
Quetiapine	−	+	−	+	−	_	+++	++	+	−	+
Risperidone	+	++++	+++	++++	−	++	+++	+	−	−	−
Aripiprazol	+	++++	++	++	+++	++	−	+	−	−	?
Sulpiride	?	++	−	−	−	?	−	−	−	−	++
VPA	−	−	−	−	−	−	−	−	−	−	+++
LY2140023	−	−	−	−	−	−	−	−	−	+++	?

* Data from Fig. 4, Table 4, and Dong et al., 2008

Table 2

Time course of the increase of S-adenosyl methionine (SAM) and of its metabolite S-adenosyl homocysteine (SAH) in the cortex of mice receiving methionine (6.6 mmol/kg s.c.)

Treatment	Hours after treatment	SAM	SAH
		pmol/mg protein	
Vehicle	1–4	200 ± 7.1	70 ± 10.0
Methionine	1	305 ± 4.0*	142 ± 8.0*
	2	280 ± 3.8*	120 ± 4.0
	4	295 ± 2.1*	100 ± 2.0*

Each value is the mean ± SE of 4–5 mice

*P <0.01 vs vehicle

Table 3

VPA at a dose that blocks HDAC fails to reduce DNMT1 and DNMT3a mRNA expression in the mouse frontal cortex.

	mRNA (fmol/0.1 pmol NSE)	
	VEH	**VPA**
DNMT1	3.2 ± 0.3	3.1 ± 0.2
DNMT3a	4.8 ± 0.4	4.6 ± 0.8

Mice were treated with vehicle (VEH) or with VPA (2.2 mmol/kg s.c. twice a day for 3 days) and were killed 2 hr after the last injection. Total mRNA isolated from the frontal cortex was analyzed using quantitative competitive PCR. The value was corrected by NSE mRNA, which was co-amplified with DNMT mRNA. Each value use the mean ± SE of five mice.

Table 4

Effects of VPA and various antipsychotics on GAD_{67} or reelin promoter demethylation in the mouse brain (frontal cortex).

Drugs	mg/kg	GAD_{67} or reelin promoter cytosine demethylation	
		−VPA	+VPA
Vehicle	−	+/−	+
Clozapine	1.25	+	+++
Risperidone	10	inactive	inactive
Haloperidol	1.5	inactive	inactive
Olanzapine	10	+	++
Quetiapine	10	+	+++

VPA: 70 mg/kg
Drugs were given s.c. twice a day for three days

Table 5

Clozapine and valproate (VPA) co-administration reverses the methionine-induced downregulation of frontal cortex GAD_{67} expression in a methionine-induced epigenetic mouse model of schizophrenia

Treatments	GAD_{67} protein (vs β actin)
Methionine	0.15 ± 0.020*
Methionine + clozapine	0.19 ± 0.015
Methionine + clozapine + VPA	0.35 ± 0.032**
Methionine + VPA	0.29 ± 0.070
VEH	0.38 ± 0.030

GAD_{67} protein levels (OD ratio of GAD_{67} to β actin) were determined by Western blot analyses in the frontal cortex of mice treated with methionine (5.2 mmol/kg s.c./twice a day/for 7 days)
VEH, VPA (70 mg/kg), clozapine (5 mg/kg) were administered to mice s.c./twice a day/for 3 days after methionine treatment termination.
Data represent the mean ± SE of 4 mice

* P<0.01 for methionine vs VEH

** P<0.01 for methionine vs methionine + clozapine + VPA