

Chapter 2

Epigenetic Regulation of GABAergic Targets in Psychiatry

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Abstract One of the more consistent findings observed in post mortem tissue from schizophrenia (SZ) patients is that the genes encoding reelin and glutamate decarboxylase 67 (*GAD67* or *GAD1*) are downregulated in cortex and other brain regions. Reelin is important for cortical migration during development and for synaptic plasticity and memory acquisition in the adult. *GAD1* is one of two enzymes that synthesize the inhibitory neurotransmitter GABA in the central nervous system. Those neurons that make GABA are GABAergic and they serve a role in dampening excitatory neurotransmission throughout the brain. In addition, reports also show that NMDA receptor subunit expression and excitatory neurotransmission are reduced in cortical GABA neurons of SZ patients. Conditional knockout mice in which the NR1 subunit of the NMDA receptor is selectively reduced in GABA neurons of the brain show a downregulation of *GAD67* and parvalbumin (*PV*) mRNAs and also exhibit behaviors characteristic of SZ. These findings allow us to conceptually integrate two major schools of thought regarding the neurotransmitter deficit responsible for the symptoms of this psychiatric disorder. That is, if reduced glutamatergic neurotransmission occurs on GABAergic interneurons, the net effect would be reduced GABA output impacting the neuronal synchronization of pyramidal cell firing. Since it has also been shown that in GABAergic neurons, the mRNA encoding DNA methyltransferase 1 (*DNMT1*) is increased in SZ patients, this and other data suggest an epigenetic mechanism by which certain genes may be selectively downregulated contributing to SZ symptomatology. We propose that enzymes that methylate DNA and selectively reduce gene expression are hyperactive in patients with SZ and that this may be related to the pathogenesis of the disease. Here, we discuss these concepts in more detail and present our integrated view of synaptic transmission in SZ.

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2.1 Etiopathogenetic Mechanisms Underlying Schizophrenia and Bipolar Disorder

For several decades, monoamines (e.g., dopamine, serotonin, and norepinephrine) (Gray and Roth 2007; Hayman 2008; Lieberman et al. 2008) were the primary biochemical targets used to monitor molecular changes in the brain associated with psychoses. However, since the available antipsychotics that act on monoamine transmission fail to improve the negative and cognitive symptoms associated with psychosis, it is conceivable that an alteration of the monoaminergic system may not be the only source of the spectrum of clinical symptoms in SZ and bipolar disorder (BPD) patients. In fact, a compelling argument based on a comprehensive review of the literature suggests that dopaminergic signaling may represent the final common pathway impacted in SZ (Howes and Kapur 2009). Moreover, these authors suggest that future drug development should focus more on neurotransmitters such as GABA and glutamate, which act upstream of dopamine and that are likely to represent the primary defects associated with the disease. This concept was shaped in part by the notion that dopamine dysregulation, which is more closely linked with psychosis than to SZ, is associated with an altered release presynaptically rather than at the level of dopamine receptors. Presynaptic hyperdopaminergic release in particular brain regions is often linked to excessive excitatory receptor stimulation that is often modulated by GABAergic interneurons (Lisman et al. 2008).

Recent studies have identified abnormalities in GABA, glutamate, and acetylcholine neurotransmitter systems associated with SZ and BPD morbidity (Benes et al. 2007; Akbarian et al. 1995; Breese et al. 2000; Guidotti et al. 2000, 2005; Woo et al. 2005; Lewis et al. 2005). However, it is unclear whether these anomalies cause or are a consequence of these psychiatric disorders.

A number of factors may be responsible for the slow progress in identifying etiopathogenetic mechanisms underlying SZ and BPD. Factors that hamper progress in the discovery of satisfactory drug treatments include (1) the inability to identify alleles that confer increased risk for these diseases in the majority of patients, (2) the poorly understood neurobiological bases of cognition, emotion, and executive brain function that are disrupted in SZ and BPD; (3) the fact that SZ and BPD are diseases exclusively expressed in the human brain; thus any animal models will not represent all the symptoms and signs of the disease; and (4) the scarcity of well-validated accessible objective brain biomarkers that can reveal psychotic dysfunction in patients. The lack of objective testing means that diagnosis of SZ or BPD has to be made on the basis of phenomenological criteria established by clinical experts originating from the neuropsychiatric symptoms at presentation, subsequent drug responsiveness, symptom progression, and illness epidemiology.

Thus, this approach will not enable the identification of new drug targets or novel therapeutic mechanisms.

A recent theory is that these disorders may be the outcome of synergistic interactions between multiple susceptibility genes and environmental (neuroepigenomic) factors (Costa et al. 2007; Mellios et al. 2009; Mill et al. 2008; Ptak and Petronis 2008). However, this concept is still new and should be supported by studies identifying the underlying molecular events. Accordingly, the discovery of the primary epigenetic molecular mechanisms that induce downstream abnormalities of the multiple genes and neurotransmitters that are altered in brains of patients with SZ and BPD is essential.

2.2 Dysfunctional GABA/Glutamate Interactions in SZ and BPD

Several lines of evidence suggest that telencephalic GABAergic transmission may be defective in psychosis (Benes et al. 1992; Akbarian et al. 1995; Guidotti et al. 2000, 2005; Lewis et al. 2005). As a case in point, the expression of glutamic acid decarboxylase (*GAD67*, *GAD1*), reelin, *Reln*, *N*-methyl-D-aspartate receptor subunits (*GRIN*), tyrosine kinase-B (*TRKB*), and other genes such as GABA-transporter-1 (*GAT-1*), nicotinic-acetylcholine receptors (*nAChRs*), somatostatin (*SST*), and numerous calcium binding proteins (Akbarian et al. 1995; Breese et al. 2000; Guidotti et al. 2000, 2005; Woo et al. 2005; Lewis et al. 2005; Costa et al. 2007; Hashimoto et al. 2008; Mellios et al. 2009; Mill et al. 2008) is decreased in GABAergic neurons of the *Stratum oriens* and *Stratum reticularis* of the hippocampus, or the basal ganglia, or in GABAergic neuronal populations located either in the upper cortical layers or proximal to layer III/V pyramidal neurons of SZ patients (Ruzicka et al. 2007; Veldic et al. 2007). However, not all genes expressed in GABAergic neurons are downregulated in psychosis. In fact, the expression of *GAD65* mRNA and protein is unchanged in GABAergic neurons of SZ and BPD patients (Guidotti et al. 2000). This suggests that the downregulation of GABAergic function is not due to a loss of GABAergic neurons.

There is also abundant evidence that glutamatergic transmission may be altered in SZ (Homayoun and Moghaddam 2007; Lisman et al. 2008). Recent data support the concept that insufficient stimulation of NMDA-selective glutamate receptors (GRIN1/GRIN2A receptor containing assemblies) expressed by GABAergic interneurons leads to insufficient GABA release at synapses on cortical pyramidal neurons (Belforte et al. 2010). This could explain why NMDA receptor antagonists such as phencyclidine and dizocilpine (MK-801) induce psychotic episodes when these compounds are ingested (Lisman et al. 2008). It also explains, in part, why metabotropic glutamate receptor (mGluR) agonists that facilitate glutamate release onto GABAergic interneurons may prove beneficial to SZP (Conn et al. 2008). Because most of the observed cortical mRNA changes in SZP have been localized to GABAergic interneurons (at least in the cortex), our

hypothesis is consistent with a disruption of pyramidal neuron synchronization mediated by an inhibitory hypofunction occurring at the level of presynaptic interneurons.

This model was also suggested by recent molecular and behavioral findings in which the NR1 subunit gene (*Grin1*) of the NMDA-selective glutamate receptor complex was selectively knocked out in cortical and hippocampal GABA interneurons of mice (Belforte et al. 2009). Using the cre-loxP system, these investigators created conditional mutant mice in which the NR1 subunit of the NMDA-selective glutamate receptor was ablated during early postnatal development. Interestingly, the loss of the NR1 subunit in ~50% of cortical and hippocampal inhibitory interneurons resulted in the onset of SZ-related symptoms in adolescence. This included novelty-induced hyperlocomotion, mating and nest-building defects, anhedonia, and anxiety-like behaviors. In addition, social memory, spatial working memory, and prepulse inhibition were also impaired. Ultimately, these mice showed reduced expression of Gad67 and parvalbumin accompanied by disinhibition of cortical pyramidal neurons and reduced neuronal synchronization (Belforte et al. 2010). These data are consistent with the hypothesis that reduced glutamatergic stimulation of cortical GABA interneurons results in decreased GABA release and hence inhibitory hypofunction.

This hypothesis (Fig. 2.1) also accounts for both the GABA neuron deficits and the glutamatergic deficits that have thus far been described in SZ and BPD (Lisman et al. 2008). We suggest that NMDA-selective glutamate receptors on cortical

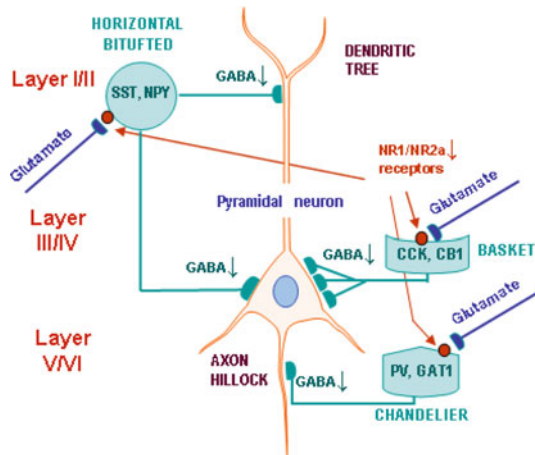


Fig. 2.1 Cortical circuitry links multiple neuronal subtypes. Phenotypically distinct neurons reside in different cortical layers. GABAergic interneurons (aqua) function to modulate the output of pyramidal (tan) and other neurons. We propose that the reduced expression of GAD1 (also known as GAD67) and other GABAergic markers (SST, NPY, CCK, CB1, PV, and GAT1), along with reduced levels of GRIN1- and GRIN2A-containing glutamate receptors, contributes to reduced GABA release. This GABA hypofunction causes decreased pyramidal neuron synchronization

GABA neurons are reduced in SZ due to an epigenetic defect in these GABA neurons arising from upregulation of DNA methyltransferase 1 gene (*DNMT1*). As described below, the hyperexpression of DNMT1 results in promoter methylation and mRNA downregulation of selected genes expressed in GABA neurons of affected individuals.

2.3 Downregulation of *GAD67* and Reelin Promoters in SZ and BPD

GAD67 is the most important enzyme regulating GABA synthesis and function (Soghomonian and Martin 1998; Guidotti et al. 2005). Consequently, it has been hypothesized that the reduced expression of GAD67 in telencephalic GABAergic neurons of psychotic patients markedly reduces the effectiveness of the GABAergic inhibitory neurotransmission that impinges on the dendrites and on the initial axon segments of pyramidal neurons (Fig. 2.1). This deficit of inhibitory neurotransmitters disrupts the intermittent synchronization of pyramidal neuron firing that is critical to the most advantageous performance of cognitive function (Gonzalez-Burgos and Lewis 2008).

In psychosis, not only GAD67 but also reelin is markedly downregulated (Impagnatiello et al. 1998; Fatemi et al. 2000; Guidotti et al. 2000; Ruzicka et al. 2007; Veldic et al. 2007). Reelin is a large (400 kDa) extracellular matrix protein synthesized in large quantities in hippocampal GABAergic interneurons and in GABAergic neurons of cortical layers I and II. Upon secretion from GABAergic interneurons, reelin adheres to postsynaptic densities located on dendritic spines and shafts of pyramidal neurons (D'Arcangelo et al. 1995; Costa et al. 2001). As a result, reelin is believed to play a significant role not only in dendritic spine formation and maturation but also in glutamate receptor homeostasis (Costa et al. 2001; Levenson et al. 2008). On the basis of this role, reduced GAD67 and reelin signaling is almost certainly a reason for the reduced number of dendritic spines (Costa et al. 2001), decreased numbers of glutamate receptors (Levenson et al. 2008), increases in muscimol binding (Benes et al. 1992), and the compensatory increase of postsynaptic $\alpha 5$ (Guidotti et al. 2005) and $\alpha 2$ (Lewis et al. 2005) GABA_A receptor subunits present in pyramidal neurons of prefrontal cortex (PFC) in SZ and BPD patients.

Because of this evidence, we and others (Benes et al. 1992; Akbarian et al. 1995; Guidotti et al. 2000, 2005; Lewis et al. 2005) have suggested that SZ or BPD are diseases characterized predominantly by a deficit of GABAergic function. Not only that, we have postulated that GABAergic neurotransmission may become an important primary research target in the development of more efficient and less toxic treatments for major psychosis. For instance, a positive allosteric modulator of GABA action at GABA_A receptors that is devoid of tolerance liability and sedative action is, in our judgment (Guidotti et al. 2005), a significant candidate for use in therapeutic interventions in psychosis and should be further investigated.

2.4 Epigenetic Mechanisms Are Probably a Component in the GABAergic Dysfunction Expressed by SZ and BPD Patients

Since the deficit of GAD67 and reelin mRNA expression in GABAergic neurons of psychotic patients can be ascribed to genetic abnormalities (copy number variations, deletions, or polymorphisms) in only a few cases, the inheritance and selective pathology of GABAergic neurons may be better understood in the context of epigenetic factors acting on susceptible candidate genes (Costa et al. 2007; Mill et al. 2008; Ptak and Petronis 2008). This hypothesis is supported by data from SZ and BPD patients that show (1) an increased number of cortical GABAergic neurons that stain positive for DNMT1 and DNMT3a mRNA and protein (Veldic et al. 2007; Zhubi et al. 2009); (2) an increased PFC level of *S*-adenosyl-methionine (SAM), the universal methyl donor utilized by DNA-methyltransferases (DNMTs) (Guidotti et al. 2007); and (3) the promoter hypermethylation in reelin and other genes observed in GABAergic neurons (Chen et al. 2002; Abdolmaleky et al. 2005; Grayson et al. 2005; Mill et al. 2008).

2.5 Upregulation of DNA-Methyltransferases and Downregulation of GABAergic Promoters

In our laboratory, studies of postmortem brain have shown that the downregulation of GAD67 and reelin mRNAs in the PFC and basal ganglia of psychotic patients is associated with a significant increase of DNMT1 and DNMT3a in GABAergic neurons (Veldic et al. 2004, 2005, 2007; Ruzicka et al. 2007; Zhubi et al. 2009). DNMTs are the enzymes that transfer a methyl group from SAM to cytosines, most often in a CpG dinucleotide context (Van Emburgh and Robertson 2008). It is notable that in the neocortex, DNMT1 and DNMT3a, which are the most abundant DNA methyltransferases present in the brain, are highly expressed in GABAergic interneurons and are expressed at very low levels in pyramidal neurons and in glial cells (Veldic et al. 2004, 2005, 2007; Ruzicka et al. 2007; Zhubi et al. 2009).

In primary cultures of mouse cortical GABAergic neurons, we showed that an antisense-mediated reduction of *Dnmt1* protein was accompanied by a reduction of *Reln* and *Gad67* promoter methylation and by increased reelin and *Gad67* mRNA expression. These data support the importance of *Dnmts* as an epigenetic regulator of gene expression in GABAergic neurons (Noh et al. 2005). More significantly, an antisense-induced knockdown of *Dnmt1* blocks the methionine-induced downregulation of reelin and *Gad67* mRNA expression (Noh et al. 2005). We recently reported (Satta et al. 2008) that the decrease (~50%) of cortical *Dnmt1* mRNA and protein induced in mice following protracted subcutaneous nicotine administration (0.75–2.5 mg/kg, four times a day for 4 days) is associated with a comparable

decrease of Dnmt1 protein, a 40–50% decrease of *Gad*₆₇ promoter methylation, and an overexpression of Gad67 protein in GABAergic interneurons. The increase of DNMT1 and to a lesser extent of DNMT3a mRNA in the brains of SZ and BPD patients is probably specific to these illnesses. In contrast, in the brains of suicidal/depressed patients, an increase of DNMT3b but not DNMT1 or DNMT3a mRNA has been reported (Poulter et al. 2008). It appears that the overexpression of DNMT1 and DNMT3a mRNA in the brains of SZ and BPD patients cannot be ascribed to demographic factors (gender, onset of illness, or duration of illness) or to the type, dose, or duration of antipsychotic administration. In fact, there was no change in cortical Dnmt1 mRNA content in mice that received haloperidol (1 mg/kg) or clozapine (5 mg/kg) subcutaneously for 21 days (Satta, this laboratory, personal communication). These data are in keeping with the concept that the downregulation in the expression of various genes in GABAergic neurons of SZ and BPD patients may be related to a DNMT1/DNMT3a-mediated methylation of the corresponding promoters (Costa et al. 2007; Mill et al. 2008).

This conclusion may be related to clinical reports dating to the late 1960s indicating that daily administration of high doses of methionine exacerbated or even triggered psychotic episodes in a SZ patient subpopulation (Wyatt et al. 1971). Since the administration of high doses of methionine increases the brain content of SAM (Tremolizzo et al. 2002, 2005), it may be inferred that the increased cortical availability of SAM associated with the increased expression of *DNMTs* may be responsible for this hypermethylation of promoters in SZ and BPD patients (Guidotti et al. 2007).

Taken together, these data suggest the hypothesis that the GABAergic mRNA downregulation occurring in SZ and BPD is likely the consequence of transcriptional repression due to promoter hypermethylation directly induced by DNMT overexpression. An alternative hypothesis is that the inhibitory action of DNMTs on GABAergic gene expression could be mediated by an interaction of DNMT with specific chromatin repressor proteins (e.g., methyl-CpG binding domain proteins, SIN3A, and histone deacetylases [HDACs]), which repress transcription via a modification of chromatin structure (i.e., shifting chromatin from a permissive open conformation to a repressive closed conformation).

Previous studies from our laboratory have shown that in the PFC, reelin expression in humans is inversely related to *DNMT1* expression (Veldic et al. 2004, 2005, 2007; Ruzicka et al. 2007; Zhubi et al. 2009) and in human neuroprogenitor (NT2) cells is regulated through changes in methylation status and chromatin structure in the vicinity of the promoter (Kundakovic et al. 2009). A goal of our recent research has been to establish the precise molecular mechanism(s) responsible for the DNMT-mediated epigenetic regulation of genes encoding reelin and GAD67. Here, we report our findings from in vitro studies that (1) confirm the coordinated regulation of the human reelin and *GAD67* promoters and (2) elucidate the molecular mechanisms that underlie this regulation.

An appreciation of this information provides a mechanistic rationale for our epigenetic hypothesis of SZ and BPD and for developing new pharmacological treatments for these diseases.

2.6 Epigenetic Drugs Coordinately Upregulate Reelin and *GAD67* mRNA Expression

We first tested the hypothesis that human reelin and *GAD67* genes are coordinately regulated through epigenetic mechanisms. We treated NT2 cells with two different classes of epigenetic drugs, DNMT inhibitors and HDAC inhibitors. Our studies demonstrated that three distinct DNMT inhibitors: a DNA intercalator doxorubicin (DOXO) and two nucleoside analogues, 5-aza-2'-deoxycytidine (AZA) and zebularine (ZEB), are able to significantly increase reelin and *GAD67* mRNA levels (Kundakovic et al. 2007, 2009). Similarly, three structurally unrelated HDAC inhibitors: MS-275 (benzamide derivative), valproic acid (VPA; aliphatic acid), and trichostatin A (TSA; hydroxamate) induce reelin and *GAD67* mRNA expression in our cell system (Kundakovic et al. 2009).

To strengthen the conclusion that reelin and *GAD67* promoters are coordinately regulated, we further examined the expression patterns of these mRNAs following treatment with the DNMT inhibitor DOXO and the HDAC inhibitor MS-275 (Figs. 2.2 and 2.3). Importantly, the detailed studies with DOXO and MS-275 produced similar findings and demonstrated that reelin and *GAD67* mRNA induction occurs (a) in a comparable dose-dependent manner (as shown by the very

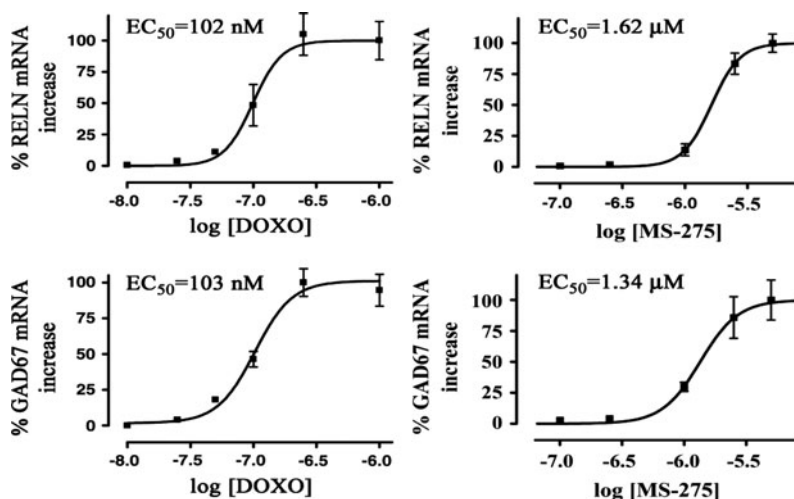


Fig. 2.2 DOXO and MS-275 lead to dose-dependent increases in reelin and *GAD67* mRNAs. Results of quantitative analysis of reelin (RELN) and *GAD67* mRNA levels following 48-h treatments of NT-2 cells with (a) DOXO (0.01–1 μM) and (b) MS-275 (0.1–10 μM) are presented as dose–response curves. RT-PCR data are plotted as a percent of maximal (reelin or *GAD67*) mRNA increase (y-axis) as a function of log [drug concentration] (shown on the x-axis). EC₅₀ is the concentration of drug that leads to 50% of maximal reelin or *GAD67* mRNA increases. Results are expressed as the mean ± S.E.M. of three independent experiments. Dose–response curves are generated using Prism version 5 (GraphPad Software, San Diego, CA), and EC₅₀ values are compared by *F*-test. Panels a (Kundakovic 2009) and b (Kundakovic et al. 2007) are reprinted with permission of the American Society for Pharmacology and Experimental Therapeutics

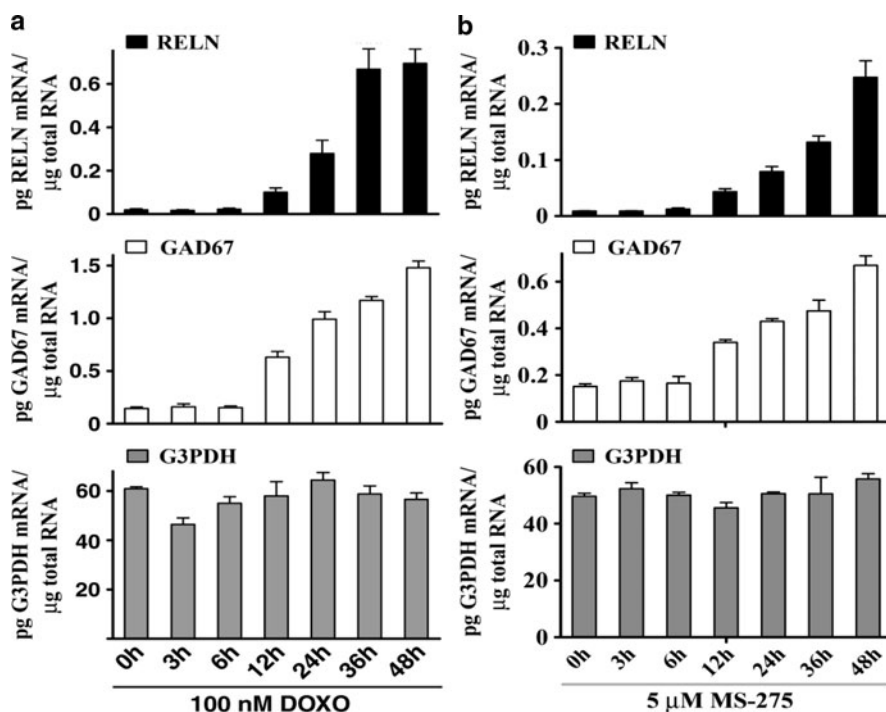


Fig. 2.3 Both DOXO and MS-275 increase reelin and GAD67 mRNA expression in a similar time-dependent manner. Results of quantitative analysis of reelin (RELN), GAD67, and G3PDH mRNA levels in NT-2 cells treated for various times with (a) 100 nM DOXO or (b) 5 μM MS-275. Data (mean ± S.E.M. from at least three independent experiments) are presented as amounts (pg) of reelin, GAD67, and G3PDH mRNA per 1 μg of total RNA at the indicated time points (x-axis). Panels a (Kundakovic 2009) and b (Kundakovic et al. 2007) are reprinted with permission of the American Society for Pharmacology and Experimental Therapeutics

similar EC_{50} and EC_{100} values for the induction of both mRNAs for each drug) (Fig. 2.2); and (b) within the same time frame (both mRNAs begin to be induced ~12 h after starting individual DOXO or MS-275 treatments) (Fig. 2.3). The similar concentration-dependence and temporal activation patterns of reelin and GAD67 mRNAs by drugs that target the epigenome strongly support our hypothesis that these two genes are coordinately regulated through epigenetic mechanisms.

2.7 Activation of the Reelin and GAD67 Promoters Is Associated with Downregulation of the DNMT1, DNMT3A, and DNMT3B Proteins

It is of interest that both groups of drugs (DNMT and HDAC inhibitors) down-regulate DNMT1 protein, suggesting a link between DNMT1 content and reelin gene and GAD67 mRNA expression (Figs. 2.4a, b). Interestingly, the DNMT

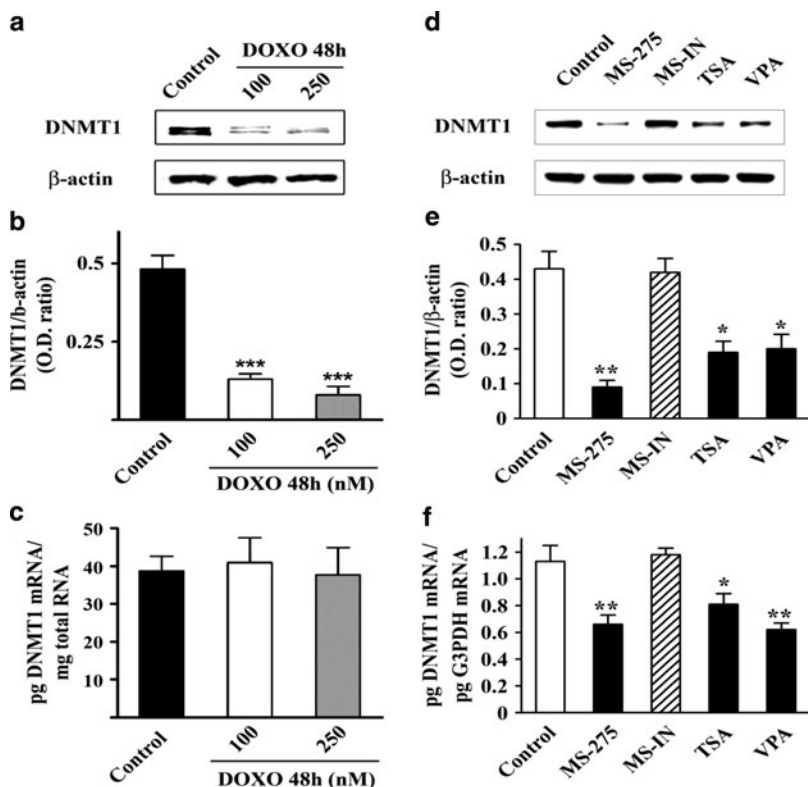


Fig. 2.4 DNMT inhibitors and HDAC inhibitors downregulate DNMT1 protein expression. The representative Western immunoblots and the ratio of the DNMT1 band over the area of the β -actin band in nuclear extract protein samples from untreated cells (control) and cells treated with either (a and b) DNMT inhibitor DOXO (100 and 250 nM, 48 h) or (d and e) HDAC inhibitors MS-275 (5 μ M, 48 h), TSA (0.3 μ M, 24 h), VPA (5 mM, 24 h), and an inactive stereoisomer of MS-275 (MS-IN, 5 μ M, 48 h); bar graphs show DNMT1 mRNA levels in the same DOXO-treated (c) and HDAC inhibitor-treated cells (f) obtained using a competitive RT-PCR assay. Data are presented as amount of DNMT1 mRNA (in pg) per 1 μ g total RNA and normalized to the corresponding G3PDH mRNA levels. Data represent the mean \pm SEM. *** p < 0.001; * p < 0.05 vs. control group. (One-way ANOVA followed by Bonferroni test.) Panels b, c (Kundakovic 2009) and d, e (Kundakovic et al. 2007) are reprinted with permission of the American Society for Pharmacology and Experimental Therapeutics

inhibitor DOXO does not change DNMT1 mRNA levels (Fig. 2.4c), implying that DNMT inhibitors reduce DNMT1 posttranscriptionally. On the other hand, HDAC inhibitors decrease DNMT1 mRNA expression under the same conditions (Fig. 2.4d–f), suggesting that these drugs downregulate *DNMT1*, at least in part, by affecting its mRNA synthesis and/or degradation. Importantly, the downregulation of DNMT1 precedes reelin and *GAD67* mRNA induction (Kundakovic et al. 2009), implicating a role for this protein in regulating reelin and *GAD67* promoters. Importantly, the activation of reelin and *GAD67* mRNA expression with both

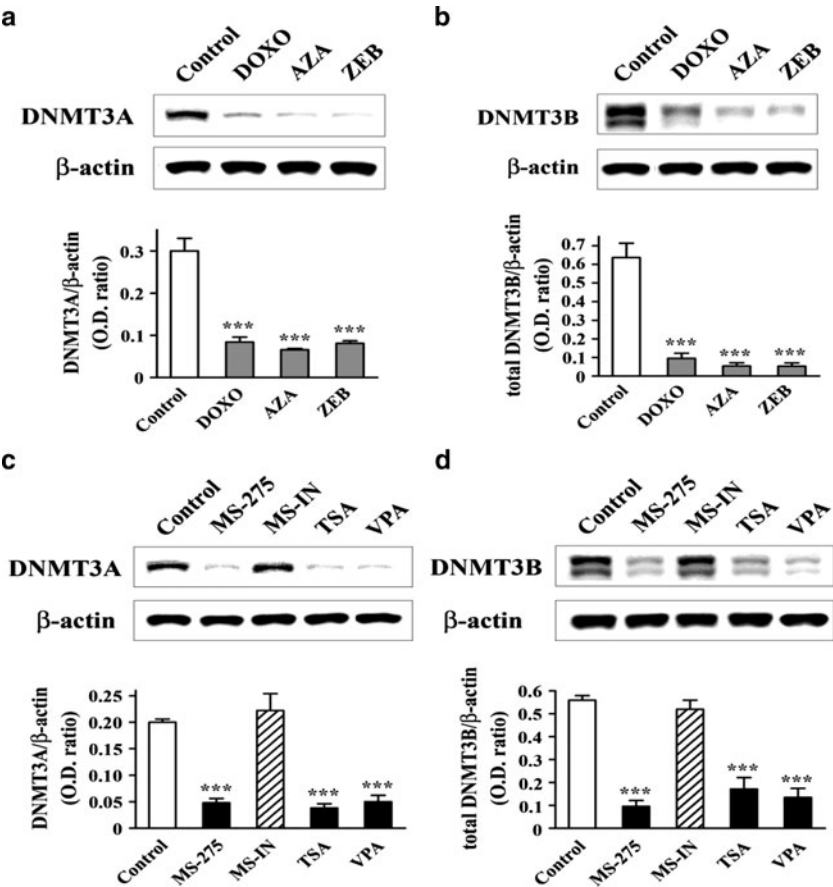


Fig. 2.5 DNMT and HDAC inhibitors decrease DNMT3A and DNMT3B protein levels. Western blot analyses of DNMT3A and DNMT3B proteins were performed using nuclear extracts prepared from control cells and cells treated with either DNMT inhibitors (**a** and **b**) or HDAC inhibitors (**c** and **d**). For assays with DNMT inhibitors, cells were treated with 250 nM DOXO (48 h), 5 μ M AZA (48 h), or 500 μ M ZEB (48 h followed by 48-h incubation with untreated medium). HDAC inhibitor treatments were performed as follows: 5 μ M MS-275 (48 h), 5 μ M MS-IN (48 h), 0.3 μ M TSA (24 h), and 5 mM VPA (24 h). In all cases (**a–d**), representative Western blots are shown (*upper panels*) together with the graphs depicting the ratio of the DNMT3A or DNMT3B (both isoforms) band over the area of the β -actin band (*lower panels*). Data represent the mean \pm S.E.M. of three independent experiments. *** $p < 0.001$ vs. control group (one-way ANOVA followed by Bonferroni test). Reprinted (Kundakovic et al. 2007) with permission of the American Society for Pharmacology and Experimental Therapeutics

DNMT and HDAC inhibitors was also associated with the downregulation of two other DNMT enzymes, DNMT3A and DNMT3B, in nuclei of NT-2 cells (Fig. 2.5). Figure 2.5a shows the effects of DNMT inhibitors on DNMT3A protein levels, while Fig. 2.5c shows the same for various HDAC inhibitors. Similarly, Fig. 2.5b

shows the results obtained using selected DNMT inhibitors on DNMT3B protein with comparable results using various HDAC inhibitors shown below (Fig. 2.5d). The inactive enantiomer of MS-275 had no effect on the level of either protein.

2.8 Epigenetic Drugs Facilitate the Dissociation of DNMT-Containing Repressor Complexes from Reelin and *GAD67* Promoters

Our studies provide evidence that all three DNMT proteins, DNMT1, DNMT3A, and DNMT3B, might participate in the formation of transcriptional repressor complexes at the *reelin* and *GAD67* promoters (Fig. 2.6). These complexes also include MeCP2 and HDAC1 proteins. Our data support the concept that treatment with DNMT inhibitors (Kundakovic et al. 2009) and HDAC inhibitors (Fig. 2.6) results in the dissociation of all three DNMT proteins, together with MeCP2 and HDAC1, from both promoters. Increased local histone acetylation was also observed, implying that both classes of drugs facilitate the relaxation of chromatin

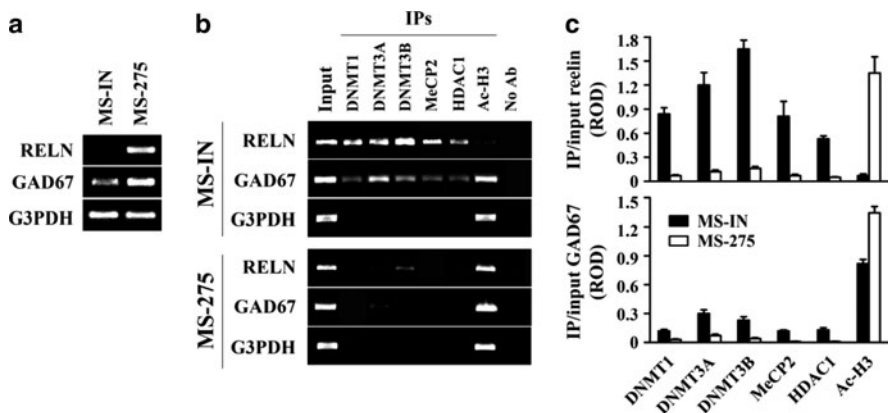


Fig. 2.6 Reelin and *GAD67* gene activation is accompanied by dissociation of repressor complexes from the corresponding promoters. NT-2 cells were treated with either 5 μ M of HDAC inhibitor MS-275 or 5 μ M of its inactive stereoisomer MS-IN for 48 h. (a) RT-PCR analysis confirmed that reelin and *GAD67* mRNAs were induced by MS-275 compared to MS-IN treatment. (b) The corresponding chromatin preparations were immunoprecipitated with DNMT1, DNMT3A, DNMT3B, MeCP2, HDAC1, and Ac-H3 antibodies (IPs). Nonimmunoprecipitated samples were used as negative controls (No Ab). DNA isolated from inputs, Ips, and control samples were PCR-amplified using primers specific for the reelin, *GAD67*, and *G3PDH* promoter regions. Relative optical densities (RODs) of the bands derived from ethidium bromide-stained gels were quantified. (c) Graphs show the results (mean \pm S.E.M. of three independent experiments) of semiquantitative analysis of the occupancy of DNMT1, DNMT3A, DNMT3B, MeCP2, HDAC1, and Ac-H3 on the reelin (*upper panel*) and *GAD67* (*lower panel*) promoters in MS-IN- and MS-275-treated cells, normalized to input DNA (each comparison MS-IN vs. MS-275 showed a statistical significance of at least $p < 0.05$, t -test). Reprinted (Kundakovic et al. 2007) with permission of the American Society for Pharmacology and Experimental Therapeutics

surrounding the *reelin* and *GAD67* promoters. In addition, each inhibitor reduces total nuclear DNMT enzyme activity and facilitates a reduction in DNA methylation in the same *reelin* and *GAD67* promoter regions that are associated with changes in chromatin structure (Kundakovic et al. 2009). These data imply that the formation of the repressor complexes is likely DNA-methylation dependent. Furthermore, we suggest that promoter demethylation might not be required for a slight to moderate induction of *reelin* and *GAD67* transcription, but is likely relevant for maximal activation of these two promoters.

2.9 Discussion

Results from our studies provide evidence that the human *reelin* and *GAD67* promoters are coordinately regulated through epigenetic mechanisms and also suggest an underlying molecular mechanism to understand this regulation. Our data imply that both promoters are negatively regulated through methylation-dependent recruitment of transcriptional repressor complexes containing DNMT1, DNMT3A, DNMT3B, MeCP2, and HDAC1 proteins. These complexes reduce the transcriptional activity of the promoters by shifting the surrounding chromatin into a more compact state, thus resulting in decreased transcription factor accessibility (Fig. 2.7). While our data are directly applicable to the epigenetic regulation of the *reelin* and *GAD67* promoters in neuronal progenitor cells, an increasing body of evidence suggests that similar regulatory mechanisms are operative in adult GABAergic neurons (Costa et al. 2004, 2006; Szyf et al. 2008).

Further, this study gives new insight into the molecular mechanisms that underlie the downregulation of *reelin* and *GAD67* mRNAs in the brains of SZ. It has been reported that the reductions in *reelin* and *GAD67* transcripts correlate with increased DNMT1 and HDAC1 expression in the same GABAergic neurons (Hayes 1989; Ruzicka et al. 2007; Benes et al. 2007). Therefore, we propose that the upregulation of DNMT1 mRNA could promote downregulation of the *reelin* and *GAD67* genes by inducing promoter hypermethylation (Abdolmaleky et al. 2005; Chen et al. 2002; Grayson et al. 2005) and increased binding of DNMT1- and HDAC1-containing corepressor complexes to the *reelin* and *GAD67* promoters. According to our data, these complexes most likely also contain additional DNMTs (DNMT3A and DNMT3B) and MeCP2 and other proteins as well. However, further studies with postmortem human brains will be necessary to confirm this hypothesis.

Additionally, we would like to suggest a new approach for the treatment of SZ that focuses on the reactivation of mRNA expression profiles that are downregulated due to modifications in the epigenome. We report that treatment of neuronal progenitor cells with various DNMT and HDAC inhibitors leads to a robust induction of *reelin* and *GAD67* mRNAs. Furthermore, we demonstrate that both classes of epigenetic drugs target DNMT1 and HDAC1, which are aberrantly expressed in SZ (Fig. 2.7). These drugs downregulate DNMT1 and directly or indirectly inhibit the repressor activity of HDAC1. Moreover, the same drugs induce

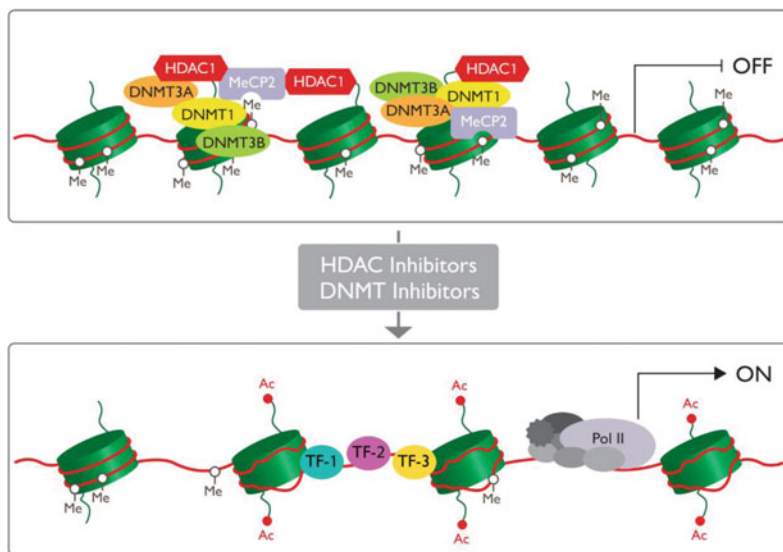


Fig. 2.7 Hypothetical model for promoter activation by HDAC and DNMT inhibitors. For simplicity, promoters are shown as either silent (repressed – OFF) or fully active (ON). The transcriptionally inactive chromatin structure surrounding the indicated promoter region (*upper panel*) is the consequence of cytosine methylation and subsequent recruitment of repressor proteins, including DNMT1, DNMT3A, DNMT3B, MeCP2, and HDAC1 (most likely others, also). The downregulation of DNMT proteins (by DNMT inhibitors and HDAC inhibitors), together with the inhibition of HDAC enzymatic activity and the decrease in MeCP2 expression (in the case of the HDAC inhibitors), results in dissociation of these repressor complexes. This leads to DNA demethylation, histone acetylation, and a relaxation of the chromatin surrounding the respective regulatory regions (*lower panel*). The more open chromatin configuration allows the recruitment of specific transcription factors (TFs), such as Sp1 and the general transcriptional machinery (*gray shapes*) to the promoters. Collectively, this results in the drug-induced epigenetic changes leading to promoter activation (as we have proposed for the reelin and GAD67 genes)

changes in the methylation status of the CpG-island-containing reelin promoter that is hypermethylated in the brains of SZ patients (Veldic et al. 2004, Abdolmaleky et al. 2005, Grayson et al. 2005). Therefore, these data provide a mechanistic rationale for our hypothesis that HDAC inhibitors and DNMT inhibitors used either individually or in combination may represent a novel pharmacological approach for correcting reelin and GAD67 mRNA levels, and the GABAergic deficits associated with SZ (Guidotti et al. 2005; Levenson 2007).

A recent study examined alterations in GABA-related mRNAs in the PFC of subjects with SZ (Hashimoto et al. 2008). Reduced mRNA levels corresponding to presynaptic regulators of GABA function, numerous neuropeptides, and GABA-A receptor subunits were detected. In addition, studies show that the downregulation of NMDA receptors in GABA neurons could be the consequence of a primary epigenetic defect (Belforte et al. 2010). These data are consistent with the GABA deficit hypothesis proposed by us (Guidotti et al. 2005) and others (Lewis et al. 2005;

Benes et al. 2007). Additional work will identify which mRNAs are activated by various HDACs, in which brain regions and in which neurons. As the collection of these compounds increases, it should not be too long before researchers are able to design these drugs to target subclasses of HDACs showing selective expression patterns in the desired brain regions (Broide et al. 2007).

Preliminary data in rodents support the notion that MS-275 crosses the blood–brain barrier and increases histone acetylation in the frontal cortex of treated animals (Simonini et al. 2006). However, a recent pharmacokinetic study using positron emission tomography (PET) showed that MS-275 exhibits poor brain penetration (Hooker et al. 2010). So while the levels of MS-275 that cross into the brain may be sufficient for some assays, this issue needs additional clarification in terms of new drug design. Increasing attention is being paid to various HDAC inhibitors in the context of therapeutic intervention for certain cancers. It seems prudent that a similar approach may prove beneficial in reactivating genes that are downregulated as a consequence of promoter hypermethylation, as we have suggested occurs in SZ (Grayson et al. 2010). For example, in addition to *reelin* and *GAD67*, the promoter corresponding to the NMDA receptor subunit 2A is embedded in a CpG island and is downregulated in the postmortem cortex of SZ subjects (Woo et al. 2005, 2008). Interestingly, this reduced expression is in parvalbumin-expressing neurons of the cortex that also contain *GAD67* and *reelin* (Noh et al. 2005). Clearly, the glutamatergic input onto cortical GABA interneurons plays a key role in determining synaptic GABA release onto efferent pyramidal neurons. This suggests that the two major conceptual schools that characterize the dysfunctional neural circuits that define SZ may contribute to an integrated hypothesis. That is, glutamatergic hypofunction and GABAergic hypofunction are linked provided they function at the level of the same cortical GABA neurons. Thus, it is possible that drugs acting at the level of chromatin remodeling could induce each of the mRNAs downregulated in SZ, thereby correcting the GABA neuron deficits. As such, by increasing the GABA interneuron content of *GAD67*, *reelin*, and NMDA receptor subunits, relevant neurons may more appropriately respond to uncompromised afferent NMDA receptor stimulation by releasing more GABA.

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References

- Abdolmaleky HMK, Cheng A, Russo CL, Smith SV, Faraon M, Wilcox R, Shafa SJ, Glatt G, Nguyen JF, Ponte S, Thiagalingam TMT (2005) Hypermethylation of the *reelin* (*RELN*) promoter in the brain of schizophrenic patients: a preliminary report. *Am J Med Genet B Neuropsychiatr Genet* 134:60–66
- Akbarian S, Kim JJ, Potkin SG, Hagman JO, Tafazzoli A, Bunney WE, Jones EG (1995) Gene expression for glutamic acid decarboxylase is reduced without loss of neurons in prefrontal cortex of schizophrenics. *Arch Gen Psychiatry* 52:258–266

- Belforte JE, Zsiros V, Sklar ER, Jiang Z, Yu G, Quinlan Li Y, Nakazawa EMK (2010) Postnatal NMDA receptor ablation in corticolimbic interneurons confers schizophrenia-like phenotypes. *Nat Neurosci* 13:76–83
- Benes J, Vincent SL, Alsterberger G, Bird ED, San Giovanni JP (1992) Increased GABAergic receptor binding in superficial layers of cingulate cortex in schizophrenia. *J Neurosci* 12: 924–929
- Benes FM, Lim B, Matzilevich D, Walsh JP, Subburaju S, Minns M (2007) Regulation of the GABA cell phenotype in hippocampus of schizophrenics and bipolars. *Proc Natl Acad Sci USA* 104:10164–10169
- Breese CR, Lee MJ, Adams CE, Sullivan B, Logel J, Gillen KM, Marks MJ, Collins AC, Leonard S (2000) Abnormal regulation of high affinity nicotinic receptors in subjects with schizophrenia. *Neuropsychopharmacology* 23:351–364
- Broide RS, Redwine JM, Aftahi N, Yound W, Bloom FE, Winrow CJ (2007) Distribution of histone deacetylases 1–11 in the rat brain. *J Mol Neurosci* 31:47–58
- Chen Y, Sharma RP, Costa RH, Costa E, Grayson DR (2002) On the epigenetic regulation of the human reelin promoter. *Nucleic Acids Res* 30:2930–2939
- Conn PJ, Lindsley CW, Jones CK (2008) Activation of metabotropic glutamate receptors as a novel approach for the treatment of schizophrenia. *Trends Pharmacol Sci* 30:25–31
- Costa E, Davis J, Grayson DR, Guidotti A, Pappas GD, Pesold C (2001) Dendritic spine hypoplasticity and downregulation of reelin and GABAergic tone in schizophrenia vulnerability. *Neurobiol Dis* 8:723–742
- Costa E, Davis JM, Dong E, Grayson DR, Guidotti A, Tremolizzo L, Veldic M (2004) A GABAergic cortical deficit dominates schizophrenia pathophysiology. *Crit Rev Neurobiol* 16:1–23
- Costa E, Dong E, Grayson DR, Ruzicka WB, Simonini MV, Veldic M, Guidotti A (2006) Epigenetic targets in GABAergic neurons to treat schizophrenia. *Adv Pharmacol* 54: 95–117
- Costa E, Dong E, Grayson DR, Guidotti A, Ruzicka WB, Veldic M (2007) Reviewing the role of DNA (cytosine-5) methyltransferase overexpression in the cortical GABAergic dysfunction associated with psychosis vulnerability. *Epigenetics* 2:29–36
- D'Arcangelo G, Miao GG, Chen SC, Soares HD, Morgan JI, Curran T (1995) A protein related to extracellular matrix proteins deleted in the mouse mutant reeler. *Nature* 376:292–293
- Fatemi SH, Earle JA, McMenomy T (2000) Reduction in Reelin immunoreactivity in hippocampus of subjects with schizophrenia, bipolar disorder and major depression. *Mol Psychiatry* 5:654–663
- Gonzalez-Burgos G, Lewis DA (2008) GABA neurons and the mechanisms of network oscillations: implications for understanding cortical dysfunction in schizophrenia. *Schizophr Bull* 34: 944–961
- Gray JA, Roth BL (2007) The pipeline and future of drug development in schizophrenia. *Mol Psychiatry* 12:904–922
- Grayson DR, Jia X, Chen Y, Sharma RP, Mitchell CP, Guidotti A, Costa E (2005) Reelin promoter hypermethylation in schizophrenia. *Proc Natl Acad Sci USA* 102:9341–9346
- Grayson DR, Kundakovic M, Sharma RP (2010) Is there a future for HDAC inhibitors in the pharmacotherapy of psychiatric disorders? *Mol Pharmacol* 77(2):126–135
- Guidotti A, Auta J, Davis JM, Dwivedi Y, Gerevini VD, Impagnatiello F, Pandey G, Pesold C, Sharma R, Uzunov DP, Costa E (2000) Decrease in reelin and glutamic acid decarboxylase₆₇ (GAD₆₇) expression in schizophrenia and bipolar disorder, a postmortem brain study. *Arch Gen Psychiatry* 57:1061–1069
- Guidotti A, Auta J, Davis JM, Dong E, Grayson DR, Veldic M, Zhang X, Costa E (2005) GABAergic dysfunction in schizophrenia: a new treatment on the horizon. *Psychopharmacology (Berl)* 180:191–205
- Guidotti A, Ruzicka W, Grayson DR, Veldic M, Davis J, Costa E (2007) S-Adenosyl methionine and DNA-methyltransferase-1 overexpression in psychosis. *Neuroreport* 18:57–60

- Hashimoto T, Arion D, Unger T, Maldonado-Aviles JG, Morris HM, Volk DW, Mirnics K, Lewis DA (2008) Alterations in GABA-related transcriptome in the dorsolateral prefrontal cortex of subjects with schizophrenia. *Mol Psychiatry* 13:147–161
- Hayes SG (1989) Long-term use of valproate in primary psychiatric disorders. *J Clin Psychiatry* 50:35S–39S
- Hayman S (2008) A glimmer of light for neuropsychiatric disorders. *Nature* 455:890–893
- Hooker JM, Kim SW, Alexoff D, Xu Y, Shea C, Reid A, Volkow N, Fowler JS (2010) Histone deacetylase inhibitor MS-275 exhibits poor brain penetration: pharmacokinetic studies of [^{11}C] MS-275 using positron emission tomography. *ACS Chem Neurosci* 1:65–73
- Homayoun H, Moghaddam B (2007) NMDA receptor hypofunction produces opposite effects on prefrontal cortex interneurons and pyramidal neurons. *J Neurosci* 27:11496–11500
- Howes OD, Kapur S (2009) The dopamine hypothesis of schizophrenia: version III—the final common pathway. *Schizophr Bull* 35:549–562
- Impagnatiello F, Guidotti A, Pesold C, Dwivedi Y, Caruncho H, Pisu MG, Uzunov DP, Smalheiser NR, Davis JM, Pandey GN, Pappas GD, Tueting P, Sharma RP, Costa E (1998) A decrease of reelin expression as a putative vulnerability factor in schizophrenia. *Proc Natl Acad Sci USA* 95:15718–15723
- Kundakovic M, Chen Y, Costa E, Grayson DR (2007) DNA methyltransferase inhibitors coordinately induce expression of the human reelin and glutamic acid decarboxylase 67 genes. *Mol Pharmacol* 71:644–653
- Kundakovic M, Chen Y, Guidotti A, Grayson DR (2009) The reelin and GAD67 promoters are activated by epigenetic drugs that facilitate the disruption of local repressor complexes. *Mol Pharmacol* 75:342–354
- Levenson JM, Qiu S, Weeber EJ (2008) The role of reelin in adult synaptic function and the genetic and epigenetic regulation of the reelin gene. *Biochim Biophys Acta* 1779:433–431
- Lewis DA, Hashimoto T, Volk DW (2005) Cortical inhibitory neurons and schizophrenia. *Nat Rev Neurosci* 6:312–324
- Lieberman JA, Bymaster FP, Meltzer HY, Deutch AY, Duncan GE, Marx CE, Aprille JR, Dwyer DS, Li XM, Mahadik SP, Duman RS, Porter JH, Modica-Napolitano JS, Newton SS, Csernansky JG (2008) Antipsychotic drugs, comparison in animal models of efficacy, neurotransmitter regulation, and neuroprotection. *Pharmacol Rev* 60:358–403
- Lisman JE, Coyle JT, Green RW, Javitt DC, Benes FM, Heckers S, Grace AA (2008) Circuit-based framework for understanding neurotransmitter and risk gene interactions in schizophrenia. *Trends Neurosci* 31:234–242
- Mellios N, Huang HS, Baker SP, Galdzicka M, Ginns E, Akbarian S (2009) Molecular determinants of dysregulated GABAergic gene expression in the prefrontal cortex of subjects with schizophrenia. *Biol Psychiatry* 65:1006–1014
- Mill J, Tang T, Kaminsky Z, Khare T, Yazdanpanah S, Bouchard L, Jia P, Assadzadeh A, Flanagan J, Schumacher A, Wang SC, Petronis A (2008) Epigenomic profiling reveals DNA-methylation changes associated with major psychosis. *Am J Hum Genet* 82:696–711
- Noh JS, Sharma RP, Veldic M, Salvacion AA, Jia X, Chen Y, Costa E, Guidotti A, Grayson DR (2005) DNA methyltransferase 1 regulates reelin mRNA expression in mouse primary cortical cultures. *Proc Natl Acad Sci USA* 102:1749–1754
- Poulter MO, Du L, Weaver IC, Palkovits M, Faludi G, Merali Z, Szyf M, Anisman H (2008) GABAA receptor promoter hypermethylation in suicide brain, implications for the involvement of epigenetic processes. *Biol Psychiatry* 64:645–652
- Ptak C, Petronis A (2008) Epigenetics and complex disease: from etiology to new therapeutics. *Annu Rev Toxicol* 48:257–276
- Ruzicka WB, Zhubi A, Veldic M, Grayson DR, Costa E, Guidotti A (2007) Selective epigenetic alteration of layer I GABAergic neurons isolated from prefrontal cortex of schizophrenia patients using laser-assisted microdissection. *Mol Psychiatry* 12:385–397
- Satta R, Maloku E, Zhubi A, Pibiri F, Hajos M, Costa E, Guidotti A (2008) Subcutaneous nicotine decreases DNMT1 expression and GAD67 promoter methylation in selected populations of mouse telencephalic GABAergic neurons. *Proc Natl Acad Sci USA* 105:16356–16361

- Simonini MV, Camargo LM, Dong E, Maloku E, Veldic M, Costa E, Guidotti A (2006) The benzamide MS-275 is a potent, long-lasting brain region-selective inhibitor of histone deacetylases. *Proc Natl Acad Sci USA* 103:1587–1592
- Soghomonian JJ, Martin DL (1998) Two isoforms of glutamate decarboxylase: why? *Trends Pharmacol Sci* 19:500–505
- Szyf M, McGowan P, Meaney MJ (2008) The social environment and the epigenome. *Environ Mol Mutagen* 49:46–60
- Tremolizzo L, Carboni G, Ruzicka WB, Mitchell CP, Sugaya I, Tueting P, Sharma RP, Grayson DR, Costa E, Guidotti A (2002) An epigenetic mouse model for molecular and behavioral neuro-pathologies related to schizophrenia vulnerability. *Proc Natl Acad Sci USA* 99:17095–17100
- Tremolizzo L, Doueiri MS, Dong E, Grayson DR, Davis J, Pinna G, Tueting P, Rodriguez-Menendez V, Costa E, Guidotti A (2005) Valproate corrects the schizophrenia-like epigenetic behavioral modifications induced by methionine in mice. *Biol Psychiatry* 57:500–509
- Van Emburgh BO, Robertson KD (2008) DNA methyltransferases and methyl CpG binding proteins as multifunctional regulators of chromatin structure and development in mammalian cells. In: Tost J (ed) *Epigenetics*, Kaister. Academic, Norwich, UK, pp 23–62
- Veldic M, Caruncho HJ, Liu WS, Davis J, Satta R, Grayson DR, Guidotti A, Costa E (2004) DNA-methyltransferase 1 mRNA is selectively overexpressed in telencephalic GABAergic interneurons of schizophrenia brains. *Proc Natl Acad Sci USA* 101:348–353
- Veldic M, Guidotti A, Maloku E, Davis JM, Costa E (2005) In psychosis, cortical interneurons overexpress DNA-methyltransferase 1. *Proc Natl Acad Sci USA* 102:2152–2157
- Veldic M, Kadriu B, Maloku E, Agis-Balboa RC, Guidotti A, Davis JM, Costa E (2007) Epigenetic mechanisms expressed in basal ganglia GABAergic neurons differentiate schizophrenia from bipolar disorder. *Schizophr Res* 91:51–61
- Woo TUW, Walsh JP, Benes FP (2005) Density of glutamic acid decarboxylase 67 messenger RNA-containing neurons that express the N-methyl-D-aspartate receptor subunit NR2A in the anterior cingulate cortex in schizophrenia and bipolar disorder. *Arch Gen Psychiatry* 61: 649–657
- Woo TUW, Kim AM, Viscidi E (2008) Disease-specific alterations in glutamatergic neurotransmission on inhibitory interneurons in the prefrontal cortex in schizophrenia. *Brain Res* 1218: 267–277
- Wyatt RJ, Termini BA, Davis JM (1971) Biochemical and sleep studies of schizophrenia. A review of the literature 1960–1970. *Schizophr Res* 4:10–44
- Zhubi A, Veldic M, Puri NV, Kadriu B, Caruncho H, Loza I, Sershen H, Lajtha A, Smith RC, Guidotti A, Davis JM, Costa E (2009) An upregulation of DNA-methyltransferase 1 and 3a expressed in telencephalic GABAergic neurons of schizophrenia patients is also detected in peripheral blood lymphocytes. *Schizophr Res* 111:115–122

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