Characterization of the action of antipsychotic subtypes on valproate-induced chromatin remodeling

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Recent advances in schizophrenia (SZ) research indicate that the telencephalic γ-amino- butyric acid (GABA)ergic neurotransmission deficit associated with this psychiatric disorder probably is mediated by the hypermethylation of the glutamic acid decarboxylase 67 (GAD$_{67}$), reelin and other GABAergic promoters. A pharmacological strategy to reduce the hypermethylation of GABAergic promoters is to induce a DNA-cytosine demethylation by altering the chromatin remodeling with valproate (VPA). When co-administered with VPA, the clinical efficacy of atypical antipsychotics is enhanced. This prompted us to investigate whether this increase in drug efficacy is related to a modification of GABAergic-promoter methylation via chromatin remodeling. Our previous and present results strongly indicate that VPA facilitates chromatin remodeling when it is associated with clozapine or sulpiride but not with haloperidol or olanzapine. This remodeling might contribute to reelin- and GAD$_{67}$-promoter demethylation and might reverse the GABAergic-gene-expression downregulation associated with SZ morbidity.

Introduction

Recent advances in schizophrenia (SZ) research indicate that a deficit of brain γ-amino-butyric acid (GABA)ergic function detected in these patients is related to the downregulation of several GABAergic genes, including glutamic acid decarboxylase 67 (GAD$_{67}$) and reelin. This downregulation could be a pathogenic mechanism underlying the complex symptomatology of SZ [1–4]. This includes positive psychotic (e.g. hallucination, delusion and thought disorders), negative (i.e. anhedonia) and cognitive (e.g. attention, executive and memory dysfunctions) symptoms.

In the prefrontal cortex (PFC) and basal ganglia GABAergic neurons of SZ patients, an increase of DNA methyltransferase 1 (the enzyme that transfers a methyl group from S-adenosylmethionine to carbon 5 of the cytosine pyrimidine ring embedded in cytosine-phospho guanine [CpG] islands containing promoters) hypermethylates selected GABAergic promoters [5]. This increase is probably among the leading causes of GAD$_{67}$, reelin and other gene-expression downregulation. Hence, a pharmacological intervention that normalizes GABAergic neurotransmission is emerging as novel target in the strategy to treat SZ morbidity [2,3].

The antipsychotic medications presently used were not designed to target GABAergic transmission. In fact, the current antipsychotics are meant to target monoamine neurotransmitter receptors such as dopamine (D$_{2}$) receptors (targeted by typical antipsychotics including chlorpromazine, haloperidol and perphenazine), serotonin (5-HT$_{2A}$) or other monoamine receptors (targeted by atypical antipsychotics including clozapine [CLZ], olanzapine [OLZ], risperidone and quetiapine) [6,7]. Indeed, a majority of the available antipsychotics, with the exception of CLZ and sulpiride (SULP), failed to ameliorate the negative and cognitive symptoms associated with SZ [6–8]. This indicates that a monoamine receptor antagonism might not fully account for the larger spectrum of the clinical beneficial action of CLZ or SULP and for the unique efficacy of CLZ in patients resistant to other antipsychotic treatments.

The discovery of antipsychotics with improved clinical efficacy has been hampered by our incomplete understanding of the cellular mechanisms underlying SZ morbidity. For example, the use of valproate (VPA) as a drug that facilitates the action of CLZ [9,10] is based on empirical observations and not on a complete understanding of the underlying molecular and cellular pathologies. Here, we hypothesize that when CLZ or SULP are co-administered with VPA (a histone deacetylase inhibitor [11]) these antipsychotics might facilitate an open state conformation of chromatin (Box 1), which reduces the epigenetic GABAergic neuron modifications that mediate psychotic symptoms.

The role of GAD$_{67}$ and reelin in SZ

The reduced expression of GAD$_{67}$, reelin and other genes expressed in GABAergic interneurons is among the most consistent molecular alteration underlying the frontal cortex circuit dysfunction believed to be operative in SZ [1–5]. GAD$_{67}$ is the most important enzyme regulating GABA synthesis because it catalyzes ~70% of brain GABA turnover [12]. Hence, a large reduction (60% or more) of GAD$_{67}$ expression in specific GABAergic interneurons of the PFC and hippocampus of SZ patients might elicit a marked reduction of the potency of GABAergic inhibitory neurotransmission [13].
In the cortex and hippocampus, GABA is released from fast-spiking GABAergic presynaptic terminals that impinge on post-synaptic GABA_\text{A} receptors located on dendrites, somata or initial axon segments of pyramidal neurons. This release of GABA is efficient at synchronizing pyramidal neuron firing rates and could be crucial for optimizing cognitive functions [13]. The GABAergic neurotransmitter deficit measured in the brains of SZ patients could disrupt the intermittent synchronization pattern of pyramidal neuron firing and thereby induce cognitive function impairments [13].

In patients with psychosis, not only GAD_{67} but also reelin expression is markedly downregulated in the same neurons [2,5] (Figure 1). Reelin is a large (400 kDa) protein that is synthesized at a high rate in the GABAergic neurons of cortical layers I and II, hippocampus, caudate and putamen and is secreted in the extracellular matrix [5,14,15]. Upon the extracellular secretion from distinct cortical or hippocampal GABAergic interneurons, reelin adheres to post-synaptic densities located on dendritic spines and shafts of pyramidal neurons [13,14,16] (Figure 1). This protein binds to specific receptors (e.g. apolipoprotein E2 [ApoE2], very low density lipoprotein and integrin), harmonizing local dendritic protein synthesis attending: (a) dendritic spine formation, (b) spine maturation and (c) regulation of glutamate receptor structure and function [14,15]. Hence, reduced GAD_{67} and reelin signaling is most likely to be the cause for the reduced number of dendritic spines [14–16] and glutamate receptors in the PFC of SZ patients [17]. This reduced signaling

Box 1. Epigenetic regulation of CpG-island-containing promoters

Genes that contain CpG islands embedded in their promoters are often regulated by changes in methylation. These promoters exist in various states depending on the status of the surrounding chromatin. For example, transcriptional activation is associated with an open chromatin state that is characterized by low levels of DNA methylation, histone acetylation (facilitated by histone acetyl transferases) and other histone modifications (trimethylation of H3Lys4) (Figure I). By contrast, transcriptional silencing is characterized by a closed state in the vicinity of the promoter. Closed chromatin is characterized by increased DNA methylation (at CpG sites), methyl DNA binding proteins (including Dnmt1, Dnmt3a and methyl CpG binding protein 2), histone deacetylation, which causes compaction of the nucleosomes and occludes transcription factor binding (Figure I). Recently, an enzyme activity, DNA demethylase, which is present in nuclear extracts prepared from mouse cortex, has been described that removes the methyl group from methylated CpG sites. This activity (DNA demethylase) is thought to reverse methylation and is correlated with transcriptional activation.

Figure I. Schematic representation of chromatin remodeling showing the reversible interconversions that occur when promoters transition from an active (expressed) to an inactive (silenced) state. In GABAergic neurons, chromatin remodeling is responsible for activation or repression of genes, such as GAD_{67} or reelin, that have an important role in regulating neuronal circuit activity underlying complex behaviors. DNMT1 and HDAC1 hyperactivity lead to promoter methylation (Me) and histone deacetylation. This, in turn, is associated with the binding of methyl CpG binding proteins, such as MeCP2, DNMTs and HDAC1. The net result is the formation of a large repressor complex and a compact chromatin architecture in the vicinity of the RNA start site. The functional consequence is to block expression of the corresponding transcription unit. Abbreviations: DNA DMase, DNA demethylase; DNMT1, DNA methyl transferase 1; DNMT3A, DNA methyl transferase 3A; HAT, histone acetyl transferase; HDAC1, histone deacetylase 1; MeCP2, methyl CpG binding protein 2; SAH, S-adenosyl homocysteine; SAM, S-adenosyl methionine.
might also participate in mediating a functional GABAergic transmitter deficit that triggers the compensatory increase of postsynaptic $\alpha_5$ [2] and $\alpha_2$ [3] GABAA-receptor subunits expressed in PFC pyramidal neurons of SZ patients.

These functional abnormalities of GABAergic neurons are not limited to GAD 67 and reelin downregulation. Importantly, levels of mRNAs encoding the GABA membrane transporter 1 (GAT-1), N-methyl-D-aspartic acid (NMDA)-receptor subunit NR2A, somatostatin, parvalbumin [3,16] and nicotinic acetylcholine receptor (nAChR) subunits $\alpha_4b_2$ and $\alpha_7$ [18] are all downregulated in PFC GABAergic interneurons of SZ patients. These multifunctional abnormalities occur in the absence of a reduction in the number of GABAergic neurons, estimated by measures of the expression of GAD65, which is unaffected in SZ [2].

Role of epigenetics in SZ vulnerability

In SZ, the downregulation of GAD67, reelin and other genes expressed in GABAergic neurons could reflect genetic abnormalities. Although a highly conserved single nucleotide polymorphism (SNP) has been identified in the vicinity of the regulatory region of GAD67 [19] and reelin genes [20,21], these polymorphisms increase risk of psychotic symptoms in sporadic familial cases [22,23].

Several lines of evidence [5,24–26] indicate that an altered epigenetic mechanism could be responsible for a functional GABAergic neurotransmission downregulation co-expressed with the SZ morbidity.

‘Epigenetic’ is a term that defines mechanisms for the modulation of gene expression, which operate upon the inherited genetic blueprints. These involve the marking of DNA (i.e. DNA methylation) and the associated chromatin proteins (i.e. covalent histone tail modifications) (Box 1) in a systematic way that coordinates the intensity of the neuronal responses with the transcriptional machinery.

In the mammalian brain, DNA methylation is catalyzed by a family of DNA-methyltransferases including DNA-methyltransferase (DNMT)1, 3a and 3b [27]. However, only DNMT1 and DNMT3a are highly expressed in the central nervous system whereas DNMT3b is almost undetectable in adult human post-mitotic neurons [27,28]. The hypothesis that an epigenetic pathology of GABAergic promoters is operative in the transcriptional downregulation of several GABAergic genes in SZ patients is supported by evidence that DNMT1 is increased in GABAergic caudate and/or putamen medium spiny neurons and in GABAergic interneurons of the PFC (layers I and II) [5,28,29]. This DNMT1 upregulation (an increase of 2–3-fold over control) occurs in the same neurons expressing a decrease of reelin and GAD67 [28,29].

Based on this evidence, one could infer that, in the future, SZ morbidity treatment should include the administration of specific inhibitors of DNMT1 expression or
function. Unfortunately, the most effective inhibitors of DNMT1 activity available today are nucleoside analogues, which fail to cross the blood–brain barrier and might act only in dividing cells [30]. Hence, when given systemically, these drugs might be ineffective in reducing promoter hypermethylation in GABAergic neurons that are terminally differentiated.

**nAChR agonists target DNMT1**

An exciting development in the regulation of DNMT1 expression in GABAergic neurons is that the administration of nicotine and other synthetic nAChR agonists can improve both cognitive and negative symptoms in SZ patients [31,32]. It is known that a large number of SZ patients abuse tobacco, and because the active component inhaled in cigarette smoking is nicotine the high level of smoking in SZ patients could be linked to self medication in an attempt to correct or minimize the cholinergic receptor abnormalities associated with this disease [32]. When mice are injected with nicotine or the specific α4β2 nAChR agonist azetidinylmethoxy-pyridine (A-85380), DNMT1 expression is downregulated in PFC and hippocampal GABAergic interneurons [31]. This reduction is associated with a reduction of GAD67-promoter methylation and an increase of GAD67 protein levels [31]. These data indicate that the frequent nicotine abuse present in SZ patients might be considered an attempt to use this alkaloid as self medication to reduce the cognitive consequences of the overexpression of DNMT1 in cortical GABAergic neurons.

**Histone deacetylase inhibitors acting on the histone code promote DNA demethylation in GABAergic neurons**

There is now increasing evidence that, in adult neurons, DNA methylation patterns might change rapidly under the influence of environmental factors, toxins or drugs that modulate chromatin remodeling [30,33,34].

The elementary unit of chromatin is the nucleosome that is formed by an octamer of histone proteins [35]. The N-terminal tails of lysines in these histones might be extensively modified by acetylation or methylation. It has been proposed that the pattern of histone N-terminal tail modifications creates a ‘histone code’ that marks the changes in transcriptionally active or repressive chromatin conformations [35].

For example, one of the most consistent histone modifications, which defines an active chromatin remodeling conformation and marks DNA demethylation of specific promoters in the brain, is the acetylation of histone 3 (H3) lysine residues in positions 9 and 14 (H3Lys9 and H3Lys14). H3Lys4 methylation is another characteristic histone modification associated with an active chromatin remodeling conformation. By contrast, histone methylation at lysine residues 9 and 27 defines a repressive chromatin inducing promoter inactivity and perhaps preventing DNA demethylation [26,35–37].

Whether hypermethylated reelin and GAD67 promoters can be demethylated by the action of a putative DNA-cytosine demethylase activated by drugs acting on the histone code was tested in mice. In these experiments, VPA administered at doses comparable to those used clinically increased the amount of nuclear acetylated H3Lys9 or H3Lys14 bound to the reelin and GAD67 promoters and dramatically accelerated reelin- and GAD67-promoter demethylation [36,37]. This indicates that a covalent histone modification induced by VPA might modulate DNA demethylation of reelin and GAD67 promoters. Similar results were obtained in mice receiving the highly selective histone deacetylase (HDAC) inhibitor MS-275 [36].

The search for an active DNA demethylation activity in mammals has been characterized by the identification of several different mechanisms. Interestingly, reports indicate that DNA demethylation is initiated by molecules that in other studies were found to stabilize (methyl binding domain 2) or to facilitate (DNMT3α) the DNA-demethylation marks in the first place [38].

Because the chemical identity of the catalytic process mediating DNA demethylation in the mammalian brain remains unclear [37,38], we cannot conclude whether the accelerated reelin- and GAD67-promoter demethylation elicited by HDAC inhibitors in the mouse PFC is the result of a direct induction of a DNA demethylase activity or if it is associated with an indirect recruitment of a demethylation mechanism related to changes in histone code remodeling. Obviously, the identification of the biochemical nature of brain DNA-demethylation mechanisms is crucial to the development of a new line of pharmacological interventions to treat SZ.

**Modulation of the histone code by antipsychotics**

The symptomatic benefits elicited by a combination of VPA with antipsychotics in the treatment of SZ [9,10] prompted us to study whether this drug combination could mediate changes in specific chromatin remodeling processes, including histone-tail acetylation and demethylation of hypermethylated GABAergic promoters (i.e. modulating reelin and GAD67 expression).

As proof of concept, experiments were carried out with various antipsychotics including haloperidol (HAL; a selective D2-receptor antagonist), CLZ and OLZ (two 5-HT2A-preferring receptor antagonists) and SULP (a D2/D3-receptor antagonist) [37].

We found that reelin- and GAD67-promoter hypermethylation in the frontal cortex (FC) and striatum of mice induced by a 7-day methionine administration could be reversed in a dose-related manner by a treatment with clinically relevant doses of CLZ or SULP but not by HAL or OLZ given either alone or with VPA (Table 1).

Direct measurements of reelin- and GAD67-promoter demethylation in nuclear extracts obtained from the FC of mice treated with VPA or CLZ alone and with VPA plus CLZ indicate that the reduction of promoter methylation elicited by these drugs might include an increase of a nuclear DNA-demethylation activity [37]. This action seems to be independent from any interaction of the antipsychotics with specific monoamine receptors (Table 1). Moreover, the data of Table 1 indicate that H3Lys9 and H3Lys14 acetylation at reelin and GAD67 promoters is synergistically potentiated after treatment with VPA plus CLZ or SULP but not after VPA plus HAL or OLZ.
The precise mechanism whereby CLZ and SULP can modulate the histone code or induce DNA demethylation in GABAergic neurons remains to be elucidated. To investigate the mode of SULP action, we must consider that we are dealing with a benzamide and that some benzamides (i.e. MS-275) are potent inhibitors of selective classes of brain HDACs [39]. However, we find it difficult to associate the chemical structure of CLZ with that of any known HDAC inhibitor.

Recently, it was reported that H3Lys4 methylation, a histone modification associated with an active chromatin transcription, is increased at GAD67 and other promoters in FC neurons of mice treated with CLZ but not with HAL [26]. This increase is probably caused by an activation of a mixed-lineage leukemia-1 (MLL-1) histone methyltransferase [26]. Hence, it could be possible that a nuclear action of CLZ via H3Lys9 and H3Lys14 acetylation is mediated by a molecular mechanism that differs from the one mediated by VPA.

### Conclusions

The findings discussed here indicate that the overexpression of DNMT1 in telencephalic GABAergic neurons of SZ patients might be responsible for an epigenetic hyper-methylation of specific GABAergic gene promoters, including those regulating GAD67 and reelin [5,25,28,29]. The transcriptional downregulation of both genes is likely to lead to a downregulation of GABAergic transmission, resulting in a disinhibition of pyramidal neurons that presumably have an important role in the pathogenetic mechanisms underlying the cognitive and behavioral impairments operative in SZ patients. In fact, overactive pyramidal neurons activating dopaminergic cells in the ventral tegmental area might also drive a hyperdopaminergic state that further increases pyramidal neuron excitability and induces psychotic symptoms in SZ [40].

The efficacy of nAChR agonists or HDAC inhibitors (i.e. VPA) alone or in combination with CLZ or SULP to reduce (a) GABAergic-promoter methylation, (b) GABAergic neurotransmission deficits and (c) psychotic, cognitive and negative symptoms indicates their utility in combination therapy in SZ. In the future it will be desirable to design specific pharmacological interventions that reverse the complex promoter hypermethylation present in GABAergic neurons of SZ patients, which are known to express an excess of DNMT1 [5,25,28,29].

On the one hand, it is possible that, by reversing the GABAergic neurotransmission deficit impinging on pyramidal neurons, nAChR agonists enhance the inhibitory control on pyramidal neurons, thereby contributing to the synchronization of cortical and hippocampal neuronal activity required for optimal cognitive function. On the other hand, by reversing the deficit in GABAergic neurotransmission with co-administration of either VPA plus CLZ or VPA plus SULP, the pyramidal neuronal stimulation and consequently the hyperactivity of monoaminergic neurons can be effectively reduced, thus producing an actual reduction of monoamines available to these receptors. This might increase the monoaminergic antagonist action of CLZ or SULP in a complementary manner.

Taken together, these results encourage the study of new generations of nAChR agonists with selectivity for α4β2 or α7 brain nAChRs or a new generation of HDAC inhibitors with a different selectivity for the numerous HDAC families that are known to regulate cortical function and that can be altered in SZ. For example, the mRNA expression levels of HDAC1 were higher in the PFC of SZ versus nonpsychiatric subjects and were strongly and negatively correlated with the mRNA levels of GAD67 [41,42].

The molecular mechanisms whereby nAChR stimulation might decrease the expression of DNMT1 are unknown. By contrast, the molecular mechanism underpinning the chromatin remodeling caused by the co-administration of VPA with CLZ or SULP on reelin and GAD67 promoters might include an activation of a putative DNA-cytosine demethylase. This might be expressed in the nuclei of selected populations of cortical or striatal GABAergic neurons [37]. Hence, the induction of DNA demethylation by CLZ or SULP but not by HAL or OLZ should be further investigated because it might elucidate a unique epigenetic mechanism mediating the antipsychotic action of CLZ or SULP that seems not be shared by HAL or OLZ.

If successful, an ‘epigenetic neuroleptic treatment’ will shift the emphasis in SZ treatment from the use of drugs acting exclusively at membrane dopaminergic and other
neurotransmitter receptors to drugs that can modify the putative chromatin remodeling disorder present in SZ.

Considering that an epigenetic origin of cortical GABA-ergic dysfunction has a role in SZ morbidity, the identification of pharmacological agents targeting epigenetic mechanisms might usher a new approach to modify GABA-ergic dysfunction without targeting GABA_A or GABA_B receptors with pharmacological agents that might be inevitably accompanied by sedation, amnesia, tolerance and dependence liabilities.

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