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Review

From trans-methylation to cytosine methylation

Evolution of the methylation hypothesis of schizophrenia

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Abbreviations: CpG, cytosine phosphoguanosine; Dnmt, DNA methyltransferase; GABA, γ-aminobutyric acid; GAD67, glutamate decarboxylase 67; MeCP2, methyl CpG binding protein 2; Mat2A, methionine adenosyltransferase 2A; MET, methionine; NT2, Ntera 2, neuronally committed human teratocarcinoma cells; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; Sz, schizophrenia

Key words: schizophrenia, epigenetics, CpG island, reelin, GAD67, methionine

The role of methylation in the history of psychiatry has traversed a storied path. The original trans-methylation hypothesis was proposed at a time when chlorpromazine had been synthesized but not yet marketed as an antipsychotic (Thorazine). The premise was that abnormal metabolism led to the methylation of biogenic amines in the brains of schizophrenia patients and that these hallucinogenic compounds produced positive symptoms of the disease. At the time, some psychiatrists were interested in drugs such as mescaline and lysergic acid diethylamide that replicated clinical symptoms. They understood that these compounds might provide a biological basis for psychosis. The amino acid methionine (MET) was given to patients in the hopes of confiriming the transmethylation hypothesis. However with time, many realized that the hunt for an endogenous psychotropic compound would remain elusive. We now believe that the MET studies may have produced a toxic reaction in susceptible patients by disrupting epigenetic regulation in the brain. The focus of the current review is on the coordinate regulation of multiple promoters expressed in neurons that may be modulated through methylation. While certainly the identification of genes and promoters regulated epigenetically has been steadily increasing over the years, there have been few studies that examine methylation changes as a consequence of increased levels of a dietary amino acid such as methionine (MET). We suggest that the MET mouse model may provide information regarding the identification of genes that are regulated by epigenetic perturbations. In addition to our studies with the reelin and GAD67 promoters, we also have evidence that additional promoters expressed in select neurons of the brain are similarly affected by MET administration. We suggest that to expand our knowledge of epigenetically-responsive promoters using MET might allow for a better appreciation of global methylation changes occurring in selected brain regions.

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Epigenetics and Neurons

Multiple mRNAs, including those corresponding to reelin and GAD67, are downregulated in postmortem cortical and hippocampal regions of schizophrenia (Sz) patients.[1-4] Recent studies have shown that a large number of these mRNAs are associated with compromised neuronal function in various brain regions.[5] Several comprehensive studies have shown that many of the downregulated mRNAs are expressed in GABAergic neurons.[6-8] Our laboratories have focused on the regulation of two mRNAs expressed in GABAergic neurons, reelin and GAD67.[9] Based on a series of studies, we propose that the observed downregulation of various transcripts in GABAergic neurons may be due to the concomitant increased expression of DNA methyltransferase (Dnmt) 1 mRNA and protein observed in the cortex of Sz patients.[10,11] The role that Dnmt 1 plays in mediating alterations in de novo methylation patterns in the nervous system is still poorly understood. However, we would like to suggest the possibility that increased Dnmt1 expression leads to increased promoter methylation in GABAergic neurons which is associated with altered gene expression in brains of patients. While this hypothesis remains to be fully tested, it represents a viable mechanism that might explain some aspects of the neuronal circuitry dysfunctions that underlie psychosis.

Alterations in methylation during development can result in mental retardation. Mutations that occur in genes encoding methyl CpG binding proteins, such as MeCP2, have severe postnatal consequences.[12] Recent studies have highlighted deficits in

synaptic function associated with loss of MeCP2 in post-mitotic neurons.[13] While for many years MeCP2 was thought to act by binding methylated cytosines and repressing transcription, recent data suggest that its action may be more complex.[14,15] There is evidence that the reelin and GAD67 promoters are targets of MeCP2 binding. As elaborated on in more detail below, administration of methionine (MET) to animals in vivo increases promoter methylation and (reelin and GAD67) mRNA down-regulation. This is accompanied by increased binding of MeCP2 to the promoters.[16,17] Secondly, when methylation is reduced by treating neuronal precursor cells (NT2 cells) with DNA methyltransferase inhibitors, MeCP2 binding is reduced in vitro.[18] Curiously, reduced MeCP2 binding to the promoters also occurs when HDAC inhibitors are co-administered, suggesting an interplay between methylation and histone acetylation.[16,19] It seems likely that the alterations in chromatin accessibility and changes in gene expression that occur in disorders such as Rett syndrome, autism and Sz could arise from the altered expression of DNA methyl transferases (Dnmts) and methyl-CpG-binding (MeCP2, MBD2, etc.,) proteins that influence chromatin access (open vs. closed). The details regarding how these events are orchestrated remain obscure. There is evidence that the phenotypes associated with complex psychiatric behaviors such as addiction are likely to involve the epigenetic regulation of a wide variety of promoters in numerous cell types.[20,21]

MET Administration and Schizophrenia

It was not so long ago that one in five hospital beds were occupied by patients with schizophrenia. Deep insulin (insulin shock) was one of a few treatments that led to transient remissions of schizophrenia symptoms and this remedy was based largely on empirical observations.[22] Psychoanalysts were persistent in their beliefs that the illness was psychogenic. Walter Freeman, amongst others, was advocating the use of psychosurgery to 'cure' patients. After some initial apparent success, it eventually became clear that the prefrontal lobotomy bordered on negligence at best. However, given the treatments available at the time, the prospect of doing something promising for intractable patients in the absence of compelling evidence to the contrary may have been viewed with some degree of hope. With some hindsight, this chapter in psychiatry is now viewed with remorse as the failure to resist this 'new' approach resulted in the irreversible treatment of many thousands of mental patients, the vast majority of which had been committed to state psychiatric facilities.[23] More importantly, the lesson we have learned from episodes such as these is the need for objective and reliable clinical trails that ensure adequate protection of human subjects.[24]

In parallel, numerous groups of biological psychiatrists began to ask whether endogenous compounds were produced in the brains of schizophrenia patients that might induce psychotic episodes.[22] It appears that the controlled and intermittent use of mescaline by some psychiatrists was at one point used as an argument against this hypothesis. However, it was subsequently pointed out that chronic psychedelic ingestion might be a more appropriate means of approximating the schizophrenia condition in humans.[25] A comparison of the structures of endogenous amines with various hallucinogenic compounds (e.g., norepinephrine and mescaline) led to the proposal that alterations in biochemical trans-methylation might be responsible for some forms of schizophrenia.[22] This suggested the possibility that compounds likely to increase the levels of O-methylated amines or hallucinogenic derivatives should exacerbate schizophrenia symptoms whereas those that reduce the levels of these compounds might prove beneficial.[26]

Taking as a starting point numerous clinical studies that took place in the 1960s and 1970s (reviewed in ref. 26), we argue that exogenously administered MET perturbs methylation patterns in post-mitotic neurons of the brain. Ten clinical trials were carried out in the above referenced time frame in which large doses of MET were co-administered with a monoamine oxidase inhibitor (MAOI). While the dosage of MET, choice of MAOI, dose and duration of MAOI varied between studies, evaluations of these trials concluded that MET has profound pharmacologic effects on mood, perception and function in patients diagnosed with schizophrenia.[27] In spite of the different co-therapies and different dosages and means of MET administration (powder, liquid, tablet or capsule), nearly 64% of patients given MET (74 of 116) exhibited a worsening of their symptoms.[27] The reaction of patients to the amino acid varied in severity and included an increase in psychosis, auditory and visual hallucinations, confusion and disorientation, autonomic responses and increased psychomotor retardation. For example, one clinical team administered large doses of MET along with typical doses of iproniazid to chronic schizophrenia patients and four of twelve patients showed an intensification of the psychotic state.[28] Another study compared the effects of two doses of MET and another MAOI, isocarboxazid. These authors reported marked alterations in behavior in seven of nine patients.[29] In yet another study using MET administration without a MAOI, an increased functional psychosis in seven of eleven patients was reported. MET was expected to increase the activity of enzymes that transmethylate biogenic amine precursors through O-methylation. Some anticipated that the net effect would be the endogenous generation of methylated hallucinogens. However, there was no biochemical data to show increased O-methylation of catecholamine metabolites.[30] In addition, of the postulated methylated hallucinogens, dimethyltryptamine is the only one which has the demonstrated potential for being produced endogenously in man. Clinicians had also tried to initiate psychotic episodes in patients by giving exogenous tryptamine or tryptophan without success. Along these lines, another study demonstrated that there was no difference in the levels of biogenic amines in chronic schizophrenia patients in remission as compared to normal subjects.[31]

In summary, a review of these studies shows that of the patients treated with MET, there were two clear groups: reactors and nonreactors. The reactors responded with an exacerbation of their symptoms, while the nonreactors were relatively unaffected by the treatments. Interestingly, there were no obvious diagnostic differences that might explain these groupings.[30] Over the years, these results have yet to be explained. It seems clear that MET does not act to increase total catecholamine metabolism. We would like to suggest that MET, after conversion to S-adenosyl

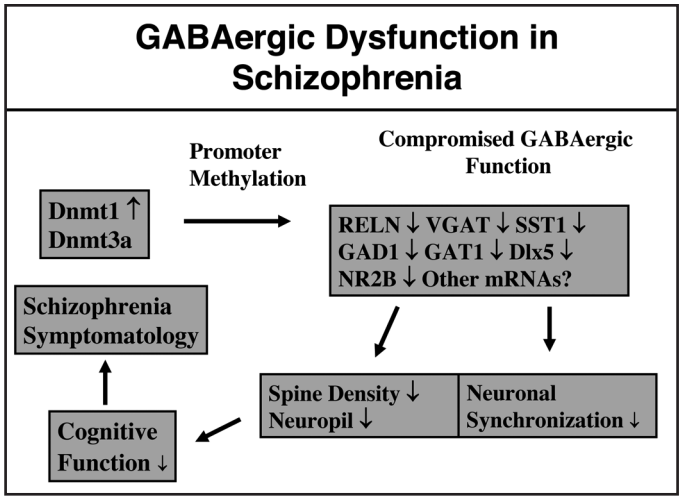

Figure 1. The GABAergic hypothesis of schizophrenia. The box shows a hypothetical link between some of the observations that have been made regarding mRNA downregulation and the expression of genes in GABAergic neurons of schizophrenia patients. We propose this epigenetic mechanism that links the increased expression of Dnmt1 (and possibly Dnmt3a) with the downregulation of sets of genes expressed in the same neurons (Costa et al. 2007). Some of these observations are based on data obtained from post-mortem human brain while additional information has come from animal studies in vivo and in vitro. The underlying hypothesis presumes that the reduced expression of mRNAs is due to an increased promoter methylation mediated by the robust expression of Dnmt1 in GABAergic neurons of schizophrenia patients.

methionine (SAM) increases promoter methylation in the brain. The subsequent promoter methylation and mRNA downregulation may have been responsible for the acute episodes of psychosis that developed in some 60% of patients (reactors) treated with the amino acid.

MET Mouse Model of Sz

As noted above, it was initially thought that exogenously administered MET induced the formation of a dopamine metabolite capable of initiating a psychotic episode in schizophrenia patients. However, the levels of various dopamine metabolites remained largely unchanged.[30] The mechanism for this MET-induced recrudescence of psychotic symptoms remained elusive. Both dietary[32] and subcutaneously injected[33] MET is converted to SAM by MET adenosyltransferase, which is subsequently used as the methyl donor for several metabolic reactions including DNA methylation. SAM has been proposed to increase the methylation of sensitive promoters by either activating DNA methylating enzymes or by reducing DNA demethylation.[34] Interestingly, a recent study from our group has shown that SAM levels in the prefrontal cortex (BA 9) of patients with schizophrenia and bipolar illness is increased approximately two-fold compared to non-psychiatric subjects.[35] Moreover, the increased levels of SAM correlate with an increased expression of Dnmt1 in BA area 9 GABAergic neurons. We have also shown that MET (5.2 mmol/kg injected subcutaneously, twice per day for seven days) facilitates changes in mRNA expression and promoter methylation in mice, both in vivo and in vitro.[16,17,36,37] Interestingly, MET was shown to increase brain S-adenosyl-methionine (SAM) levels in treated mice and this increase coincided with changes in promoter methylation and in mRNA expression.[33] In addition, we observed that there was increased binding of methyl CpG binding proteins, such as MeCP2, to these promoters.[17,19] Moreover, MET-induced methylation is reversible. That is, MET washout reverses the effects seen with chronic MET administration and HDAC inhibitors accelerate the process.[17] Prepulse inhibition of startle declines at a faster rate in MET-treated mice.[36] Interestingly, all of these effects are ameliorated when the HDAC inhibitor valproic acid (VPA) is co-administered, suggesting an interplay between histone modifications and DNA methylation.[38] Of particular relevance to the mechanism of MET action is that the MET-induced reelin mRNA downregulation can be prevented using antisense oligos that reduce Dnmt1 protein levels.[37] This implicates a role for Dnmt1 in the changes that occur in response to exogenous MET administration.

Heterozygous *reeler* mice (HRM) replicate many of the dendritic spine and GABAergic deficits described in Sz.[39] Recent studies show that the hippocampal-dependent plasticity and cognitive function of HRM are defective compared to control animals.[40,41] More precisely, the HRM exhibits reduced field excitatory post-synaptic potentials, a lowered paired pulse facilitation ratio, and impaired long term depression and long term potentiation. In addition to the cortical and cerebellar defects previously described, specific hippocampal-dependent learning is altered which may be the result of impaired inhibitory innervation. The nature of this reduced inhibitory innervation in the HRM is not fully characterized. The similarities between various physiological cortical measures in patients with Sz and HRM have been reviewed.[39] The similarities between the HRM model and MET-induced recrudescence of psychosis may not be coincidental. As noted in the previous paragraph, MET induces the downregulation of reelin, GAD and other mRNAs expressed in cortical GABAergic neurons. These same neurons overexpress high levels of Dnmt1 and reduced levels of reelin and GAD67 mRNAs in postmortem human brain of schizophrenia patients.[9,42] The phenotypes associated with the HRM, and MET treated mice show important similarities. While it will never be known with absolute certainty, schizophrenia patients treated with large doses of MET also may have undergone similar changes with respect to the molecular events operative in regulating their epigenome.

DNA Methyltransferase Expression in the Brain

Alterations in the expression of Dnmts (Dnmt1, 3a and/or 3b) could lead to changes in genomic methylation resulting in changes in the expression of large numbers of promoters. It seems that while Dnmt1, 3a and 3b are expressed in post-mitotic cells, Dnmt1 is highly expressed in GABAergic neurons and not in pyramidal neurons.[10,11,43] Dnmt1 is overexpressed in GABAergic neurons of cortical layers I and II and in medium spiny neurons of the caudate and putamen of Sz patients.[11] The role of these proteins in post-mitotic neurons is not entirely clear and numerous

studies have shown that each of these work together with methyl CpG binding proteins and histone deacetylases to effect their function.[44] Consistent with the increased expression of Dnmt1 in cortical GABAergic neurons of Sz patients, we[45] and others[46] have shown that portions of the reelin promoter are hypermethylated in Sz patients. We propose that the reduced expression of the mRNAs encoding reelin, GAD67, and other mRNAs results in a decrease in the GABAergic interneuron inhibitory tone (GABAergic hypo function) that has been described in Sz patients and that appears to be linked to a disruption of pyramidal neuron firing rates.[47-50] This could explain many of the observations linking inappropriate inhibition of hyperexcitable auditory and visual circuits resulting in the perception of hallucinations (inappropriate sensory information) observed in patients suffering from psychosis. The relevant filtering circuits for extraneous sensory information processing are GABAergic and hence not likely to be functioning effectively.[51,52]

Methylation and Gene Expression in Neurons

While the precise functions of key epigenetic regulatory proteins in distinct neuronal populations are not currently well understood, it seems clear that a role for methylation in brain function is emerging.[10,53] For example, in some rats, maternal grooming behaviors alter the methylation status of the hippocampal glucocorticoid receptor gene in their offspring.[54-56] Using contextual fear conditioning, changes in reelin and protein phosphatase I (PP1) promoter methylation have been linked to memory consolidation in mice.[57] Some researchers argue that methylation patterns might be reversible during chromatin remodeling. It appears that promoter methylation is intimately linked to histone deacetylation.[19,34,58] In fact, the histone deacetylation inhibitor valproic acid which is readily prescribed to psychotic patients as a mood stabilizer, is thought to act in part by increasing DNA demethylation.[17,59-61] Although the expression of DNA demethylases is still controversial,[62] it seems clear that histone deacetylase inhibitors such as valproic acid and MS-275 act to inhibit deacetylases and alter cellular DNA promoter methylation patterns. The concept of demethylation in the regulation of neuronal gene expression has been suggested in the context of cognitive development[63] and memory formation.[50]

We propose the scheme outlined in Figure 1 to explain the epigenetic origins of Sz as it relates to concepts presented thus far. As shown, increased expression of Dnmt1 is thought to be associated with selective promoter hypermethylation and mRNA downregulation. Some of the downregulated mRNAs in GABAergic neurons include reelin, GAD67, somatostatin (SST), vesicular GABA transporter (VGAT), GABA neurotransmitter transporter (Gat-1), NMDA receptor subunit 2 (NR2) and the distal-less 2 (Dlx2) homeobox transcription factors.[6,48,64] The observation that Dnmt1 is overexpressed in GABAergic neurons of Sz patients is consistent with the idea that a promoter hypermethylation mechanism may underlie Sz.[10,11] In addition, using Dnmt1 antisense oligos, we have shown that Dnmt1 protein knock-down prevents the Met-mediated decrease in reelin and GAD67 mRNAs in cortical neurons maintained in vitro.[37] While the role of Dnmt-mediated changes in promoter-specific methylation is well known in the context of cancer biology,[65] much less is known regarding

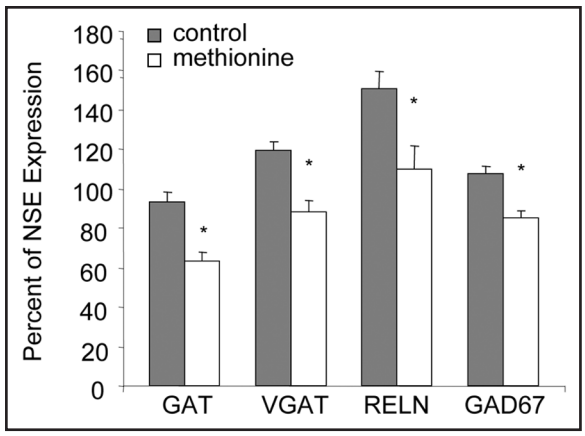

Figure 2. Methionine downregulates the expression of mRNAs expressed in GABAergic neurons in vivo. MET (6.6 mmol/kg, subcutaneous) was administered to mice twice a day for seven days. After the last injection, frontal cortices were harvested for RNA isolation. The indicated mRNAs were quantitated using competitive RT-PCR[71] and are expressed as a percent neuron specific enolase (NSE) mRNA with and without MET. Reverse transcription was performed and primers were derived from the following NCBI sequences: GAT (neurotransmitter transporter, GABA) NM_178703; VGAT (GABA vesicular transporter) NM_00958; RELN (reelin) NM_011261 and GAD67 (also known as Gad1) NM_008077.

methylation as a regulatory mechanism in neurons. Currently, we do not understand what causes this (Dnmt1) increase in Sz but it could be related to an as yet unknown upregulation of the Dnmt1 promoter in response to hormonal surges that occur during or just after puberty (possibly estrogen or other hormones). This would account for the observations that (1) reelin downregulation from birth leads to a more severe pathological phenotype[66] and (2) the onset of Sz symptoms very rarely occurs before puberty. While this idea remains unproven, it provides an appropriate framework for testing hypotheses related to the temporal expression of Dnmt1 in humans. If Dnmt1 expression could be linked to various hormonal influences, it might provide a mechanism as to why the mRNA remains elevated in patients with Sz. This suggestion also implies the possibility that Dnmt inhibitors may be appropriate in the context of drug discovery for psychosis.

Neuron-specific and developmental expression patterns are accompanied by distinct alterations in chromatin structure and DNA methylation status.[67,68] For this reason, it is important to focus on how the methylation of critical cytosines in these sequences affects the access of transcription factors to their recognition sites. We suggest that methylation represents a switch that can be used to regulate promoter expression under appropriate conditions.[18,19] An important concept that has been reinforced by many studies is that increased methylation increases the recruitment of methyl CpG binding proteins to the promoter, inducing a repressed or silenced promoter state.[16,69] Whether the so-called 'methylation switch' is reversible is still open to exploration. Recent data suggest that developmental changes induced in the absence of MeCP2 from birth are reversible and the implications are that these alterations may be consistent with methylation acting dynamically to modulate promoter expression.[70] It will now be important to

address whether other promoters known to be downregulated in Sz share a similar sensitivity to changes in methylation. We know that in addition to reelin and GAD67, that other mRNAs expressed in GABAergic neurons such as the vesicular GABA transporter (VGAT) and the GABA transporter (GAT-1) are reduced by ~50% following MET treatment in vivo (see Fig. 2). In addition to the in vivo studies, we also have preliminary data showing that selected Bdnf promoters, Dlx2, GAT-1 and VGAT are similarly reduced by MET administration in primary neuronal cultures in vitro (Chen Y, unpublished data). It seems appropriate to expand our knowledge of epigenetically responsive promoters so as to better appreciate the dynamic changes occurring in the brain. The hypothesis that MET perturbs neuronal methylation patterns in vivo is new and requires additional study on a larger scale. The possibility that MET administration may help to identify methylation-sensitive promoters in the brains of mice has applications that extend to learning and memory, cognition, aging, autism spectrum disorders, addictive behaviors and schizophrenia. The extent to which these altered methylation patterns lead to psychotic behaviors is still far from settled. However, based on the arguments presented above, this hypothesis provides a viable rationale for understanding how MET administration in the 1960s and early 1970s led to the re-emergence of psychotic symptoms in schizophrenia patients. Finally, the hypothesis opens the door for additional scrutiny of the role of one carbon metabolism and the origins of schizophrenia.

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