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The human reelin gene: Transcription factors (+), repressors (−) and the methylation switch (+/−) in schizophrenia

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Abstract

A recent report suggests that the down-regulation of reelin and glutamic acid decarboxylase (GAD_{67}) mRNAs represents 2 of the more consistent findings thus far described in post-mortem material from schizophrenia (SZ) patients [reviewed in Torrey, E. F., Barci, B. M., Webster, M. J., Bartko, J. J., Meador-Woodruff, J. H., & Knable, M. B. (2005). Neurochemical markers for schizophrenia, bipolar disorder amd major depression in postmortem brains. *Biol Psychiatry* 57, 252–260]. To study mechanisms responsible for this down-regulation, we have analyzed the promoter of the human reelin gene. Collectively, our studies suggest that SZ is characterized by a gamma-amino butyric acid (GABA)-ergic neuron pathology presumably mediated by promoter hypermethylation facilitated by the over-expression of the methylating enzyme DNA methyltransferase (Dnmt) 1. Using transient expression assays, promoter deletions and co-transfection assays with various transcription factors, we have shown a clear synergistic action that is a critical component of the mechanism of the trans-activation process. Equally important is the observation that the reelin promoter is more heavily methylated in brain regions in patients diagnosed with SZ as compared to non-psychiatric control subjects [Grayson, D. R., Jia, X., Chen, Y., Sharma, R. P., Mitchell, C. P., & Guidotti, A., et al. (2005). Reelin promoter hypermethylation in schizophrenia. *Proc Natl Acad Sci U S A* 102, 9341–9346]. The combination of studies in cell lines and in animal models of SZ, coupled with data obtained from post-mortem human material provides compelling evidence that aberrant methylation may be part of a core dysfunction in this psychiatric disease. More interestingly, the hypermethylation concept provides a coherent mechanism that establishes a plausible link between the epigenetic misregulation of multiple genes that are affected in SZ and that collectively contribute to the associated symptomatology.

Keywords: Schizophrenia; Methylation; Migration; GABA; Gene; Promoter; Histone deacetylase; DNA methyltransferase; Glutamate decarboxylase; Drug development; Neuron; Psychiatry

Abbreviations: AZA, azadeoxycytidine; BP, bipolar disorder; Dnmt, DNA methyltransferase; GABA, gamma-amino butyric acid; GAD, glutamic acid decarboxylase; HDAC, histone deacetylase; HP1, heterochromatin protein 1; LTP, long term potentiation; MeCP2, methyl CPG binding protein 2; MBD2, methyl binding domain protein 2; SV40, Simian virus 40; SZ, schizophrenia; TSA, Trichostatin A; VPA, valproic acid.

Contents

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1. Introduction

The starting point for the studies presented herein was the seminal observations regarding the down-regulation of reelin and glutamic acid decarboxylase (GAD_{67}) expression in cortical gamma-amino butyric acid (GABA)-ergic interneurons of post-mortem brain samples from patients diagnosed with schizophrenia (SZ) compared to similar material obtained from patients with other psychiatric diagnoses (Guidotti et al., 2000). These initial studies which showed some selectivity for psychoses were independently replicated at the immunocytochemical level in the hippocampi of patients with SZ and bipolar disorder (BP) (Fatemi et al., 2000). The observation that reelin mRNA is reduced in patients with SZ was also established using in situ hybridization histochemistry of cortical slices prepared post-mortem (Impagnatiello et al., 1998; Eastwood & Harrison, 2003). While beyond the context of the current review, reelin mRNA and protein levels have also been examined in autistic patients and these studies suggest a common link relating the down-regulation of reelin to abnormal neurobiological function (Fatemi et al., 2005a). In fact, these data suggest the possibility that psychosis and autism might share common features related to information and or sensory processing with a major difference being the age of onset at which these deficits occur.

The above studies establish that reelin and GAD_{67} mRNA and protein levels are reduced by approximately 50% in nearly every region of the cerebral cortex, as well as, in the hippocampus and basal ganglia of patients diagnosed with SZ (Impagnatiello et al., 1998; Fatemi et al., 2000; Guidotti et al., 2000; Eastwood & Harrison, 2003). The reduced levels of cortical reelin and GAD_{67} in SZ and BP patients are probably not due to a loss of GABAergic neurons but are more likely due to a decreased expression in defective GABAergic neurons. This is supported by the lack of concomitant changes in the levels of GAD_{65} immunoreactivity and mRNA which are expressed in the same GABAergic interneurons that express the reelin and GAD_{67} downregulation. In addition, the levels of neuron specific enolase mRNA are not reduced in SP (Guidotti et al., 2000). This finding supports the concept that the reduced reelin and GAD_{67} are not the result of a reduced number of neurons. This also suggests the possibility that the genes encoding reelin and GAD_{67} may be coordinately regulated and that a defect in a 'master' regulatory protein may compromise the expression of sets of genes expressed in these neurons. This master regulator may be involved in controlling the expression of multiple promoters contributing to the GABAergic neuron phenotype in SZ. In fact, the compromised promoters could easily be extended to include the NR2A (Woo et al., 2004) GAT1 and GAT 3 (Schleimer et al., 2004) and parvalbumin promoters and other promoters expressed in GABAergic neurons (Lewis et al., 2005). While the main focus of this review is the study of the reelin promoter and its regulation, in this context, many of the observations quoted apply equally well to other genes expressed in GABAergic neurons including GAD_{67}.

We have begun to decode the reelin gene regulatory program by focusing our attention on the reelin promoter (Chen et al., 2002). Our long-term goal has been to elucidate mechanisms operative in regulating the expression of the human reelin promoter, so as to provide an appropriate framework for the development of hypotheses relevant to the reduced reelin mRNA expression documented in various psychiatric disorders (see Guidotti et al., 2000; Costa et al., 2003a, 2003b, 2004 for recent reviews). Data obtained thus far indicate that multiple sequence elements within the human reelin promoter are important for expression in various human cell lines (Chen et al., 2002). We have reported data showing that the human reelin promoter is sensitive to methylation and that when the promoter is hypermethylated, reelin expression is silenced. More recently, we have shown that DNA methyltransferase (Dnmt) 1 mRNA is overexpressed in those same GABAergic neurons in which reelin, as well as, GAD_{67} are reduced suggesting a common negative regulatory mechanism that likely involves the coordinated hypermethylation of the corresponding promoters (Veldic et al., 2004, 2005). Central to our hypothesis is that biologically accurate patterns of reelin and also GAD_{67} expression are due to the positive action of specific transcription factors and that access of these factors to their recognition sites is modified through temporal- and spatial-dependent modifications in the methylation status of the corresponding promoter. In addition, it seems equally likely that methyl CpG binding proteins play a role by binding sites within the methylated promoter(s) and further reinforcing the negative regulation.

2. Reelin expression in the CNS

Reelin is expressed in Cajal-Retzius neurons of the marginal zone during cortical development and when secreted, the protein interacts with the products of several other genes, including the cell membrane receptors VLDL/Apo ER2 and mouse disabled 1, and serves to regulate neuronal positioning during telencephalic development (reviewed in Rice & Curran,

2001). The mouse reelin gene encodes a large (388 kD) glycoprotein expressed mainly in the central nervous system (CNS). The homozygous null mice, which fail to express this protein, display the posture and mobility characteristic of *reeler* phenotype (D'Arcangelo et al., 1995). A key component of this phenotype is the extensive disruption of the organization of laminated brain structures that utilize reelin-guided neuronal positioning (for a review see Curran & D'Arcangelo, 1998; Rice & Curran, 2001). During critical periods of neuronal migration, reelin interacts with the alpha3/beta1 integrin (Dulabon et al., 2000; Schmid et al., 2005) as well as to the VLDLR/ApoER2 complex. This latter interaction is thought to be responsible for the downstream phosphorylation of mouse disabled 1 which is associated with the termination of neuronal migration (Howell et al., 1997; Hiesberger et al., 1999; Howell et al., 1999). The null mutant reeler mouse expresses anomalies of cortical/hippocampal and cerebellar development that are responsible for the *reeler* phenotype. The heterozygous, haploinsufficient *reeler* mouse fails to express the *reeler* phenotype but shows several of the neuroanatomical, neurochemical and behavioral characteristics of patients with SZ (Tueting et al., 1999; Costa et al., 2001; Qiu et al., in press).

Reelin is expressed not only in the developing brain, but also in GABAergic neurons of the cortex and hippocampus and in glutamatergic granule cells in the cerebellum of adult humans and rodents (Ikeda & Terashima, 1997; Pesold et al., 1998). In the adult neocortex, reelin is synthesized and secreted from GABAergic interneurons (Alcantara et al., 1998; Pesold et al., 1998; Costa et al., 2001) predominantly of layers I, II and IV (Veldic et al., 2004). In the cortex of adult non-human primates, reelin co-localizes with integrin receptors in the vicinity of dendritic spine post-syanptic densities (Rodriguez et al., 2000). In cerebellar granule neurons in vitro, reelin is synthesized and secreted constitutively (Lacor et al., 2002). In cortex and hippocampus, adult reelin, released from GABAergic neurons, functions by signaling to the dendritic spines of pyramidal neurons through a discrete molecular cascade that ultimately promotes binding and phosphorylation of NMDA receptors resulting in increased translation of spine resident mRNAs and a stimulation in local protein synthesis (Dong et al., 2003). This process is intimately linked to the maturation of the dendritic spines of these pyramidal neurons and to their function as neuronal signaling components. In this way, reelin has been linked to processes of synaptic plasticity, learning and memory formation (Weeber et al., 2002; Larson et al., 2003; Carboni et al., 2004; Beffert et al., 2005; D'Arcangelo, 2005; Qiu et al., in press).

Very little is currently known with respect to how the expression of reelin is regulated in these adult neuron subtypes. However, we have reported that reelin, GAD_{67} and Dnmt1 mRNAs co-localize in cortical GABAergic neurons of the adult human cortex. A common interest amongst many in the field resides in clarifying and understanding those determinants that target the active expression of these promoters in these cortical GABAergic neurons. Equally important is an understanding of temporally selective switches that may be operative in defining the developmental changes in the patterns of reelin, GAD_{67} and Dnmt1 in these same neurons. Our data suggest that methylation and demethylation are very likely critical regulators of these dynamic changes. In addition, it seems likely that common effectors of transcription operate in the regulation of each of these genes in a spatially restricted manner allowing for coordinated patterns of gene/mRNA expression.

3. Structure of the reelin gene

The gene encoding reelin was identified and characterized after a transgene insertion into the *reeler* locus produced a phenotype identical to that of the *reeler* mice (Miao et al., 1994; D'Arcangelo et al., 1995). Subsequent work led directly to the isolation of a large mRNA that was disrupted in these transgenic mice and it was subsequently shown that the mRNA encodes the protein reelin. The murine reelin protein is translated from a mRNA that contains an open reading frame of 10,383 bp in length and is followed by an additional 1 kb of untranslated sequence. The mRNA is encoded by a gene that contains 65 exons which spans approximately 450 kb of genomic DNA (Royaux et al., 1997). The exons range in size from 6 bp (exon 64) to 1104 bp (exon 65). The first exon contains 282 bp of 5′ untranslated sequence and the ATG start codon. The complete intron/exon structure of the gene has been mapped and virtually all intron/exon boundary sequences conform to consensus donor/acceptor sequences (for a detailed delineation of the intron/exon structure of human *reln*, see Chen et al., 2002, supplementary material at http://nar.oxfordjournals.org/cgi/data/30/13/2930/DC1/1 and DeSilva et al., 1997). We have mapped the intron/exon structure of the human reelin gene and have found that the intron/exon boundaries are remarkably conserved between human and mouse, as are the sizes of the corresponding introns. In the Orleans allele of reeler ($Reln^{rl\text{-}Orl}$), a 220 nucleotide deletion occurs producing a truncated form of reelin that is not secreted, consistent with a role for the C-terminus of the protein in cell secretion (De Bergeyck et al., 1997). These studies concerning the functional relevance of the 3′ portion of the reelin cDNA prompted our initial search for polymorphisms within terminal exons of the reeler gene, exons 57–65 (Uzunov et al., 1999).

With respect to positive molecular determinants responsible for regulating reelin gene expression, evidence is accumulating that key transcription factors play a role in recruiting RNA polymerase complexes to the promoter. The human and mouse cDNAs share 88% nucleotide identity. Compared with the mouse promoter, the human gene (BAC clone RG126M09 from human 7q21-q22) shows a high degree of sequence similarity surrounding the start site of transcription. The sequence flanking the human RNA start site is very GC rich (75%) and together with the first exon forms a large CpG island (Gardiner-Garden & Frommer, 1987). This CpG island extends from approximately −1200 bp upstream to about 200 bp downstream of the RNA start site (Chen et al., 2002). As with many others genes that show this high GC rich content, the presence of this island is consistent with the hypothesis that the reelin promoter may be regulated through changes in DNA methylation (see Newell-Price et al., 2000; Tucker, 2001). In contrast to the vast

majority of CpG dinucleotides in the genome which are generally methylated, CpG islands tend to be undermethylated and this pattern can vary in both a temporal and spatially selective manner (Newell-Price et al., 2000; Costello & Plass, 2001). There are 120 CpG sites contained within sequences flanking the reelin minimal promoter (from −514 bp to +322 bp). Our data show that a small fraction of these CpG sites are methylated in neural progenitor cells that do not express reelin while these sites are not methylated in differentiated neurons that express reelin (Chen et al., 2002; Mitchell et al., 2005).

4. Analysis of the human reelin promoter–transcription factors

As a starting point for our studies, we mapped the exon/intron structure of the human reelin gene to various BAC sequences present in the human database (Chen et al., 2002). The 5′ portion of the human mRNA and upstream sequence maps to human BAC clone RG126M09 which contains 163 kb of human genomic DNA. We obtained this DNA (Research Genetics) and verified the location of many restriction sites. The first exon maps to a region that is located near the terminal 3′ end of the BAC. We have since subcloned a 4.2 kb EcoRI fragment which contains the entire first exon, 255 bp of the first intron and 3.7 kb of 5′ flanking DNA. Various unique restriction sites in this clone have been used to subclone portions of the region upstream of the ATG start codon (see Fig. 1 for a representation of the human reelin promoter; Chen et al., 2002). Comparison of this region with a corresponding region of the mouse promoter (Royaux et al., 1997) shows a stretch of sequence identity that is quite high. This region contains several recognition sites for the zinc-finger transcription factor Sp1 (Chu & Ferro, 2005). Sp1 belongs to a family of related transcription factors which bind and act through the so-called GC box (Harrison et al., 2000). Interestingly, Sp1 factors are also regulated through phosphorylation which can affect the affinity of the factor for its recognition sequence (Chu & Ferro, 2005). We created reelin expression vectors by first amplifying a region which extends from −514 bp to +76 bp relative to the human transcriptional start site. A series of promoter/reporter fusions were derived and expressed using transient transfection assays. Transient transfection analyses of these expression vectors indicate that there are likely to be multiple regions of the reelin promoter that operate in modulating transcription of this gene (Chen et al., 2002). Fig. 1 also shows the presence of a cyclic AMP responsive element binding protein (CREB) binding site which has not been functionally characterized at this point. There are also sites recognized by T brain 1 (Tbr1) and Paired Homeobox 6 (Pax6) which are discussed below.

Based on an analysis of a series of promoter deletion constructs, we established that high levels of activity were evident with as little as −303 bp of the promoter (Chen et al., 2002). Because of the high activity associated with sequences contained within the −303 bp construct (see Fig. 1, enclosed in box), we assessed whether this region might activate expression of a heterologous promoter (Grayson et al., 1988). In other words, these sequences appeared to have properties

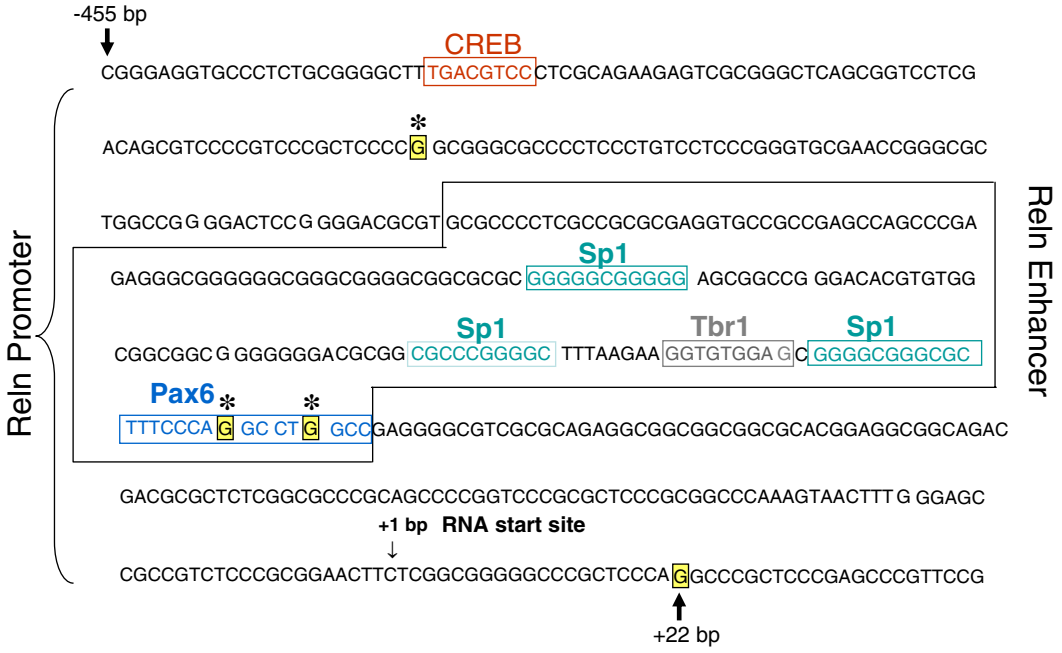

Fig. 1. Reelin promoter summary. This cartoon shows the promoter proximal and enhancer sequences of the reelin promoter as currently defined. The RNA start site is denoted as +1. All sequences upstream of these sites are negative relative to this position. Putative transcription factor binding sites are indicated for those sites in which there are some data to support a role for the indicated factor. G residues marked in yellow represent bases where significant methylation has been found in SZ patients and not in non-psychiatric pateints (Grayson et al., 2005). On the opposite strand, these bases would be methylated cytosines. The enhancer region (shown in the box) represents a portion of the promoter that was previously characterized functionally as described (Chen et al., 2002). Interestingly, the two G bases within the Pax 6 recognition site are the most sensitive to loss of function by transient transfection assays.

similar to those ascribed to enhancer elements. Enhancers contain transcription factor binding sites and generally operate in an orientation independent manner (Grayson et al., 1988). Moreover, enhancers are able to trans-activate heterologous promoters. Sequences between −304 and −137 bp were cloned upstream of the Simian virus 40 (SV40) promoter in both sense and antisense orientations. We assessed activity by transient transfection into 5 different human cell lines. Some of these cell lines express the endogenous gene (such as the SHSY5Y, hNT and HepG2) while others express endogenous reelin only at very low levels (NT2 and 293A). The promoter proximal sequences increased expression of the SV40 promoter from 2- to 8-fold as compared to the SV40 promoter/luciferase vector alone. Interestingly, the region of high sequence similarity between the human and mouse promoters is contained within the enhancer region. This part of the promoter contains 2 of the 3 Sp1 sites found in the human reelin upstream sequence (see Fig. 1). Our data using DNase I treatment of nuclei prepared from expressing and non-expressing cell types identified 3 hypersensitive sites, the most proximal of which maps to the enhancer region (Chen et al., 2002).

More recently, we have extended our studies of the human reelin promoter in vitro (Chen et al., 2004). Using chromatin immunoprecipitation assays (ChIP), we have been able to demonstrate that in non-treated NT2 cells, at least 1 protein, methyl CPG binding protein 2 (MeCP2), is physically associated with the promoter. This protein is known to bind methylated cytosines within various promoters and form complexes (Jones et al., 1998) to repress transcription (see Burgers et al., 2002; Fan & Hutnick, 2005 for recent reviews). This is supported by data which show that extracts prepared from these cells contain nuclear proteins that bind to methylated sequences with a higher affinity than the corresponding non-methylated counterparts (Grayson et al., 2005).

Retinoic acid (RA) is a vitamin A derivative which has powerful effects on cell growth and differentiation through its action at the RA and retinoid X receptors (Mangelsdorf & Evans, 1995; Chambon, 1996). Activation of the respective receptors induces a cascade of events that ultimately result in changes in transcriptional patterns that reflect the modified phenotype of the differentiated state. Many studies have implicated the action of the transcription factors Sp1 (Suzuki et al., 1999; Husmann et al., 2000; Safe & Kim, 2004; Lee et al.,

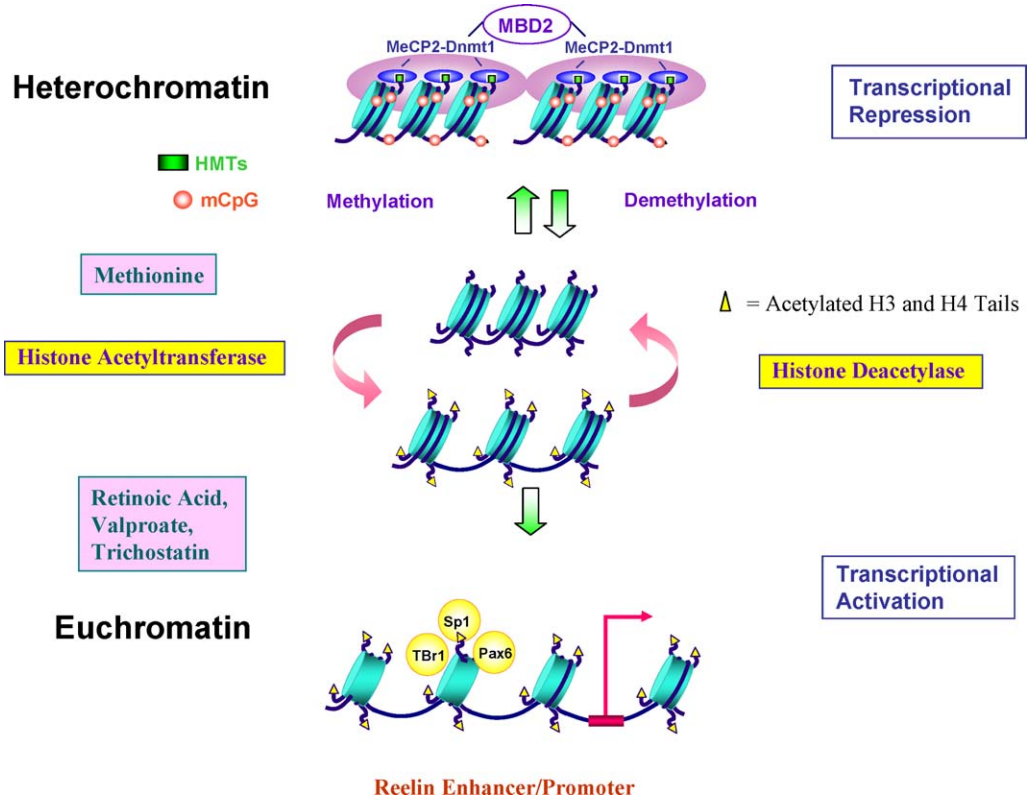

Fig. 2. The reelin promoter chromatin undergoes compaction/relaxation transitions. This cartoon schematically depicts the reelin promoter in various stages of activation. The heterochromatic state is shown at the top and represents the promoter in the off or non-transcribed state (Transcriptional Repression). Arrows show transitions that are induced by various manipulations. For example, methionine is known to turn off expression of the reelin promoter and hence is shown shifting the promoter to the closed state. In contrast, RA, VPA and TSA activate the promoter (transcriptional activation) albeit through different mechanisms. These transitions involve changes either in the accessibility of various transcription factors such as Tbr1, Sp1 and Pax6 or in the concomittant acetylation of the histone tails in this region. Histone deacetylases remove these acetyl groups shifting the chromatin into a more compact or closed state. This is further enhanced by the action of DNA methyltransferases which methylate the promoter completing the shut down. It is also known that certain methyl DNA binding proteins such as MBD2, MeCP2 and Dnmt1 are associated with the silenced reelin promoter while it is transcriptionally inactive. In NT2 cells, reelin is initially not transcribed. When retinoic acid is added to the cultures, we see a disassociation of MBD2, Dnmt1 and MeCP2 from the promoter and increases in the transcription factor Pax6. This then binds to its recognition site which is then followed by a relaxation changes in local chromatin conformation resulting in transcriptional activation.

2005) and also the paired homeobox protein, Pax 6 (Gajovic et al., 1997; Przyborski et al., 2003). following neuronal differentiation by RA. Interestingly, mutants in selected transcription factors expressed in the brain, such as Pax6 (Tarabykin et al., 2001) and Tbrain 1 (Tbr1, Bulfone et al., 1995), show phenotypes that overlap with the *reeler* phenotype (Rice & Curran, 2001). In Tbr1-deficient mice, the Cajal-Retzius cells express decreased levels of reelin resulting in a reeler like cortical migration disorder (Hevner et al., 2001). This suggests that factors such as Tbr1 and Pax6 (also shown in Fig. 1) may be associated with regulating reelin expression and may be indirectly responsible for the partial cortical malformation phenotypes observed in these mutant mice.

When RA is added to cultures of neural progenitor (NT2) cells in vitro, reelin mRNA begins and continues to accumulate over a 6-day time frame. Quantitative studies show that reelin mRNA levels increase by nearly 130-fold or so. During this period, Pax6 mRNA begins to accumulate some 48 hr before the onset of reelin mRNA. This is followed by an increase in the respective protein and its binding to the reelin promoter (Chen et al., 2004). There are also increases in the amounts of Tbr-1 mRNA and Sp1 binding that coincides with the activation of the reelin promoter and the subsequent transcriptional increase that ensues. Interestingly, these changes occur prior to the onset of neuronal differentiation which accompanies RA treatment of neural progenitor cells after prolonged treatment (Leypoldt et al., 2001; Katoh, 2002). Additional changes that occur in this initial time frame (see Fig. 2), including alterations in chromatin structure are likely mediated by changes in local acetylation and/or methylation status of histone H3 (and to a lesser extent H4, Mitchell et al., 2005). There are also changes in the distribution of methylated bases within the reelin promoter, and these changes may result in enhanced HDAC activity targeted to this region. Dnmt1 is capable of recruiting repressor proteins such as MeCP2 and other co-repressors (Kimura & Shiota, 2003) to promoter sequences likely initiating a subsequent shutdown in gene expression through the compaction of local chromatin. The precise timing of this sequence is not completely understood, but it seems as though the initial events involve regional opening of the chromatin and a subsequent dissociation of repressor proteins from the methylated DNA. This would then be accompanied by a decreased methylation of the DNA at specific cytosines through the action of either MBD2B or another molecule with demethylase activity (Detich et al., 2003). Whether the actual demethylase is methyl binding domain protein 2 (MBD2), a variant or another protein altogether is still not resolved. Ultimately, the addition of retinoic acid results in changes in the vicinity of the reelin gene that open the chromatin in the area of the promoter and this provides access to the requisite transcription factors that enable transcriptional activation.

5. Regulation by methylation

In the course of these studies, we conducted experiments to evaluate the role of DNA methylation in regulating reelin promoter expression. These experiments were motivated by observations that (1) the cloned reelin promoter behaves promiscuously in different human cell lines in vitro; (2) the promoter is embedded in a large CpG island that surrounds the 5′ flanking sequence and first exon of the gene (Chen et al., 2002); and (3) agents that alter methylation patterns increase reelin mRNA expression (Chen et al., 2002; Mitchell et al., 2005). We have since generated data showing that in neurons expressing reelin, the methylation status of the promoter is different from that in the progenitors not expressing the gene (see Chen et al., 2002; Mitchell et al., 2005; Noh et al., 2005). Evidence obtained thus far in vitro, in mouse neurons in primary culture (Noh et al., 2005), are consistent with a methylation mechanism involving Dnmt1. These changes in methylation, in retrospect, are subtle and do not represent a gross change in methylation along the length of the entire CpG island. Instead, selected bases within key regions responsible for regulation are methylated when the gene is silenced and then not methylated when the gene becomes active.

5.1. Role of DNA methyltransferase 1

The above considerations have prompted us to examine and study the regulation of Dnmt1 expression (Costa et al., 2003a, 2003b; Veldic et al., 2004). Dnmt1 is 1 of 3 DNA methyl transferases and is expressed at high levels in post-mitotic neurons (Inano et al., 2000; Fan et al., 2001; Veldic et al., 2004; Goll & Bestor, 2005; Robertson, 2005). The mammalian brain expresses high levels of Dnmt1 both during development and in the adult (Inano et al., 2000; Veldic et al., 2004). The levels of DNA methylation in the adult brain are higher than in other tissues (Tawa et al., 1990). More recently, it was shown that DNA hypomethylation in Dnmt1 null mice perturbs the function and survival of CNS neurons in postnatal animals (Fan et al., 2001). This was somewhat surprising as it was commonly thought that methylation patterns are established during replication and do not change in post-mitotic cells which would include most neurons. In fact, Dnmt1 has been found in association with the replication machinery suggesting that DNA methylation patterns are replicated along with the genetic information (Leonhardt & Cardoso, 2000). The expression of Dnmt1 itself is also regulated through methylation and represents a possible feedback mechanism that may provide a sensing system for determining the methylation capacity of a cell (Slack et al., 1999). While still somewhat controversial (Fan et al., 2001), several lines of evidence suggest that DNA methylation is a reversible biological signal and that specific DNA demethylases exist and can reverse changes that are established during terminal phases of cell or neuronal differentiation (Ramchandani et al., 1999). This would provide a compelling rationale for the function of Dnmt1 that is present in post-mitotic neurons (Fan et al., 2001; Veldic et al., 2004). As indicated above, Dnmt1 is known to associate with both HDAC and co-repressor proteins (Ahringer, 2000; Fuks et al., 2000; Robertson et al., 2000; Rountree et al., 2000; Burgers et al., 2002). Eukaryotic Dnmt1 show little sequence specificity other than for CpG dinucleotides and it seems likely that the

interaction between Dnmt1 and specific gene promoters occurs through the association of sequence specific DNA binding proteins.

5.2. Histone deacetylase and chromatin structure remodeling

As suggested in Fig. 2, the localized presence of Dnmt1 in a complex with other proteins at target promoter sequences involves an interaction between the Dnmt1/HDAC complex and selected transcription factors. Heterochromatin protein 1 (HP1) and MeCP2 are thought to maintain the chromatin in a closed state that is both repressed and methylated. It seems likely that Dnmt1 and HDAC associate with transcription factors or co-repressors X (Rb, E2F, Sin3, Sp1, or others) which bind to a sequence defined cis-element operative in the regulation of the corresponding gene (not depicted in Fig. 3). In addition, various methyl CpG binding proteins (HP1, MECP2, MBD1-2) could target Dnmt1 to specific promoter sequences. Fig. 3 illustrates that demethylation represents 1 epigenetic switch that could lead to the opening of condensed chromatin into a more open configuration. Histone H3 is methylated at Lys9 by a histone methyltransferase, SUV39h1 in mammals, and this site-specific modification provides a docking site for the attachment of HP1. HP1, in turn, recruits both Dnmt1 and SUV39h1 and creates a potential self-perpetuating loop that methylates DNA and additional H3-Lys9 sites, and these new modifications serve to extend the region of condensed chromatin. The Dnmt1/HDAC complex modifies the local chromatin structure through histone (H3 and H4) deacetylation (release of Ac) which in conjunction with active cytosine methylation (Me) leads to gene silencing (transcription OFF). Demethylation causes a change in histone methylation (reduced), an increase in acetylated histones and the unraveling of the chromatin in the region shown. These observations allow us to speculate that the high levels of Dnmt1 present in post-mitotic neurons may play an active role in regulating gene expression through transient but also, reversible, changes in DNA methylation and chromatin remodeling.

The histone deacetylase (HDAC) inhibitor and mood stabilizing drug, valproic acid (VPA), also induced the expression of the endogenous reelin promoter (Chen et al., 2002). Several recent studies suggest that changes in histone acetylation are accompanied by changes in local DNA methylation implying that this process is reversible (see also Detich et al., 2003; Mitchell et al., 2005; Tremolizzo et al., 2005; Weaver et al., 2005). Transcriptional activation of a silenced gene would likely involve several steps. These include (1) histone acetylation and (2) DNA demethylation which has been demonstrated in vitro (Cervoni & Szyf, 2001). These processes would then be accompanied by a change in local chromatin structure from a closed to an open state. It

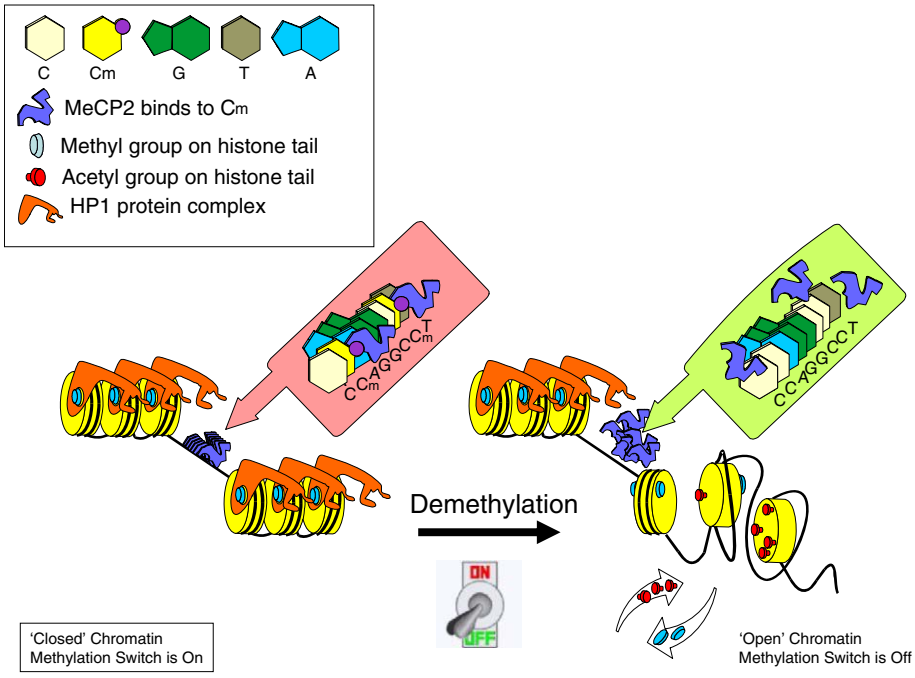

Fig. 3. Molecular events leading to chromatin relaxation. The cartoon illustrates a sequence of epigenetic events leading to the opening of condensed chromatin through the 'so-called' methylation switch (see Fuks et al., 2003; Maison & Almouzni, 2004). In the closed state (left), local chromatin is compact and restricted from access by transcription factors and the RNA polymerase machinary. This closed state is maintained by the binding of the HP1 complex (orange) and various repressor proteins such as MeCP2 (blue). Histone H3 is methylated at Lys9 by a histone methyltransferase, SUV39h1 in mammals, and this site-specific modification provides a docking site for the attachment of HP1 (orange). HP1 in turn recruits both Dnmt1 and SUV39h1 (HP1 complex), which methylates DNA and additional histone sites extending the region of condensed chromatin. When the methylation signal is switched 'off', promoter sequences presumably become accessible following demethylation and the release of MeCP2. Histone H3 becomes acetylated and themethyl histone residues are replaced either by turnover or through mechanisms that are, as yet, not well understood. The sequence shown in the box (orange) represents the region of the relin promoter found to be more highly methylated in brain regions of patients diagnosed with SZ (Grayson et al., 2005). This region also binds repressor proteins likely related to MeCP2. The box on the right (green) shows the non-methylated and non-repressed promoter sequences.

seems plausible that increases in histone acetylation and reduced DNA methylation are coupled processes that could be mediated by chromatin remodeling complexes that modify accessibility of chromatin to the transcriptional apparatus (Bowen et al., 2004).

Our published studies indicate that repeated methionine administration to the heterozygous *reeler* (+/−) mouse (HRM) results in a pronounced down-regulation of reelin and GAD_{67} mRNAs (Tremolizzo et al., 2002, 2005). We believe that this action occurs, at least in part, through a mechanism involving the hypermethylation of the corresponding promoters. The administration of methionine increases the levels of *S*-adenosylmethionine (SAM), the universal methyl donor (Tremolizzo et al., 2002). This, in turn, provides added SAM for metabolic reactions including DNA methylation and the methylation of histones (Zhang & Reinberg, 2001) and additional proteins. Interestingly, chronic methionine administration increases reelin promoter methylation and increases the binding of methyl binding domain proteins to the reelin promoter (Dong et al., 2005). We also know from early clinical studies that treating patients with methionine elicits psychotic episodes if these patients were previously symptomatic for SZ (Antun et al., 1971; reviewed in Costa et al., 2002). The mechanism for this recrudesence of psychotic symptoms remained elusive until, as indicated in the following section, we were able to demonstrate methionine-mediated changes in gene expression in mice both in vivo and in vitro (Tremolizzo et al., 2002; Dong et al., 2005; Noh et al., 2005; Tremolizzo et al., 2005). Changes in methylation status can result in pronounced changes in gene expression, particularly as methylation status correlates with transcriptional inactivation (Tucker, 2001).

5.3. Pharmacological regulation of methylation

DNA hypermethylation has been associated with Rett syndrome and fragile X syndrome (Robertson & Wolffe, 2000). Rett syndrome is a neurodevelopmental disorder that results in mental retardation due to mutations in the gene encoding methyl CpG binding protein MeCP2 (Amir et al., 1999; Caballero & Hendrich, 2005). Patients appear normal for the first 6–18 months of life, then cognitive function rapidly degenerates, leading to severe dementia and autistic-like behaviors (Chen et al., 2001). Recent data show that MeCP2 assists in transcriptional silencing by binding methylated CpG sites and associating with chromatin-remodeling complexes (Nan et al., 1998; Jones et al., 1998). In spite of the increased attention received by MeCP2, the specific targets of its action remain elusive (Amir & Zoghbi, 2000). One recent report has established that the reelin promoter is one target of MeCP2 action (Dong et al., 2005). As indicated above, mice treated with L-methionine model some of the molecular neuropathologies detected in SZ including the hypermethylation of the reelin promoter CpG island and the downregulation of reelin and GAD_{67} mRNA expression. In these mice, the reelin promoter shows an increased binding of MeCP2 indicating that not only is the promoter more heavily methylated but that the promoter is actively repressed as well. Recently published data show that DNA methylation is dynamic and that DNA demethylases may be involved in shaping patterns of methylation (Cervoni & Szyf, 2001). This allows us to speculate that the high levels of Dnmt1 present in post-mitotic neurons play an active role in regulating gene expression through transient changes in DNA methylation.

To test whether methylation might account for why the endogenous gene is silenced, we incubated NT2 cells (a neuronal precursor cell line) with the methylation inhibitor 5-aza-2′-deoxycytidine (AZA, 5 μM) for 3 days. RNA was harvested and the levels of reelin mRNA were quantitated by competitive RT-PCR (Chen et al., 2002). Treatment of NT2 cells with the methylation inhibitor increased expression of the endogenous gene by over 60-fold. These data suggest that in non-expressing cells/neurons, the reelin promoter is normally inactive and that methylation is the likely mechanism by which the gene is silenced. In contrast, in neurons that express reelin, the gene is undermethylated and the transcription factors that activate expression are able to access their recognition sites. Further, the data suggest that promoter methylation may act as a permissive switch that allows expression in selected neurons and cell types (see Figs. 3 and 4). We propose that sequence specific transcription factors recruit Dnmt to specific genes and induce changes in methylation status at the respective promoters as part of a methylation switch involved in shutting down gene expression.

Recent studies (Wade, 2001; Wade & Wolffe, 2001) show that methylated DNA is recognized and bound by a family of methyl DNA binding proteins which recruit co-repressor complexes and HDAC. Collectively, these proteins associate

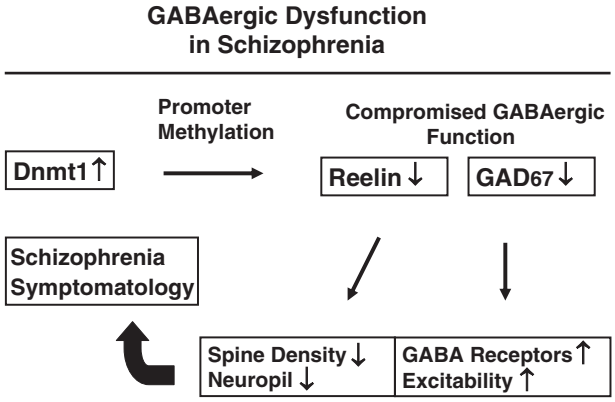

Fig. 4. The epigenetic origin of SZ. This depicts the central hypothesis addressed in the context of the review. Based on published work (Veldic et al., 2004), we have been able to show that Dnmt1 is more abundantly expressed in GABAergic neurons of the schizophrenia cortex as compared to non-psychiatric subjects. This expression is in those same neurons that show reduced levels of reelin and GAD_{67} expression. As one function of Dnmt1 is the regulation of genes through changes in methylation, the figure begs the question as to whether this might in fact be a causal relationship and not simply a correlation. One attractive facet of the proposed hypothesis is that it provides a reasonable mechanism for the down-regulation of multiple genes in the context of the schizophrenia brain. The scheme also suggests that it is the collective down-regulation of multiple mRNAs and proteins that then leads to the symptoms of the disease. In fact, one may be able to account for the multiple genes that are implicated in microarray studies of human post-mortem schizophrenia brain in terms of the GABAergic hypothesis that we have put forth.

to form multimolecular repressor complexes that maintain genes in a silenced or transcriptionally inactive state. Consistent with this model, we have shown that the HDAC inhibitor, Trichostatin A (TSA), induces reelin mRNA expression and that the extent of promoter methylation following treatment is reduced relative to non-treated cells (Mitchell et al., 2005). This suggests that by altering the acetylation status of histones in the vicinity of active promoters, one can also induce demethylation in this same region. Of relevance to the treatment of SZ and bipolar illness is a report which shows that the mood stabilizer, VPA, at therapeutic doses, acts as a HDAC inhibitor (Phiel et al., 2001). This action is comparable to that of TSA and we have shown that VPA induces reelin promoter expression both in vitro (Chen et al., 2002) and in vivo (Tremolizzo et al., 2002). Moreover, the increased expression is accompanied by alterations in reelin promoter methylation (Mitchell et al., 2005). We hypothesize that reelin expression is regulated by the coordinate interaction of selected transcription factors (Sp1, Tbr1 and Pax6) with their recognition sites and that access to these sites is limited by alterations in chromatin structure. The alterations in chromatin structure are defined by the methylation status of the promoter and the action of HDACs acting in concert to provide access to actively transcribed genes (Newell-Price et al., 2000; Robertson, 2002).

6. Gene expression in GABAergic neurons

With respect to the possible coordinate regulation of GAD_{67} and reelin expression, GAD_{67} mRNA levels are reduced in multiple brain regions of SZ patients and in the heterozygous reeler (+/−) mice (HRM; Liu et al., 2001; Veldic et al., 2004). In contrast, reelin mRNA levels are normal in GAD_{67} (+/−) mice. This finding is consistent with the possibility that reelin signaling in the prefrontal cortex might influence GAD_{67} expression. That is, alterations in the levels of reelin may impact either directly or indirectly on GAD_{67} transcription. A more likely possibility is that both reelin and GAD_{67} are coordinately regulated either epigenetically through changes in promoter methylation or through the action of transcription factors that recognize elements common to both genes. The expression of a third gene under investigation, GAD_{65}, is not known to be altered in either the reeler heterozygotes or in the GAD_{67} (+/−) mice. More importantly, GAD_{65} is not down-regulated in the brains of patients diagnosed with SZ (Guidotti et al., 2000). We predict that GAD_{65} is independently regulated by selected transcription factors and may be insensitive to regulation by methylation. As these 3 genes are selectively expressed in GABAergic neurons in the adult CNS, it remains unclear as to why reelin and GAD_{67} are coordinately regulated through factors that affect methylaton, while GAD_{65} is not (Tremolizzo et al., 2002, 2005; Noh et al., 2005). In this context, our findings that Dnmt1 is over-expressed in the same cortical GABAergic neurons where there is decreased expression of reelin and GAD_{67} in patients diagnosed with SZ is consistent with a profound defect in gene regulation in cortical inhibitory interneurons. The co-localization data suggest that Dnmt1 results in a hypermethylation of the reelin and GAD_{67} promoters causing the observed down-regulation of the corresponding mRNAs. Subsequently, the reduced inhibitory tone provided by the compromised GABAergic interneurons would likely have pronounced downstream effects in terms of genes expressed in pyramidal neurons as well. One intriguing possibility is that because GABAergic transmission impinges on the activity of glutamatergic and dopaminergic neurons, a defect in these neurons could ultimately explain the abnormalities noted in multiple neurotransmitter projections that have been observed in the etiological studies of this disease. Certainly, defects in GABAergic tone impinging onto glutamatergic pyramidal neurons would then cause them to be hyperexcitable and this might explain certain aspects of the psychosis that is generally associated with SZ and often times bipolar illness. In other words, hyperexcitatability in specific glutamatergic circuits that result in some side effects associated with SZ are consistent with a defect resulting originally in defective inhibitory neurotransmission.

7. The methylation switch and schizophrenia

More recently, it has been shown that the acetylation state of histone H4 in hippocampal pyramidal neurons is altered following pilocarpine injection providing evidence for 1 mechanism of chromatin remodeling associated with seizure-induced changes in gene expression (Huang et al., 2002). Additional findings show that histone lysine methylation at gene promoters is involved in the developmental regulation and maintenance of region-specific expression patterns of both ligand-gated and G-protein coupled glutamate receptors (Stadler et al., 2005). This establishes a link between a specific epigenetic mark, H3-(methyl)-lysine 4, with glutamatergic signaling in the human brain. High levels of H3-(methyl) arginine 17 have been associated with down-regulated gene expression in the prefrontal cortex of subjects with SZ (Akbarian et al., 2005). Histone modifications may contribute to the pathogenesis of prefrontal dysfunction in SZ. Collectively, these data show that histone modifications which induce alterations in local chromatin compaction are relevant to psychiatric disease. These modifications (Biel et al., 2005), in turn, may affect the methylation state of the promoters to which they are linked. One long term goal in molecular psychiatry is to determine the extent to which inappropriate promoter hypermethylation and histone deacetylation impact on the expression of selected genes (e.g. reelin, GAD_{67}, GAD_{65}) and to establish whether methylation-induced gene inactivation is associated with SZ (Grayson et al., 2005). As we have been arguing, these events likely affect the expression of many promoters and the common determinants that link these genes appears to go beyond simple physical proximity such as chromosomal clustering. If this common link is associated with methylation it still remains unclear as to what is the signal that targets the methylating enzyme to the set of promoters that are affected.

We (Grayson et al., 2005) and others (Abdolmaleky et al., 2005) have provided data showing that there are differences in the methylation patterns of the human reelin promoter in postmortem brain samples obtained from patients diagnosed

with SZ as compared to non-SZ subjects. These latter data suggest the very real possibility that changes in promoter methylation may arise through the overexpression of the methylating enzyme Dnmt1 (Veldic et al., 2004, 2005). Collectively, these studies establish that within the reelin promoter there is a short stretch of *cis*-acting elements that function in both positive and negative transcriptional regulation depending on whether key bases within this region are methylated or not. This finding raises the interesting possibility that the down-regulation of reelin expression documented in psychiatric patients might be the consequence of inappropriate promoter methylation. Moreover, it suggests that the polygenic origin of SZ may arise from the aberrant methylation of multiple promoters as one facet of deviant epigenetic gene regulation.

The increased methylation may interfere with the ability of certain transcription factors to interact at their cognate recognition sites and hence could hamper their ability to promote transcription of the corresponding genes. At the same time, it seems equally plausible that methylation recruits repressor proteins to the promoter that then block transcription and maintain the chromatin in a more condensed state. In either case, the increased Dnmt1 and increased methylation in cortical GABAergic neurons is consistent with current thinking that supports the concept that SZ is a primary epigenetic disorder of GABAergic neurons (Benes & Berretta, 2001; Costa et al., 2003b; Fatemi et al., 2005b; Guidotti et al., 2005; Lewis et al., 2005).

An interesting implication of the hypermethylation hypothesis is that it suggests a mechanism by which multiple genes could be affected through a common pathway and thus provides an explanation for data beginning to emerge from numerous microarray studies that are based on comparisons of RNA from SZ patients as compared to non-psychiatric subjects (see, for example, Pongrac et al., 2002; Lehrmann et al., 2003; Vawter et al., 2004). This hypothesis is presented schematically in Fig. 4 and, simply stated, suggests that over expression of Dnmt1 leads to promoter hypermethylation of selected genes in GABAergic neurons which then leads to the reduced expression of the mRNAs affected by this mechanism in these neurons. We propose that the down-regulation of multiple genes in these neurons collectively results in a GABAergic neuronal circuit dysfunction and in the disruption of the high frequency synchronization related to working memory deficits, cognitive impairment and executive function deficits associated with SZ (Perlstein et al., 2001; Spencer et al., 2004).

As suggested by Fig. 4, the reduced levels of GAD_{67} result in decreased GABA release at synapses and an upregulation of post-synaptic $GABA_A$ receptors located on dendrites, cell bodies or the initial axon segments of pyramidal neurons (reviewed in Guidotti et al., 2005; Lewis et al., 2005). The observation that there was increased $GABA_A$ receptor binding in the anterior cingulate cortex of SZ patients was first noted some years ago (Benes et al., 1992). This was followed by a series of studies that suggested alterations in GABAergic signaling as a primary cause of increased excitatory input in prefrontal areas which might underlie the pathophysiology of SZ (Benes et al., 1996, reviewed in Benes & Berretta, 2001). The increase in receptors is accompanied by changes in the expression of the GABA Transporters, GAT1 and GAT3 (Schleimer et al., 2004). These changes in inhibitory neural transmission, in turn, result in hyperexcitability and subsequent changes in genes associated with pyramidal neuron NMDA and/or AMPA receptors (Woo et al., 2004). Reduced reelin expression, in turn, is likely to reduce mRNAs that are translated post-synaptically in the vicinity of dendritic spines and also mRNAs associated with dendritic spine maturation (Dong et al., 2003). Ultimately, these processes are likely to be responsible for the profound neuropil hypoplasia associated with SZ and the reduced dendritic spine density (Selemon & Goldman-Rakic, 1999; Rosoklija et al., 2000; Glantz & Lewis, 2001) and the long term potentiation (LTP) and memory deficits that have been observed in reelin deficient mice (Weeber et al., 2002; Larson et al., 2003; Beffert et al., 2005) and in an epigenetic mouse model of SZ (Tremolizzo et al., 2002, 2005).

8. Perspectives for an epigenetic treatment of reelin dysfunction in schizophrenia

Based on the evidence that in cortical GABAergic neurons an epigenetic methylation switch may be at the heart of the neuropathologies detected in SZ, a rationale approach to correct reelin expression deficiency in this disorder should be the use of drugs that correct the DNA methylation patterns. We have reported (Tremolizzo et al., 2002, 2005; Mitchell et al., 2005) that the transcriptionally silent hypermethylated reelin promoter can be reactivated by either Dnmt1 inhibitors or HDAC inhibitors. These data suggest that a new approach for the treatment of reelin deficiency in SZ and BP disorders should be to correct the hypermethylation of gene promoters and the resulting transcription down-regulation by blocking either (a) Dnmt1 or using (b) HDAC inhibitors.

8.1. Dnmt1 inhibitors

If, in fact, there is a tripartite link between Dnmt1, reelin and GAD_{67} that forms the basis for the deficits observed in SZ, then drugs targeted at their coordinate regulation may prove useful as a therapeutic strategy in the treatment of specific symptoms. The best characterized family of Dnmt inhibitors are the nucleotide analogues of cytosine that include 5-AZA. These inhibitors rapidly reactivate the expression of reelin that has undergone transcriptional silencing in neuronal progenitor cell cultures (Chen et al., 2002). Upon entering the nucleus, these nucleotide analogues are incorporated into DNA where they block methylation at CpG islands of the replicating DNA by binding available Dnmt in an unproductive reaction. These drugs are therefore active in the S-phase of the cell cycle where they serve a powerful mechanism in the inhibition of Dnmt. Minimally, the mechanism requires at least 1 round of replication for the incorporation. For this reason, the efficacy of these inhibitors when they do not incorporate into DNA, such as occurs in neurons, has not been sufficiently examined. One might expect that if these compounds need to be incorporated

into the genome then they might be relatively inactive in up-regulating gene transcription in differentiated neurons. Thus, the nucleoside analogue inhibitors, although very potent inhibitors of DNA methylation in replicating cells in vitro and in vivo, may not represent the most viable strategy to treat the promoter hypermethylation reported to occur in cortical GABAergic neurons of SZ patients (Grayson et al., 2005). In point of fact, this class of drugs is in clinical use for the treatment of various types of cancers (El-Osta, 2003).

In addition to the utility of antipsychotics used in conjunction with HDAC inhibitors (Harwood, 2003; Sharma, 2005; Tremolizzo et al., 2005), certain Dnmt1 inhibitors warrant consideration as well. In the course of studies with NT2 cells in vitro, we have found that the Dnmt1 inhibitor doxorubicin induces both reelin and GAD_{67} within a comparable time frame. Doxorubicin is currently used in the chemotherapeutic treatment of certain cancers due to its ability to intercalate into the DNA double strand and subsequently inhibit the action of Dnmt1. Doxorubicin has been shown to inhibit the catalytic activity of Dnmt1 in enzymatic assays and kills cells via a drug-induced apoptotic cell death (Yokochi & Robertson, 2004). In our experiments, we have been able to induce both reelin and GAD_{67} mRNA 100- and 16-fold, respectively, at a concentration of 250 nM after a relatively short duration exposure of 12 hr (Kundakovic et al., 2005). While the efficacy of this compound in vivo remains unclear, it suggests the possibility that targets of Dnmt1 action need to be explored in the context of SZ.

8.2. HDAC inhibitors

As discussed above, another possible strategy for pharmacological intervention to normalize the epigenetic-reduced reelin and other gene expression deficits in cortical GABAergic neurons of SZ patients is to reduce promoter methylation by administering HDAC inhibitors (such as VPA or TSA). Although the mechanism of action of how HDAC inhibitors facilitate a reduction in promoter methylation is by no means straightforward, it is believed that HDAC inhibitors are effective by hyperacetylating nucleosomal histone tails and thus: (a) controlling Dnmt1 accessibility to promoter DNA segments and/or (b) inducing DNA demethylase activity.

In the methionine-induced mouse model of SZ (see above), VPA, in doses that enhance brain AcH3 content, also corrects the associated PPI and social interaction deficits (Tremolizzo et al., 2005). Because VPA is an inhibitor of HDAC with mmol affinity it is possible that at the high doses used, its pharmacological actions on reelin mRNA and the prevention of the behavioral deficits observed in methionine-treated mice are mediated by mechanisms that are independent of an action on histone acetylation. In testing the potency of different classes of HDAC inhibitors in increasing brain nucleosomal acetylated histone content, we observed that the benzamides (MS275, sulpiride, and amisulpiride) are 50- to 100-fold more potent than VPA in increasing AcH3 content in frontal cortex and hippocampus of mice. Moreover, these drugs like VPA prevent reelin promoter hypermethylation induced by methionine and revert the behavioral deficits in the methionine-induced epigenetic mouse model of SZ.

We suggest that the benzamides such as MS-275 and sulpiride, by blocking HDAC, may provide a unique way to treat epigenetic alterations in GABAergic neurons such as those detected in brains of SZ and BP disorder patients. Considering the epigenetic origin of the GABAergic dysfunction in SZ, the identification of an epigenetic pharmacology targeted to chromatin structure remodeling may open a new approach to relieve SZ symptomatology without using direct receptor agonists, antagonists or modulators.

9. Significance

Experiments described in this review are designed to provide information relevant to the mechanism(s) responsible for the coordinate regulation of human reelin and GAD_{67} gene expression. More specifically, we have attempted to establish a role for methylation, as well as histone acetylation in the context of transcriptional regulation of the respective promoters. As tissue-specific and developmental expression patterns are accompanied by distinct alterations in chromatin structure and DNA methylation status (Razin, 1998), it is important to focus on how methylation of critical cytosines within these sequences affects the access of transcription factors to these sites. Based on our data, we suggest that methylation represents a switch that can be used to turn off reelin expression under appropriate conditions. An important concept that has been reinforced by the discussion of our studies is that increased methylation increases the recruitment of methyl CpG binding proteins to the promoter inducing a repressed gene state (Grayson et al., 2005; Dong et al., 2005). Whether the methylation switch is reversible is still open to exploration. However, in vitro studies in which RA-induced reelin expression is accompanied by changes in the methylation of the reelin promoter suggest that this might be the case. It is not clear whether this involves demethylation directly or not. In the near future, it will be important to address whether GAD_{67}, which appears to be coordinately regulated with reelin, shares similar transcription factors and is similarly sensitive to methylation.

The role that methylation plays in regulating gene expression in the nervous system is still under explored. We know that alterations in methylation can result in mental retardation. Mutations that occur in methyl CpG binding proteins have drastic consequences that occur post-natally. We will determine whether the reelin and GAD_{67} promoters are functionally compromised in patients diagnosed with psychosis while the GAD_{65} promoter is not. It seems likely that there may be allelic variations in the recognition sequences of transcription factors that coordinately regulate the expression of these 2 genes. However, it seems more likely that alterations in expression may arise instead from aberrant methylation that alters transcription factor access leading to repressed expression. Alterations in the expression of Dnmt1 (or also 3a and/or 3b) could lead to global changes in genomic methylation resulting in changes in the expression of large numbers of genes. While it remains to be established, we would like to suggest that the

variable symptomatology associated with the SZ spectrum of disorders might be the consequence of inappropriate methylation patterns resulting from the dysregulation of this fundamental regulatory mechanism. Interestingly, an analysis of genomic DNA from monozygotic twins discordant for SZ showed discrepancies in the methylation patterns of certain genes suggesting that an epigenetic mechanism may account for differences between monozygotic twins (Tsujita et al., 1998; Petronis et al., 2003; Kato et al., 2005). In the very near future, experiments should provide insights into the regulation of specific gene products, which in the adult are located in the vicinity of pre- and post-synaptic densities of dendritic spines. Since both the numbers of dendritic spines and the levels of reelin and GAD_{67} are decreased in SZ, any understanding of this down-regulation may lead to the elucidation of mechanisms that fail to operate in psychiatric illness. In this same context, we propose the scheme outlined in Fig. 4 to explain the epigenetic origin of SZ as it relates to the concepts we've discussed thus far. The observation that Dnmt1 is over-expressed in GABAergic neurons of SZ patients is consistent with the idea that an epigenetic origin involving hypermethylation may be at the heart of the disorder. We do not, as yet, understand the mechanism of this up-regulation but it may be related to an as yet unknown regulation of the Dnmt1 gene in response to hormones likely secreted during puberty. This would account for the observations that (1) reelin down-regulation from birth leads to a more dramatic and severe disease phenotype (Hong et al., 2000) and (2) the onset of SZ symptoms occurs very rarely before puberty. While this hypothesis is as yet unproven, it provides an appropriate framework for testing hypotheses related to the temporal regulation of the Dnmt1 gene in the human as it relates to various hormonal influences that may act at the level of the corresponding promoter.

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