Chapter

DNA Methyltransferases and Schizophrenia: Current Status

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Abstract

Schizophrenia (SZ) is a complex disorder without a single cause but with multiple etiologies. Monozygotic twin studies suggesting high discordant rates provide evidence for epigenetic mechanisms among the factors that result in increased susceptibility. Among the different epigenetic modifications in mammals, DNA methylation mediated by DNA methyltransferases (DNMTs) is the most-well studied. Studies on post-mortem brain samples and blood samples of SZ patients revealed altered levels of most DNMTs. In addition, some recent studies also reported disease-associated SNPs in the DNMT genes. While the effects of dysregulation of DNMTs are beginning to be understood, many unanswered questions remain. Here, we review the current evidences that shed light on the relationship between DNMT dysregulation and SZ, and suggest the possible strategies to address some of the unanswered questions.

Keywords: Schizophrenia, DNA methyltransferases, DNA methylation, Dysregulation, Abnormal neurogenesis

1. Introduction

Schizophrenia (SZ) is a severe and chronic mental disorder with an incidence of ~1%, affecting ~20 million people worldwide [1]. The main symptoms of SZ include hallucination, delusion, abnormal disorganized behavior, disorganized speech, disturbances of emotions such as marked apathy, etc. The disorder is associated with considerable disability and can affect educational and occupational performance with 2–3 times increased likelihood of death earlier than the general population [2].

SZ is a complex disorder with no single causative factor but with multiple etiologies (**Table 1**). The five main factors that are believed to result in increased risk are: physical and chemical changes in brain [3], pregnancy or birth complications [4], childhood trauma [5], genetic [6] -and epigenetic [8]. Among these, a high risk among first-degree relatives compared to the general population and increased risk in monozygotic than dizygotic twins suggest genetic factors [7]. However, the observed concordance rates (~50%) in monozygotic twins that were much lesser than expected for a purely genetic risk (nearly 100%) suggest the contribution of epigenetic mechanisms to SZ [9].

Recent data based on brain imaging and molecular-genetic studies suggest that SZ is a form of neurodevelopmental disorder [10]. The neurodevelopmental hypothesis for SZ suggests pathological neurodevelopment during first and second

S. No	Factor	Comment	References
1	Physical and chemical changes in brain.	Subtle structural changes have been observed in post-mortem brain samples of SZ patients. Imbalances in neurotransmitters such as dopamine and glutamate have been linked to SZ.	[3]
2	Pregnancy or birth complications	Low birth weight, infection during pregnancy, asphyxia, premature labour, maternal obesity diagnosis in pregnancy, etc. have been associated with SZ in the offspring.	[4]
3	Childhood trauma	There is an increased risk to experience SZ if there is death or permanent separation of one or both parents.	[5]
4	Genetic	The risk in identical twins (1 in 2) is four times higher than non-identical twins (1 in 8). These risks are much higher than for general population (1 in 100).	[6, 7]
5	Epigenetic	Monozygotic twins show only 45–50% concordance.	[8, 9]

Table 1.
Risk factors for schizophrenia.

trimesters of pregnancy results in altered neuronal circuits which in turn result in psychosis in adolescents or young adults when exposed to increased biological or psychological stress. Evidences in support of this hypothesis comes from genetic studies that identified affected genes and risk factors during perinatal life that may disrupt the normal process of neurodevelopment. In addition, studies over the past 20 years showed that in comparison with controls, SZ patients after the onset exhibit accelerated aging-related loss of brain tissue [11]. Specifically, the patients show increased age-related reduction in the proportion of grey matter compared with controls [12]. These findings suggest that altered neurodevelopment may underlie the processes associated with SZ.

2. Epigenetic mechanisms

As mentioned above, evidence on the contribution of epigenetic mechanisms in SZ comes from monozygotic twin studies wherein the concordance rates are only ~50%. This low concordance rate suggests the interplay of genes and environment resulting in SZ. Because of this interplay, the epigenetic mechanisms have been suggested to be among the etiological factors [8]. Epigenetic mechanisms are defined as processes that can alter the patterns of gene expression without causing a change in the DNA sequence [13]. These mechanisms operate at the levels of transcription, mRNA stability and translation (**Table 2**). At the level of transcription, mammalian genes can be regulated by covalent modifications of the DNA [19], modifications of N-terminal tails of histones [15], microRNAs [20], circular RNAs [17] and long noncoding RNAs [21]. A number of modifications of RNA have been reported to influence mRNA stability and efficiency of translation. These modifications and their roles are described elsewhere [22, 23]. Because of epigenetic differences, genetically identical cells in a multicellular organism express different sets of genes that confer cell type – specific identity and function [24]. The most well studied epigenetic modification is methylation of the 5th carbon in the cytosine residues in the genomic DNA, often referred to as cytosine methylation. This modification mostly occurs in the CpG dinucleotides because of the maintenance mechanism in a post-replicative manner involving hemi-methylated DNA [see below]. As such, DNA methylation is often used as a synonym to CpG methylation

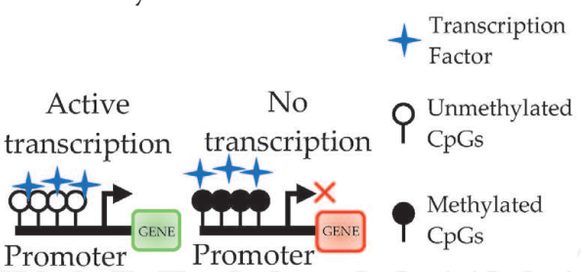
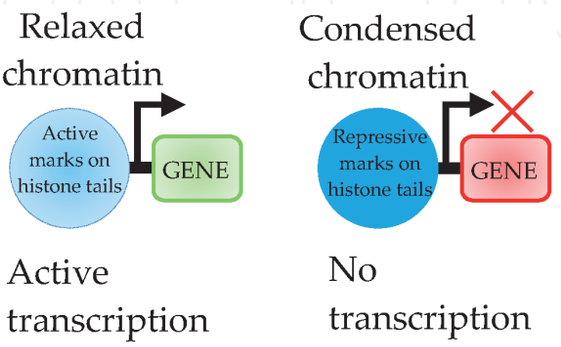
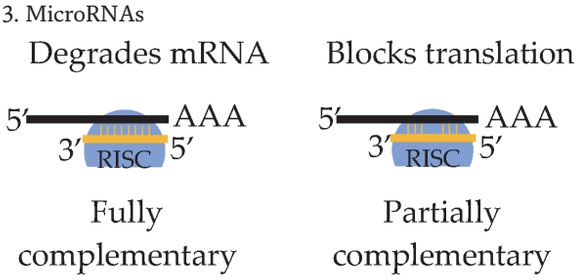
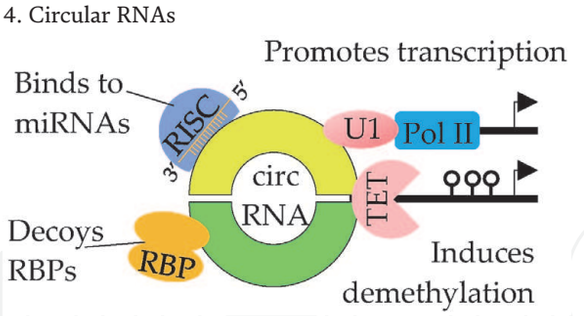
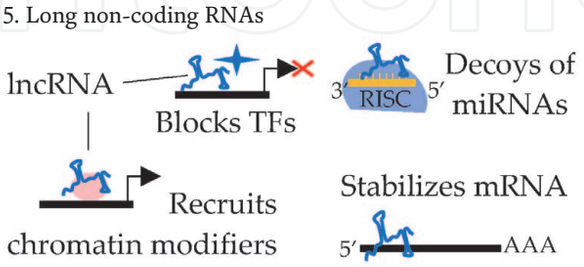
Mechanisms	Machinery	Comment
1. DNA Methylation 	Establishment and maintenance: DNMTs Erasure: TETs, AID/APOBECs.	Altered methylation patterns and gene expression patterns observed in SZ [14].
2. Histone tail modifications 	Establishment: HATs, HMTs Erasure: HDMs and HDACs.	Aberrant histone modifications were reported in SZ [15].
3. MicroRNAs 	DROSHA, EXPORTIN, DICER, RISC and ARGONAUTE	Altered miRNA profiles were reported in SZ [16].
4. Circular RNAs 	RNA binding proteins, muscleblind (MBL), quaking and adenosine deaminase.	Multiple circRNAs have been confirmed to play important roles in the occurrence and development of SZ [17].
5. Long non-coding RNAs 	PRC1: Polycomb Repressive Group proteins.	Long non-coding RNAs are associated with SZ and effects in the neuronal structure [18].

Table 2.
Epigenetic mechanisms in regulating gene expression.

in mammals. A family of enzymes, referred to as DNA methyltransferases (DNMTs) are responsible for establishment and maintenance of DNA methylation [25]. Several studies that focused on the relationship between DNA methylation and gene expression showed an inverse correlation, meaning that DNA methylation is

often associated with repressed state of the promoters [26]. In case of histones, the lysines in the N-terminal tails of core histones can either be acetylated or methylated. These modifications occur on the same lysine residues and are therefore mutually exclusive [27]. Whereas histone lysine acetylation is always associated with gene expression, histone methylation is associated with either expression or silencing depending on the residues involved [28]. For example, methylation at lysine 9 of histone H3 (H3-K9) or H3-K27 is associated with silencing. On the other hand, H3-K4 or H3-K36 methylation is associated with gene expression. Histone methyltransferases and histone acetyltransferases are two families of enzymes for imparting the two covalent modifications of the N-terminal tails of the core histones [29]. As in case of DNA methylation, histone marks are also heritable. The covalently modified nucleosomes from the parental chromatin are segregated equally among the two daughter DNA molecules so that additional nucleosomes containing histone marks identical to the parental nucleosomes are assembled [30]. Both DNA methylation and histone modifications are reversible involving different categories of enzymes and processes. The machinery of DNA methylation and demethylation is described in the next section [Section 2.1]. With regard to the histone modifications, histone demethylases (HDMs), histone methyltransferases (HMTs), histone acetyltransferases (HATs) and histone deacetylases (HDACs) together play a role in erasure and establishment of histone modification marks [31]. HDMs remove methyl groups from the lysines of the core histones so that the unmethylated lysines can be acetylated by HATs. HDACs, on the other hand, remove acetyl groups from the acetylated lysines so that the same residues can be methylated by HMTs.

Apart from covalent modifications of the genome, long noncoding RNAs (lnc RNAs), microRNAs (miRNAs) and circular RNAs (circRNAs) also play an important role in regulating gene expression. Of these, circRNAs and miRNAs regulate expression at post-transcriptional levels whereas lncRNAs can regulate at both transcriptional and post-transcriptional levels. Lnc RNAs are ≥200 nucleotides, do not encode any protein and regulate genes at the both transcriptional and post-transcriptional levels [32]. At the level of transcription, lncRNAs either can promote histone modifications and chromatin condensation or recruit transcription factors to facilitate gene expression or evict transcription factors and result in gene repression. In addition, lncRNAs are also known to influence alternative splicing, polysome recruitment to enable translation, act as decoys for microRNAs (miRNAs) and regulate mRNA stability. The miRNAs, on the other hand cause translational repression of the target mRNAs. Each miRNA is ~22 bases long and can recognize multiple targets having a few mismatches at their 3′-ends [33]. In cases, where there is no mismatch, miRNA can induce degradation of the target mRNA sequence [34]. CircRNAs are generated by back-splicing or non-colinear splicing of pre-mRNA molecules and may include both exonic and intronic sequences [35]. In addition to competing with canonical splicing and controlling the levels of the corresponding protein-coding mRNAs, circRNAs can also act as protein decoys or miRNA sponges to regulate gene expression [36].

2.1 DNA methylation and demethylation machinery

Of the different epigenetic mechanisms influencing gene expression described above, DNA methylation-mediated regulation of gene expression is the most-well studied. DNA methylation is established and maintained by DNMT family of enzymes whereas different mechanisms exist for demethylation (**Figure 1A**). Of the four members of DNMTs that facilitate DNA methylation, DNMT3L does not have an active methyltransferase (catalytic) domain. DNMT3A and 3B are *de novo* methyltransferases of which DNMT3A is mainly responsible for establishment of

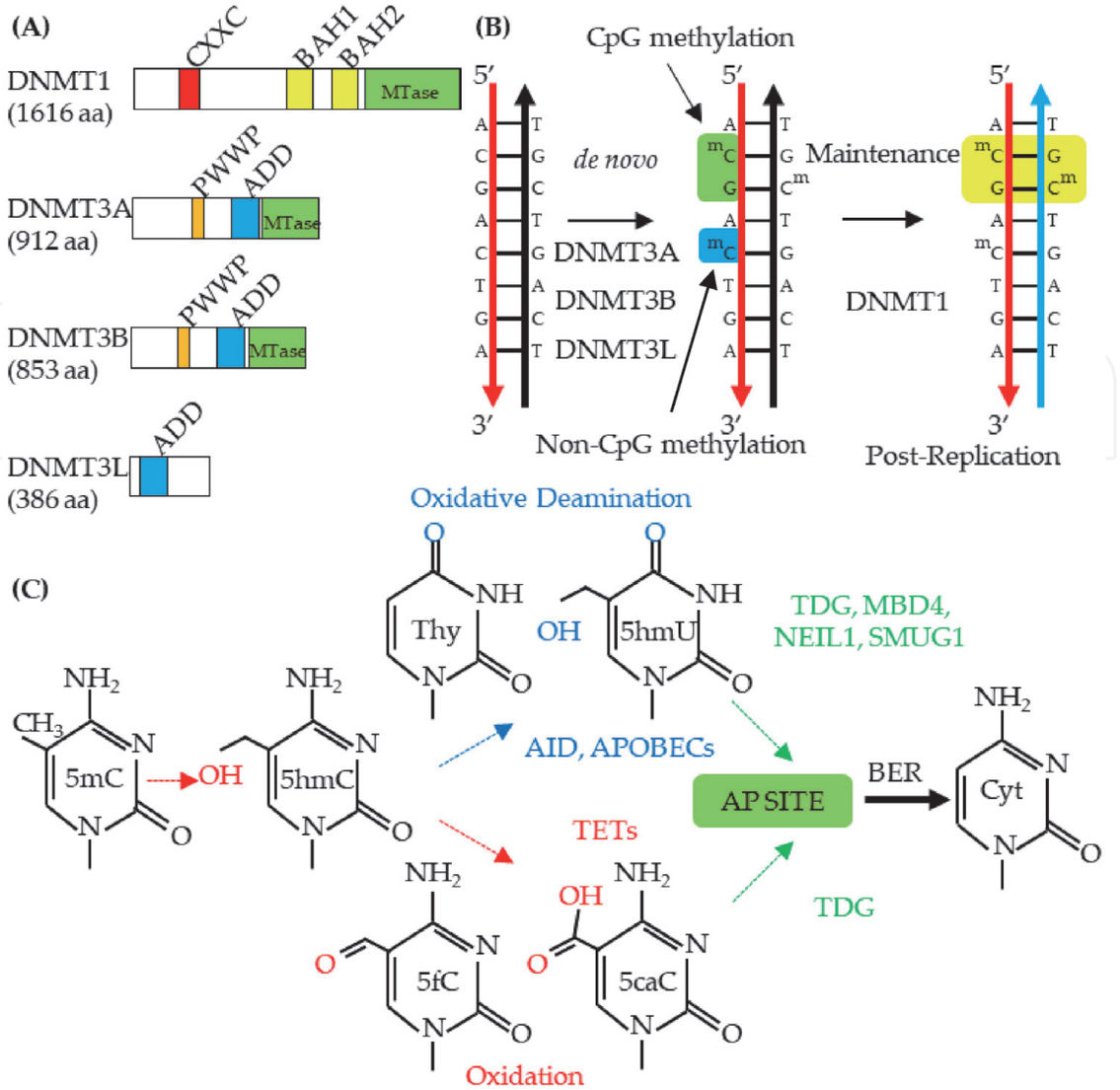


Figure 1.
DNA methylation and demethylation machinery. (A) Domains of DNMTs. CXXC: Cys-X-X-Cys domain, BAH: Bromo-Adjacent Homology domain, MTase: Methyltransferase domain, PWWP: Pro-Trp-Trp-Pro domain, ADD: ATRX-DNMT3-DNMT3L domain (B) Cytosines are methylated by de novo *methyltransferases DNMT3A and DNMT3B with the help of DNMT3L. Only methylated CpGs are maintained by DNMT1. (C) Different pathways of demethylation of methylated cytosines (5mC). TET: ten-eleven translocation (TET) proteins, AID/APOBEC: activity-induced cytidine deaminase/ apolipoprotein B mRNA editing complex, Thy: thymine. TDG: Thymine-DNA glycosylase, AP: apurinic/apyrimidinic site, BER: base-excision repair. 5hmU: 5-hydroxymethyluracil, 5hmC: 5-hydroxymethylcytosine, 5fC: 5-formylcytosine and 5caC: 5-carboxylcytosine.*

methylation in imprinted genes whereas DNMT3B establishes methylation in pericentric repetitive regions [37]. DNMT1 is a maintenance methyltransferase, which methylates the daughter DNA strand in the hemi-methylated DNA generated after replication (**Figure 1B**). In this process, the methylated CpG sites in the parental strands serve as information to methylate the complementary CpG sites in the daughter strand. Demethylation, on the other hand can be achieved by cytidine deaminases or Ten-Eleven Translocation (TET) enzymes [38] (**Figure 1C**). Cytidine deaminases such as activated induced cytidine deaminase (AID) and apolipoprotein B mRNA editing enzyme catalytic polypetide 1 (APOBEC1) catalyze the conversion of methylcytosine to thymine [39], leading to T:G mismatches. These mismatches are repaired by base excision repair machinery that incorporates unmodified cytosine. The TET enzymes hydroxymethylate the methylated cytosines which are further processed into oxidized forms of cytosine (5-formylcytosine and 5-carboxycytosine) that are further subjected to base excision repair resulting

in active demethylation. Hydroxymethylcytosine results in passive demethylation via DNA replication because of absence of methylgroup in the parental strand in the hemimethylated DNA.

2.2 DNA methylation studies in schizophrenia

Initial studies on DNA methylation differences between SZ patients and controls, and among discordant monozygotic twins focused on candidate genes identified by genetic studies. For example, Abdolmaleky et al. [40] by using DNA from frontal lobes of post-mortem brain samples showed ~50% increased methylation in the RELN promoter. Subsequent DNA methylation studies focused on genes involved in Dopaminergic [41], GABAergic [42], Glutamatergic [43], serotonergic pathways [44] of neurotransmission and genes such as BDNF [45]. These studies used DNAs either post-mortem brain samples or peripheral blood lymphocytes. However, the data did not always yield consistent reports. For example, in case of BDNF promoter IV, decreased DNA methylation was observed in peripheral blood in a study by Kordi et al. [46] whereas, Ikegame et al. [47] and Ümit Sertan Çöpoğlu et al. [48] reported no change in the methylation levels in the same tissue. Subsequent studies which used genome-wide methylation analysis identified many genes showing statistically significant differences in DNA methylation, but the effective values or the degree of methylation differences observed were not large enough to demonstrate a biological effect such as altered expression. For example, in one of the first studies, Mill et al. [49] by using microarrays identified genes RPL39 and WDR18 with increased methylation in the promoter upstream regions of 8% and 3%, respectively. Studies conducted after these observations used a variety of technologies such as Methylated DNA Immunoprecipitation (MeDIP) – sequencing and Illumina-27 K and 450 K arrays and reported differentially methylated sequences with low effective values. Importantly these studies identified genes with little or no overlap among the top gene hits corresponding to the most significant differentially methylated sites [50]. Nevertheless, some of these genome-wide studies also identified methylation differences in candidate genes such as COMT [51], GAD1, RELN [52] and BDNF [53]. Although these genome-wide studies did not yield common genes with significant differences in DNA methylation, bioinformatic analyses revealed common pathways. For example, methylome data using the blood DNAs revealed the involvement of functioning of the immune system [54]. This in turn is in agreement with the genome-wide association studies that identified immune-related genes including the major histocompatibility locus [55]. Another common pathway identified in both blood– and DNA- based studies is the neurodevelopmental processes [56]. The DNA methylation studies were also extended to study the effects on gene expression. In one such study, Liu *et al*. [57] identified 16 differentially methylated sites using a case–control approach. When the corresponding 16 genes were studied only five genes showed an inverse correlation of expression with methylation whereas two showed a positive correlation. The remaining genes showed no difference in the level of expression. Besides analysis of gene-related regions of the genome, bulk DNA methylation in SZ patients was also investigated. In such studies, Bonsch *et al*. [58] observed lower levels of methylation in peripheral blood monocytes of patients among discordant monozygotic twins. Meals *et al*. [59] also found a decreased global methylation levels in leukocytes of patients compared to normal individuals. However, these studies are not in agreement with Bromberg *et al*. [60] who did not observe any difference in the global methylation levels in leukocytes. Overall studies on the global methylation levels were inconclusive and likely to be influenced by factors such as age, gender, medication and smoking behavior. In summary, some but not all studies observed

significant differences in DNA methylation levels in the candidate genes whereas genome-wide studies indicated the involvement of neurodevelopmental processes and immune system function. These results are consistent with the model of etiology that SZ is a complex disorder with no single causative factor.

2.3 Dysregulated DNMTs in schizophrenia

Epigenetic processes and epigenetic modifications are tightly controlled to enable normal mammalian development. In this context, the presence of aberrant DNA methylation patterns affecting the candidate genes suggests the possibility of the role of dysregulation of epigenetic machinery in SZ. Investigations on dysregulation of DNA methylation machinery in SZ dates back to 2005 when Veldic *et al.* [61] reported increased DNMT1 levels in the GABAergic interneurons of post-mortem brain tissues of SZ patients. This increase was also correlated with increased promoter methylation and decreased expression of *REELIN,* an extracellular matrix protein and *GAD67*, an enzyme involved in production of GABA. Importantly, DNMT1 inhibitors were reported to decrease hypermethylation and increased expression of the two genes [62]. Subsequently, HDAC inhibitors were also shown to relieve the repression associated with DNMT1 overexpression to an extent similar to DNMT1 inhibitors [63]. These results suggest the potential of epigenetic drugs in ameliorating the phenotypes associated with SZ. Later experiments in brain tissues of patients revealed that at increased levels, DNMT1 binds to *REELIN*, *GAD67* and *BDNF* promoters in cortex but not cerebellum. Further, this selective cortex-specific binding is not associated with any changes in the levels of DNA methylation [64]. The authors suggested that increased DNMT1-associated downregulation of the three genes can be independent of the catalytic activity of DNMT1. As mentioned above, DNMT1 is a maintenance methyltransferase and cannot introduce new methyl groups in the DNA. Therefore, hypermethylation of *REELIN* and *GAD67* is possible only if there is *de novo* methylation followed by maintenance methylation of DNMT1. Not surprisingly, overexpression of DNMT1 as well as DNMT3A was subsequently observed in post-mortem brain samples as well as peripheral blood lymphocytes of SZ patients [65]. Further, DNMT3B overexpression was also reported in peripheral blood lymphocytes but is not reported as of date in post-mortem brain tissues of SZ patients. Since both DNMT3A and 3B are required for *de novo* methylation, it is not unexpected that DNMT3B would also be overexpressed in the brain tissues of the patients. In addition to human studies, experiments using offspring of prenatal restrained stressed mice also confirmed the association of increased DNMTs with SZ-associated phenotypes. In the progeny, DNMT1 and 3A protein levels were high with increased binding of DNMT1 and MeCP2 (Methyl-CpG binding protein 2) and repression of *REELIN* and *GAD67* promoters [66].

Taken together, there is reasonable argument for DNMT1 and DNMT3A and, possibly DNMT3B overexpression as risk factors for SZ. However, the information on the number of genes dysregulated due to DNMT1 overexpression was limited only three (*REELIN, GAD67* and *BDNF*). By taking DNMTs as risk-conferring genes, Saradalekshmi *et al*. [67] investigated whether any SNPs of DNMTs are associated with SZ. In this case–control study, minor alleles at rs2114724 and rs2228611 of *Dnmt1*, rs2424932 and rs1569686 of *Dnmt*3B and rs2070565 in *Dnmt3L* showed significant association with SZ. The authors also reported that rs2424932 showed an association in male patients whereas rs1569686 was associated with an earlier onset in patients with family history. Bioinformatic analysis on the effects of these SNPs suggested that the minor alleles affect the splicing of *Dnmt1* or *Dnmt3L*

transcript or reduce the levels of expression of *Dnmt3B*. However, functional studies on these SNPs were not reported yet.

2.4 Models of dysregulated DNMTs

In the light of reports suggesting increased DNMT1 and/or DNMT3A levels as risk factors for SZ, it is important to understand the effects of their overexpression on neurodevelopment. Unfortunately, overexpression of DNMT1 results in mid-gestational lethality in mice [68] making it impossible to generate animal models with constitutive overexpression. In addition, reduction of DNMT1 protein levels, but not its absence, appears to be an essential step for differentiation [69]. In this context, it is also difficult to generate mice conditional alleles of *Dnmt1* that enable neurogenesis-specific overexpression. Therefore, we proposed that cell-based models that either over express DNMT3A or DNMT1 or together serve as useful tools for studying the effects on neurogenesis. Specifically, embryonic stem cells (ESCs) are attractive because they provide opportunities to investigate the effects of DNMT1 and /or DNMT3A overexpression at different stages of neural differentiation. For instance, during the induction of neuronal differentiation, the ESCs are first differentiated into embryoid bodies (EBs) to obtain progenitor cells with ectoderm, endoderm and mesoderm specification. From EB stage, the cells can be differentiated into neuronal progenitor cells (NPCs) and subsequently into neurons.

In order to study the effects of DNMT1 overexpression on neurogenesis, D'Aiuto *et al*. [70] utilized *Dnmt1*$^{tet/tet}$ (*Tet/Tet*), a mouse embryonic stem cell line that overexpresses DNMT1 (**Figure 2A**). This cell line was generated by insertion of *tet-off* cassettes between the *Dnmt1* promoters and the start codons of both chromosomes [71]. As a result, the endogenous *Dnmt1* promoter expressed tTA, a transactivator that binds to the CMV-*tet operator* (*TetO* + *CMV* sequence present at the 3′-end of the *tet-off* cassettes. This resulted in increased expression of DNMT1 in the *Tet/Tet* ESCs. When doxycycline is added to this cell line, tTA became inactive and could not express *Dnmt1* and making the genome hypomethylated. When the *Tet/Tet* ESCs were used for neuronal differentiation by the authors, there was reduction in DNMT1 levels in embryoid bodies with no difference between the wild-type (*R1*) and *Tet/Tet* cells. However, neurons differentiated from the *Tet/Tet* cells showed abnormal dendritic branching (**Figure 2B**), increased activity of N-methyl-D-aspartate (NMDA) receptor (**Figure 2C**) and increased levels of the NR1 subunit of the receptor. In this study, the authors reported that increased DNMT1 levels did not result in any hypermethylation of *Reelin* or *Gad67* promoters. This finding was not surprising because DNMT1 was only a maintenance methyltransferase and new methylation marks are established only by the *de novo* methyltransferases. Although this study indicated that DNMT1 overexpression results in abnormal neurogenesis, the effects on the levels of SZ-associated gene transcripts, particularly on genes such as *Gad67*, *Reelin* and *Bdnf* were not investigated.

In a recent study, Saxena *et al*. [72] used a modified neuronal differentiation method that resulted in increased expression of DNMT1 in *Tet/Tet* neurons (**Figure 2D**). These results suggested that *Tet/Tet* neurons were suitable for studying the expression levels of SZ-associated genes in presence of increased DNMT1 levels [73]. When 15 SZ-associated genes were tested between the *Tet/Tet* and *R1* neurons, 13 showed significantly altered transcript levels of which, 11 showed identical patterns of dysregulation as in patients (**Figure 2E**). Eight of these 11 also showed significantly altered transcript levels in *Tet/Tet* ESCs but the patterns were similar to *Tet/Tet* neurons in only five cases. These results suggested that the dysregulation patterns of the SZ-associated genes varied during the stages of

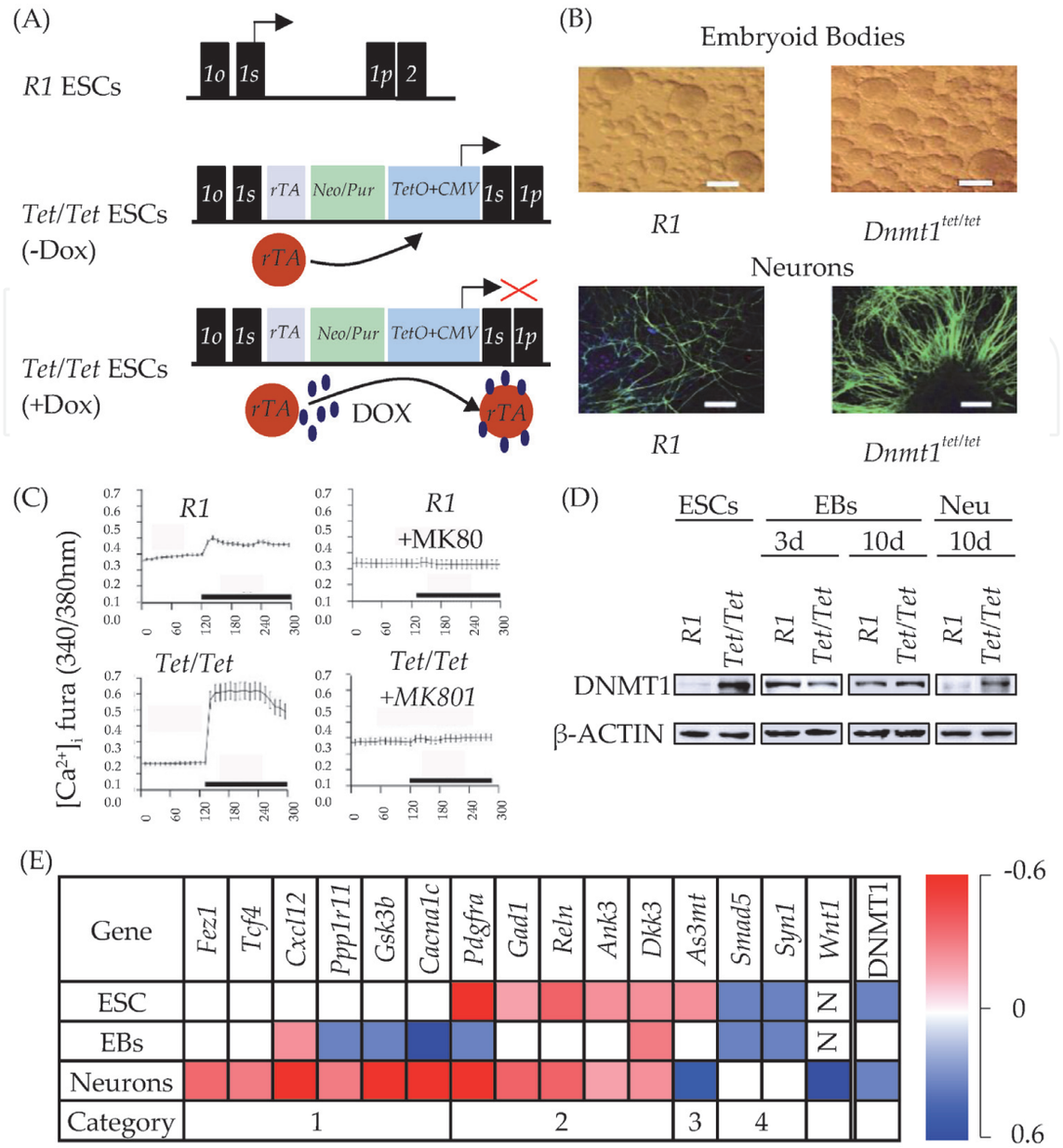


Figure 2.
(A) Generation of Tet/Tet *ESC line.* R1*: wild-type. Oocyte (1o), somatic cell (1s) and pachytene spermatocyte (1p) promoters are shown. (B) Embryoid bodies (EBs) and neurons differentiated from* R1 *and* Tet/Tet *ESCs. Neo/Pur: Neomycin and puromycin selection markers. (C) Increased NMDA receptor activity in* Tet/Tet *neurons. Compared to* R1 *neurons, when glutamate was added, the calcium uptake is higher in* Tet/Tet *neurons. This uptake is inhibited when MK801 (inhibitor of NMDA receptor) was used. (D) Western blot analysis of DNMT1 in* Tet/Tet *ESCs, EBs and neurons. (E) Four distinct categories of the 15 SZ-associated gene transcripts studied in* Tet/Tet *and* R1 *cells. Direction of change is indicated as per the color key. Red color indicates decreased transcript levels whereas increased transcript levels are shown in blue. Absence of color indicates no change.*

pluripotency and neuronal differentiation. The authors then used doxycycline to turn off *Dnmt1* and studied whether dysregulation observed in *Tet/Tet* ESCs could be reversed. Out of the eight genes tested in ESCs, the direction of transcript dysregulation for only four genes was reversed. These results suggested that by using DNMT1 inhibitors, it may not be possible to reverse DNMT1 overexpression-associated dysregulation of certain SZ-associated genes. Importantly, in this study, the authors did not observe any significant difference in the levels of methylation of the promoters of the affected genes either in ESCs or neurons. These results indicated that dysregulation of the genes studied in *Tet/Tet* neurons could be due to catalytic activity-independent effects of DNMT1. While the results on the *Tet/Tet*

cells undoubtedly revealed the effects of DNMT1 overexpression on a wider set of SZ-associated genes, details on the global effects of increased DNMT1 levels at the transcriptome and methylome levels are still awaited.

3. Conclusions

In conclusion, molecular details that connect DNMT1 overexpression with abnormal neurogenesis are beginning to emerge. With the availability of genome-wide methylation and transcriptome analysis methods, it is now possible to investigate the effects of DNMT1 overexpression in post-mortem brain samples of SZ patients. However, this effort requires an understanding on the incidence of DNMT1 overexpression in these samples. Of particular interest is to compare the effects of overexpression of DNMT1 or DNMT3A or both during the process of neuronal differentiation and the nature of the altered transcript levels. Whether the genes affected are only related to SZ or other neuropsychiatric disorders or neurodevelopmental disorders is an important question that needs to be addressed. Such information is useful to explore the contribution of epigenetic mechanisms in a wider spectrum of neurological disorders. In addition, improvement in the methods for generating genetically modified ESCs, their differentiation into specific types of neurons and development of brain organoids should help advance our understanding of the relationship between dysregulation of DNA methyltransferases and neurodevelopmental disorders such as schizophrenia.

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Conflict of interest

The authors declare no conflicts of interest.

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