Published in final edited form as:

Differentiation. 2011 July ; 82(1): 9–17. doi:10.1016/j.diff.2011.03.003.

Mouse ES cells overexpressing DNMT1 produce abnormal neurons with upregulated NR1

Leonardo D'Aiuto^{1,§}, Roberto Di Maio^{2,3,§}, K. Naga Mohan^{1,§}, Crescenzo Minervini⁴, Federica Saporiti², Isabella Soreca⁵, J. Timothy Greenamyre², and J. Richard Chaillet^{1,*}

Leonardo D'Aiuto: daiuto@msx.upmc.edu; Roberto Di Maio: rdimaio@hs.pitt.edu; K. Naga Mohan: kommumohan@gmail.com; Crescenzo Minervini: c.minervini@biologia.uniba.it; Federica Saporiti: federica.saporiti@ccfm.it; Isabella Soreca: sorecai@upmc.edu; J. Timothy Greenamyre: greenamyrejt@upmc.edu

¹Department of Microbiology and Molecular Genetics, University of Pittsburgh, Pittsburgh, PA 15213, USA

²Department of Neurology, University of Pittsburgh, Pittsburgh, PA 15260, USA

³Ri.MED Foundation Palermo, Italy

⁴Department of Surgery, University of Pittsburgh, 200 Lothrop St., Pittsburgh, PA 15213, USA

⁵Department of Psychiatry, University of Pittsburgh, 3811 O'Hara Street, Pittsburgh, PA 15213, USA

Abstract

High levels of DNA methyltransferase 1 (DNMT1), hypermethylation, and downregulation of *GAD67* and *reelin* have been described in GABAergic interneurons of patients with schizophrenia (SZ) and bipolar (BP) disorders. However, overexpression of DNMT1 is lethal, making it difficult to assess the direct effect of high levels of DNMT1 on neuronal development *in vivo*. We therefore used *Dnmt1^{tet/tet}* mouse ES cells that overexpress DNMT1 as an *in vitro* model to investigate the impact of high levels of DNMT1 on neuronal differentiation. Although there is down-regulation of DNMT1 during early stages of differentiation in wild type and *Dnmt1^{tet/tet}* ES cell lines, neurons derived from *Dnmt1^{tet/tet}* cells showed abnormal dendritic arborization and branching. The *Dnmt1^{tet/tet}* neuronal cells also showed elevated levels of functional N-methyl D-aspartate receptor (NMDAR), a feature also reported in some neurological and neurodegenerative disorders. Considering the roles of *reelin* and *GAD67* in neuronal networking and excitatory/inhibitory balance respectively, we studied methylation of these genes' promoters in *Dnmt1^{tet/tet}* ES cells and neurons. Both *reelin* and *GAD67* promoters were not hypermethylated in the *Dnmt1^{tet/tet}* ES cells and neurons, suggesting that overexpression of DNMT1 may not directly result in methylation-mediated repression of these two genes. Taken together, our results suggest that overexpression of DNMT1 in ES cells results in an epigenetic change prior to the onset of differentiation. This epigenetic change in turn results in abnormal neuronal differentiation and upregulation of functional NMDA receptor.

© 2011 International Society Of Differentiation. Published by Elsevier B.V. All rights reserved.

* Corresponding author: J. Richard Chaillet, University of Pittsburgh, Department of Microbiology and Molecular Genetics, Magee-Womens Research Institute, 204 Craft Avenue, Pittsburgh, PA 15213, Phone: (412) 641-8166, Fax: (412) 641 3899, Chaillet@pitt.edu.

§These authors contributed equally to this work

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

DNA methylation; Dnmt1; neuronal differentiation; NMDA receptor; epigenetic

Introduction

Epigenetic modifications of mammalian genomes include DNA methylation, and core histone methylation and acetylation that result in changes of gene expression through modulation of chromatin structure. Of these, DNA methylation is a covalent modification involving addition of a methyl group in CpG dinucleotides and is often inversely correlated with gene expression. During development, establishment of CpG methylation is mediated by the *de novo* DNA methyltransferases DNMT3A and DNMT3B whereas maintenance of the correct DNA methylation patterns in different genomic regions is catalyzed by the DNMT1 maintenance methyltransferase (Reinhart and Chaillet, 2005; Cirio et al., 2008).

Abnormal epigenetic modifications have been implicated as etiologic factors in major human psychiatric disorders. The protein products of a significant portion of the approximately 300 genes linked to mental disorders are directly or indirectly involved in epigenetic modulation. Examples of these include histone acetyltransferases, histone methyltransferases, histone demethylase JARID1C, histone binding protein CHD7, DNA binding proteins such as methyl DNA-binding protein MeCP2 and DNA methyltransferase Dnmt3A (Kramer and van Bokhoven, 2008). Rett syndrome (RTT) is a prime example of a disorder associated with an epigenetic modification. This neurodevelopmental disorder is caused by mutations in MeCP2, a methyl CpG binding protein involved in transcriptional repression. MeCP2 knockout mice show neurological defects and behavioral defects similar to those observed in RTT. Intriguingly, a gain in MeCP2 dosage also causes severe mental retardation and RTT features (Chahrour and Zoghbi, 2007). MeCP2 interacts with ATRX, an ATPase/helicase that is involved in regulation of gene transcription and chromosome segregation. ATRX is largely localized to highly methylated regions of condensed heterochromatin through binding to HP1, and mutations in ATRX lead to altered DNA methylation patterns. Mutations in ATRX are associated with α -thalassemia and mental retardation (Gibbons et al. 2008).

Recent evidence suggests that the pathogenesis and maintenance of complex psychiatric disorders such as major depression and schizophrenia, neurological disorders such as epilepsy, and neurodegenerative disorders such as Parkinson's disease and Huntington's disease rely on altered epigenetic mechanisms. For example, hypermethylation of *GAD67* and *reelin* promoters was observed in GABAergic interneurons of postmortem temporal cortex in schizophrenia and bipolar disorders. Such hypermethylation was correlated with reduced expression of these two genes (Chen et al., 2002; Costa et al., 2007). Interestingly, significantly high levels of DNMT1 were also observed in these samples. Considering the central role of DNMT1 in the maintenance of DNA methylation, it has been suggested that overexpression of this enzyme results in an aberrant methylation of the promoters of *reelin* and *GAD67* and a consequent down-regulation of these genes. Overall, these studies suggest that proper levels of DNMT1 may be required for normal neuronal function.

Effects of elevated levels of DNMT1 on neurogenesis and neuronal differentiation need to be further explored. *In vivo* studies pose several methodological challenges, because elevated levels of DNMT1 are lethal in mouse (Biniszkiewicz et al., 2002), and loss of DNMT1 in brain leads to neuronal cell death (Hutnick et al., 2009). Therefore, developing an *in vitro* model to explore the relationship between DNMT1 over-expression and abnormal neuronal differentiation is warranted. To this end, we used *Dnmt1^{tet/tet}* mouse ES

cell line in which the level of DNMT1 is approximately five times the level in the wild-type ES cells. In this study, we investigated the impact of high levels of DNMT1 on neuronal differentiation and on methylation of *GAD67* and *reelin* promoters in these ES cells and the resultant neurons.

Materials and Methods

Cell cultures

R1, *Dnmt1^{c/c}* and *Dnmt1^{tet/tet}* embryonic stem (ES) cell lines were cultured without feeders in ES medium containing LIF (1000 U/ml) as previously described (Smith 2001). *Dnmt1^{c/c}* cells are homozygous for a *Dnmt1* null allele that disrupts the C-terminal catalytic domain of the enzyme (Lei et al., 1996).

Neuronal differentiation from embryoid bodies

To generate embryoid bodies (EBs), R1 and *Dnmt1^{tet/tet}* ES cells were plated at 5×10^4 cells/well in non-adherent 6-well plates (Costar) in EB medium (DMEM containing 5% Knockout serum replacement (Invitrogen), $1 \times$ non-essential amino acids (Millipore), $1 \times$ nucleosides (Millipore), $100 \mu\text{M}$ β -mercaptoethanol, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 ug/ml Streptomycin and 100 U/ml Penicillin). Medium was changed every two days. On day 7 the EBs were collected, resuspended in a modified N2 medium (DMEM/F12 supplemented with N2 (Invitrogen), 1 $\mu\text{g/ml}$ mouse laminin I (R&D systems), $1 \times$ non-essential amino acids, 2 mM L-glutamine, $100 \mu\text{M}$ β -mercaptoethanol, 4 ng/ml bFGF (Invitrogen), 100 ug/ml Streptomycin and 100 U/ml Penicillin) and transferred to an adherent 6-well plate coated with 0.2% gelatin. The modified N2 medium was changed every two days.

Neuronal differentiation from neurospheres

The following procedure was used to obtain neurons from EB-derived neurospheres. EBs grown in EB medium for seven days were plated at a density of 40-50 EBs/ml in a standard, treated 10-cm tissue culture dish containing 12 ml of feeder-cell medium (DMEM supplemented with 10% FBS, $1 \times$ non-essential amino acids, 100 ug/ml Streptomycin and 100 U/ml Penicillin). Attached EBs were cultured until the attached cells reached confluence. Cells were then harvested, disaggregated to a single-cell suspension with trypsin, and one-fifth of the suspension replated. After the culture again reached a confluent state, the procedure was repeated. Afterwards, unattached spherical bodies (neurospheres) were collected by unit-gravity sedimentation in a 50-ml conical tube. Neurospheres were then cultured on poly-D-lysine-coated cover slips in Neurobasal medium supplemented with B27 (Invitrogen), 2 mM glutamine, 100 mg Streptomycin/ml and 100 U Penicillin/ml. The day of plating of neurospheres in supplemented Neurobasal medium was designated day-*in vitro* 0. The experiments were performed from day-*in vitro* 1 to day-*in vitro* 16. Neurospheres could be continually harvested from serially passaged cultures of attached EBs.

Analysis of DNA methylation

2.0 μg of genomic DNA was treated with bisulfite by using the Epitect bisulfite modification kit (Qiagen) following the manufacturer's instructions. Primers for amplifying bisulfite treated DNA were designed using BiSearch software. The following primers were used to amplify bisulfite-treated *GAD67*, *reelin* and *Dnmt1* promoters:

GAD67 F1: TTT AGA GGT AGT TAG ATA TTT GTA AAG GAG

GAD67 R1: AAA ACA AAA AAC TAA ACA AAA AAC C

Reelin F1: TAA TGT GTA GGG AAA TGA GTA TT

Reelin R1: ACT TTA AAA AAA TAC TAA AAA AAC

Dnmt1 F1: CCC TAA AAA TAT CCC CAA ACT TAT T

Dnmt1 R1: AAA ACA ACA TAT ACC ACA CAA ACA A

The bisulfite treated DNA was amplified with the primers above. Each PCR reaction contained 100ng of bisulfite-treated DNA, 100 pmol each of forward and reverse primers, 100µM each of dNTPs, 2.5U of Taq DNA polymerase (Invitrogen) in a 1× PCR buffer (20mM Tris, pH 8.4, 50mM KCl and 1.5mM MgCl₂). The reactions were carried out as follows: 4 minutes at 94°C, 2 minutes at 55°C, and 2 minutes at 72°C for two cycles, followed by 35 cycles of PCR consisting of 1 minute at 94°C, 2 minutes at 55°C, and 2 minutes at 72°C (Lucifero *et al.* 2002). The PCR products were then electrophoresed on 1.5% agarose gels, purified using Qiagen gel-extraction kits and cloned in Topo-TA vector (Invitrogen). Ten to twelve clones of each PCR product were sequenced.

Immunoblotting

Cell lysates were prepared with 10 volumes of RIPA buffer (25mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS), denatured by heating at 95°C and then separated by electrophoresis on 4-12% gradient SDS- polyacrylamide gels, transferred onto Immobilon membranes (Millipore) and probed with mouse monoclonal antibodies to DNMT1 (Abcam, ab13537), and rabbit monoclonal anti-NR1 subunit (Abcam, ab 68144). The blots were then probed with fluorescently labeled secondary antibodies (Li-Cor antibodies) to detect the immunoreactivity and quantify the fluorescent signal intensities by using an Odyssey Imaging scanner (Li-Cor).

Immunocytochemistry and Microscopy

Immunocytochemistry on cultured cells was performed as previously described (D'Aiuto *et al.*, 2008). Mouse anti-β-III Tubulin monoclonal antibody (clone TUJ1, Covance, 1:500 dilution), sheep polyclonal anti-human GFAP (R&D Systems, 1:500 dilution) and Rabbit monoclonal anti-NR1 (Abcam, 1:500 dilution) were used as primary antibodies. Fluorescently labeled secondary antibodies were used for detection. Counterstaining was done with DAPI. Images were acquired using a laser scanning confocal microscope (Fluoview 1000, Olympus) equipped with spectral detector technology that provides precise wavelength separation of emitted light. The ratio of perinuclear NR1 signal to β-III Tubulin (βIII) signal in individual neurons was determined using this confocal technology, and the difference in the ratios between R1 and *Dnmt1^{tet/tet}* neurons compared using the unpaired t test.

Fluorescence measurements of intracellular calcium concentration

To measure NMDAR function in terms of glutamate-induced calcium influx, we performed experiments using live imaging of R1- and *Dnmt1^{tet/tet}*-derived neurons at day-*in vitro* 16. Cells were loaded with 5 µM Fura 2-AM (Invitrogen) in Hepes-buffered salt solution standard (HBSS) as described by Vergun *et al.* (2003). Cells were placed into a perfusion chamber on a DM IRM inverted microscope (Leica). Fura 2 intensity was monitored in single cells with 340 and 380 nm excitation wavelengths. Acquired data were analyzed using Simple PCI software (Compix, Inc., Cranberry, PA) as ratio 340/380 nm.

Real-time PCR analysis

RNA was extracted from R1 and *Dnmt1^{tet/tet}* ES cells, and R1 and *Dnmt1^{tet/tet}* neurons (day-*in vitro* 5) using an RNeasy kit (Qiagen). Approximately 5 µg of total RNA was mixed with

genomic DNA elimination buffer (SABiosciences), incubated at 42°C and subsequently mixed with RT cocktail (RT buffer, primer and control mix, RT enzyme; SABiosciences) for cDNA synthesis. The resulting cDNA reaction was mixed with SYBR green qPCR master mix and amplified with primer sets specific for mouse *Dnmt1* and mouse β -actin. A two-step real-time PCR reaction was performed starting at 95°C (10 min) for 1 cycle and followed by 95°C (15 s) and 60°C (1 min) for 40 cycles.

Results

Abnormal neuronal differentiation of *Dnmt1^{tet/tet}* ES cells

We recently described the generation of the *Dnmt1^{tet/tet}* ES cell line by targeted mutagenesis to introduce a *tet-off* regulatory cassette into the first exon of both *Dnmt1* alleles of R1 ES cells (Borowczyk et al., 2009). In the absence of doxycycline (DOX), DNMT1 expression was approximately 5-fold higher in undifferentiated *Dnmt1^{tet/tet}* ES cells than in wild-type R1 cells (Figure 1a). tTA is a strong transcriptional activator, and tTA-mediated overexpression of a gene under the control of the tetO promoter is typical of *tet-off* gene inducible systems (Tanaka et al., 2010). Because of an elevated level of DNMT1, the *Dnmt1^{tet/tet}* ES cell line is a suitable *in vitro* model to determine if the forced overexpression of DNMT1 influences neuronal differentiation and causes hypermethylation of *reelin* and *GAD67* promoters.

R1 and *Dnmt1^{tet/tet}* ES cells were differentiated into embryoid bodies (EBs) for five days. EBs derived from *Dnmt1^{tet/tet}* cells showed a similar morphology and size as those from R1 cells (Figure 2a). Following attachment and growth of these EBs in modified N2 medium for 5–7 days, cells with a neuronal morphology began to appear within 2 days. At five days, most of the neurons were tubulin-positive, confirming the neuronal phenotype. At this point, neurons differentiated from *Dnmt1^{tet/tet}* EBs were different than R1 EB-derived neurons. Abnormal sprouting of the processes was observed in *Dnmt1^{tet/tet}*-derived neurons but not in R1-derived neurons (Figure 2b). Furthermore, few astroglial cells were seen among the differentiated *Dnmt1^{tet/tet}* cells compared to differentiated R1 cells (Figure 2b).

DNMT1 downregulation is an early step of neuronal differentiation in both *Dnmt1^{tet/tet}* and R1 ES cells

Because the targeted mutations in *Dnmt1^{tet/tet}* ES cells reside solely within the *Dnmt1* alleles, it is very likely that the differences in neuronal morphology between R1 and *Dnmt1^{tet/tet}* cells are related to the difference in DNMT1 levels. For this reason we investigated whether the high level of DNMT1 seen in *Dnmt1^{tet/tet}* ES cells persisted, in the absence of DOX, throughout neuronal differentiation. Although the level of DNMT1 in *Dnmt1^{tet/tet}* cells is five times that in R1 cells, we observed an efficient downregulation of this protein in *Dnmt1^{tet/tet}*-derived EBs after 24 hours of differentiation (Figure 1a). In R1 neurons, the level of DNMT1 was ~3.0 times lower than in the R1 ES cells whereas in *Dnmt1^{tet/tet}* neurons there is an 18-fold reduction in the levels of DNMT1 compared to *Dnmt1^{tet/tet}* ES cells (Figure 1b). As a result, the levels of DNMT1 in *Dnmt1^{tet/tet}* and R1 neurons are similar. Therefore, DNMT1 downregulation appears to be an early event in differentiation of mouse ES cells.

We also performed quantitative RT-PCR experiments to compare the levels of *Dnmt1* transcripts in R1 and *Dnmt1^{tet/tet}* neurons. As shown in Figure 1c, there is a decrease in the amount of *Dnmt1* transcripts in both *Dnmt1^{tet/tet}* as well as R1 neurons. However, as in the case of DNMT1 protein levels, there is a more pronounced decline in *Dnmt1* transcript level in *Dnmt1^{tet/tet}* neurons compared to R1 neurons. Therefore, the reduced levels of DNMT1

observed in R1 and *Dnmt1^{tet/tet}* neurons are likely due to lower steady-state concentrations of *Dnmt1* transcripts.

Downregulation of DNMT1 by *de novo* methylation of its own promoter has been recently reported in human placenta (Novakovic et al., 2010). We investigated the possibility that a similar methylation-mediated silencing could be associated with the downregulation observed during neuronal differentiation from mouse ES cells. For this purpose, we performed bisulfite sequencing of the *Dnmt1s* promoter CpG island. Both undifferentiated R1 ES cells and R1-derived neurons had low levels of promoter methylation (~3%). These results indicate that downregulation of *Dnmt1* transcripts and DNMT1 protein during ES-cell differentiation is not associated with methylation of the *Dnmt1s* promoter.

From these experiments we conclude that the extent of downregulation of both *Dnmt1* transcripts and DNMT1 protein in *Dnmt1^{tet/tet}* neurons is higher than the extent of downregulation in R1 neurons. Because similar levels of *Dnmt1* transcripts and DNMT1 protein were observed in R1- and *Dnmt1^{tet/tet}*-derived neurons, differentiation *per se* is accompanied by a tight regulation of *Dnmt1* expression. These findings suggest a model in which there is a highly efficient, methylation-independent mechanism of *Dnmt1* regulation during early neurogenesis.

Abnormal neuronal differentiation of *Dnmt1^{tet/tet}* ES cells is associated with overexpression of a regulatory subunit of NMDA receptors

Aberrant fiber sprouting similar to that observed in neurons differentiated from *Dnmt1^{tet/tet}* ES cells (Figure 2b) has been described as a specific feature in several neuropathologies (Mathern et al., 1998). Findings obtained from different neuropathological models support the hypothesis that changes in expression of glutamate receptors (NMDARs) and changes in subunit stoichiometry are associated with aberrant axon circuitry in different brain areas. Therefore, NMDARs may contribute to reorganized axon circuit receptor properties. To investigate whether there is any alteration in the levels of NMDARs in the neurons derived from *Dnmt1^{tet/tet}* ES cells, immunocytochemistry and immunoblotting were performed to detect the NR1 regulatory subunit of NMDARs. These analyses were performed on neurons differentiated from ES cell-derived neurospheres rather than ES cell-derived EBs to easily identify neurons for immunocytochemistry and physiological recording. When neurospheres attach to the bottom surface of the culture flask, cells migrate away and differentiate into neurons, astrocytes and oligodendrocytes. Thus, neurons are easily distinguishable. Conversely, EBs that undergo neuronal differentiation show only neurite outgrowth whilst the neuron soma remains hidden in the EB, thus precluding any single-neuron investigation. Neurons differentiated from *Dnmt1^{tet/tet}* neurospheres showed the same abnormal phenotype as neurons differentiated from *Dnmt1^{tet/tet}* EBs. Immunocytochemistry showed that abnormal sprouting of the processes of *Dnmt1^{tet/tet}* neurons were associated with redistribution and a dramatic increase of immunoreactivity for NR1, most notably near the soma (Figure 4a). Confocal microscopy was used to quantify perinuclear NR1 expression relative to β -III Tubulin (β III) expression in a large number of immunostained neurons. *Dnmt1^{tet/tet}* neurons exhibited a significantly higher level of NR1 expression relative to the level of expression in R1 neurons (Figure 4b). Immunoblotting showed that NR1 is expressed after day-*in vitro* 15 in both R1 and *Dnmt1^{tet/tet}* neurons and confirmed ~3-fold overexpression of the NR1 subunit in *Dnmt1^{tet/tet}* neurons (Figure 4c). We conclude from this that the increase in NR1 expression is due to an increase in NR1 expression per *Dnmt1^{tet/tet}*-derived neuron.

We also used live imaging microscopy to test whether the overexpressed NR1 in *Dnmt1^{tet/tet}* neurons is functional by measuring the calcium influx induced by administration of 10 μ M glutamate. These analyses were performed on day-*in vitro* 16 neurons differentiated using

neurospheres from R1 and *Dnmt1^{tet/tet}* ES cells. MK801, an NMDAR-specific antagonist was used to check for specificity. In both neuronal cell types, glutamate mediated Ca^{2+} influx was blocked by 10 μM MK801 (Figure 5), suggesting that NMDARs are the predominant glutamate-sensitive receptors in ES cell-derived neurons. Consistent with the difference in the levels of NR1 in *Dnmt1^{tet/tet}* and R1 neurons, we observed a significantly higher calcium influx in *Dnmt1^{tet/tet}* neurons.

Promoter methylation of *GAD₆₇* and *reelin* is not associated with abnormal neuronal differentiation of *Dnmt1^{tet/tet}* ES cells

Reelin and *GAD₆₇* play important roles in neuronal differentiation (Massalini et al., 2009) and regulation of neuronal excitability (Hyde and Weinberger, 1997; Daskalakis et al., 2002; Mattay et al., 1997), respectively. Recent studies showed that these two genes are downregulated in GABAergic interneurons overexpressing *Dnmt1* in SZ and BP brains (Veldic et al., 2005; Tueting et al., 2006). To investigate whether inappropriate methylation of *reelin* and *GAD₆₇* is associated with abnormal differentiation of *Dnmt1^{tet/tet}* neurons, we compared the extent of promoter methylation in R1 and *Dnmt1^{tet/tet}* ES cells. For *GAD₆₇* promoter, the degree of methylation was estimated as 1.76% for R1 ES cells and 1.18% for the *Dnmt1^{tet/tet}* ES cells (Figure 3b). Similarly, methylation levels for *reelin* promoter showed no difference between R1 (1.29%) and *Dnmt1^{tet/tet}* (1.6%) ES cells (Figure 3c). These results indicate that both the promoters are unmethylated in these cells. Analysis of these promoters in neurons also indicates that there is no significant increase in the level of methylation of *GAD₆₇* promoter (2.05%) and *reelin* promoter (2.27%) in R1 neurons and *Dnmt1^{tet/tet}* neurons (1.76% for *GAD₆₇* and 2.27% for *reelin*).

Realtime PCR array analysis of R1 and *Dnmt1^{tet/tet}* neurons

Realtime PCR analyses were used to test the possibility that abnormal differentiation of *Dnmt1^{tet/tet}* cells is associated with a change in the expression of genes associated with cell division, cell death and neurogenesis. These experiments were performed on mRNA from cells after two days of initiation of differentiation. Results from these experiments (data not shown) indicated that there was no significant difference between the two cell types in the level of expression of any of the genes represented in the PCR arrays.

Discussion

The main findings of this study are (1) DNMT1 overexpression in ES cells causes abnormal neuronal differentiation (2) high DNMT1 levels are not maintained into neurons because *Dnmt1* is rapidly downregulated upon differentiation of ES cells, (3) DNMT1 overexpression in ES cells predisposes cells to increased expression of functional NR1 regulatory subunit of NMDA receptors during neuronal differentiation, and (4) high levels of DNMT1 in ES cells do not result in hypermethylation of *reelin* and *GAD₆₇* promoters.

Downregulation of DNMT1 can be achieved at both transcriptional and posttranscriptional levels. In the human placenta promoter methylation has been associated with transcriptional downregulation (Novakovic et al., 2010), whereas in post-mitotic neurons differentiated from rat pheochromocytoma (PC12) cells, post-transcriptional downregulation is accomplished by destabilization of the transcripts through binding of a 40 kDa protein to the 3' UTR of *Dnmt1* mRNA (Detich et al, 2001). Downregulation of DNMT1 has been reported during epidermal differentiation (Sen et al. 2010), differentiation of mouse myoblasts (Liu et al 1996), and during rat neuronal differentiation (Singh et al 2009). From these studies, it is reasonable to propose that downregulation of DNMT1 is associated with the various differentiation programs.

Deng and Szyf (1996) hypothesized that inappropriate levels of DNMT1 may have consequences on the physiology of mature neurons and DNMT1 downregulation during neuronal differentiation was interpreted as a mechanism to ensure correct neuronal development. Reinforcing this hypothesis, the results described here show that differentiation-associated DNMT1 down regulation can be very efficient even in those ES cells that overexpress DNMT1 and as a result, the levels of DNMT1 in *Dnmt1^{tet/tet}* neurons fall to levels observed in wild-type R1 neurons.

An emerging theme in the field of neuroscience is that disruption of epigenetic mechanisms can give rise to a variety of neurological disorders associated with cognitive abnormalities (Egger et al., 2004; Jiang et al., 2004; Levenson and Sweatt, 2005). DNA methylation is essential for proper neuronal function (Fan et al., 2001; Tucker, 2001) and may be an important mechanism associated with the dynamic regulation of genes expressed in neurons, especially those involved in synaptic plasticity, such as *reelin* (Chen et al., 2002; Levenson et al., 2005). Interestingly, REELIN is an important modulator of NMDAR-mediated neurodevelopment. Furthermore, increasing evidence indicates that the dysfunctions seen in SZ and BP may be related to hypermethylation of the promoters of *reelin* and *GAD67* caused by *Dnmt1* overexpression in GABAergic interneurons (Tueting et al., 2006; Veldic et al., 2005). Although overexpression of DNMT1 in *Dnmt1^{tet/tet}* ES cells is not maintained in the resultant neurons, we were able to study the relationship between elevated levels of DNMT1 and hypermethylation of *reelin* and *GAD67* promoters in the ES cells. Our results provide evidence that increased levels of DNMT1 do not result in hypermethylation of *reelin* and *GAD67* promoters. On the basis of these observations, it is reasonable to expect that abnormal methylation of these two promoters in SZ and BP brains may not be solely due to elevated DNMT1 levels but require additional factors for their downregulation in these specific cells types. In addition, it is also possible that hypermethylation of these genes may be a part of epigenetic dysregulation of a broader spectrum of genes. In this scenario, dysregulation of DNMT1 expression may only represent an aspect of defective epigenetic machinery.

To our knowledge, the only genetic difference between the *Dnmt1^{tet/tet}* and the wild-type R1 ES cells is the overexpression of DNMT1. However, DNMT1 levels are downregulated in both *Dnmt1^{tet/tet}* and R1 neurons and these levels are equivalent. Despite such normalization of DNMT1 levels, *Dnmt1^{tet/tet}* neurons show altered phenotypes characterized by abnormal and degenerating sprouting. Taken together, these observations indicate that even after subsequent downregulation, elevated levels of DNMT1 induce an epigenetic change in the *Dnmt1^{tet/tet}* ES cells that has a long-term effect on neuronal differentiation. It is also possible that elevated levels of DNMT1 may have an impact on other differentiation programs as well.

Our studies indicate that persistent DNMT1 elevation upon induction of differentiation is not required for the abnormal neuronal phenotype. One of the consequences of this effect is an increase in the levels of ubiquitous NR1 regulatory subunit of NMDARs. This feature assumes significance because elevated NR1 levels and sustained activation of NMDARs have also been reported in previous studies on experimental conditions associated to neuronal development, degeneration and damage, including schizophrenia, epilepsy and Alzheimer's disease (Lafon-Cazal et al., 1993; Leite et al., 1996; Pacheco Otalora et al., 2006; Palop et al., 2007; Patel et al., 1996). Future studies will unravel the mechanism by which DNMT1 levels modulate the expression of NR1. This would provide insight into the possible role of defective epigenetic machinery affecting the plasticity of circuitry and connectivity in the development of several neurological diseases

Acknowledgments

We thank Guillermo Gonzalez-Burgos for critical reading of the manuscript. This project was funded from a NIH grant to JRC, and by the Ri.MED Foundation to RDM.

References

- Biniszkiewicz D, Gribnau J, Ramsahoye B, Gaudet F, Eggan K, Humpherys D, Mastrangelo MA, Jun Z, Walter J, Jaenisch R. Dnmt1 overexpression causes genomic hypermethylation, loss of imprinting, and embryonic lethality. *Mol Cell Biol*. 2002; 22:2124–2135. [PubMed: 11884600]
- Borowczyk E, Mohan KN, D'Aiuto L, Cirio MC, Chaillet JR. Identification of a region of the DNMT1 methyltransferase that regulates the maintenance of genomic imprints. *Proc Natl Acad Sci USA*. 2009; 106:20806–20811. [PubMed: 19923434]
- Chahrouh M, Zoghbi HY. The story of Rett syndrome: from clinic to neurobiology. *Neuron*. 2007; 56:422–437. [PubMed: 17988628]
- Chen Y, Sharma PP, Costa RH, Costa E, Grayson DR. On the epigenetic regulation of the human reelin promoter. *Nucleic Acids Res*. 2002; 30:2930–2939. [PubMed: 12087179]
- Cirio MC, Martel J, Mann M, Toppings M, Bartolomei M, Trasler J, Chaillet JR. DNA methyltransferase 1o functions during preimplantation development to preclude a profound level of epigenetic variation. *Dev Biol*. 2008; 324:139–150. [PubMed: 18845137]
- Costa E, Dong E, Grayson DR, Guidotti A, Ruzicka W, Veldic M. Reviewing the role of DNA (cytosine-5) methyltransferase overexpression in the cortical GABAergic dysfunction associated with psychosis vulnerability. *Epigenetics*. 2007; 2:29–36. [PubMed: 17965595]
- D'Aiuto L, Robison CS, Gigante M, Nwanegbo E, Shaffer B, Sukhwani M, Castro CA, Chaillet JR. Human IL-12 p40 as a reporter gene for high-throughput screening of engineered mouse embryonic stem cells. *BMC Biotechnol*. 2008; 8:52. [PubMed: 18522747]
- Daskalakis ZJ, Christensen BK, Chen R, Fitzgerald PB, Zipursky RB, Kapur S. Evidence for impaired cortical inhibition in schizophrenia using transcranial magnetic stimulation. *Arch Gen Psychiatry*. 2002; 59:347–354. [PubMed: 11926935]
- Deng J, Szyf M. Downregulation of DNA (cytosine-5-)methyltransferase is a late event in NGF-induced PC12 cell differentiation. *Brain Res Mol Brain Res*. 1999; 71:23–31. [PubMed: 10407183]
- Detich N, Ramchandani S, Szyf M. A conserved 3'-untranslated element mediates growth regulation of DNA methyltransferase 1 and inhibits its transforming activity. *J Biol Chem*. 2001; 276:24881–24890. [PubMed: 11335728]
- Egger G, Liang G, Aparicio A, Jones PA. Epigenetics in human disease and prospects for epigenetic therapy. *Nature*. 2004; 429:457–463. [PubMed: 15164071]
- Fan G, Beard C, Chen RZ, Csankovszki G, Sun Y, Siniaia M, Biniszkiewicz D, Bates B, Lee PP, Kuhn R, Trumpp A, Poon C, Wilson CB, Jaenisch R. DNA hypomethylation perturbs the function and survival of CNS neurons in postnatal animals. *J Neurosci*. 2001; 21:788–797. [PubMed: 11157065]
- Gibbons RJ, Wada T, Fisher CA, Malik N, Mitson MJ, Steensma DP, Fryer A, Goudie DR, Krantz ID, Traeger-Synodinos J. Mutations in the chromatin-associated protein ATRX. *Hum Mutat*. 2008; 29:796–802. [PubMed: 18409179]
- Hyde TM, Weinberger DR. Seizures and schizophrenia. *Schizophr Bull*. 1997; 23:611–622. [PubMed: 9365998]
- Hutnick LK, Golshani P, Namihira M, Xue Z, Matynia A, Yang XW, Silva AJ, Schweizer FE, Fan G. DNA hypomethylation restricted to the murine forebrain induces cortical degeneration and impairs postnatal neuronal maturation. *Hum Mol Genet*. 2009; 18:2875–2888. [PubMed: 19433415]
- Jiang Y, Bressler J, Beaudet AL. Epigenetics and human disease. *Ann Rev Genomics Hum Genet*. 2004; 5:479–510. [PubMed: 15485357]
- Kramer JM, van Bokhoven H. Genetic and epigenetic defects in mental retardation. *Int J Biochem Cell Biol*. 2008; 41:96–107. [PubMed: 18765296]
- Lafon-Cazal M, Pietri S, Culcasi M, Bockaert J. NMDA-dependent superoxide production and neurotoxicity. *Nature*. 1993; 364:535–537. [PubMed: 7687749]

- Lei H, Oh SP, Okano M, Juttermann R, Goss KA, Jaenisch R, Li E. De novo DNA cytosine methyltransferase activities in mouse embryonic stem cells. *Development*. 1996; 122:3195–3205. [PubMed: 8898232]
- Leite JP, Babb TL, Pretorius JK, Kulman PA, Yeoman KM, Mathern GW. Neuron loss, mossy fiber sprouting, and interictal spikes after intrahippocampal kainate in developing rats. *Epilepsy Res*. 1996; 26:219–231. [PubMed: 8985702]
- Levenson JM, Sweatt JD. Epigenetic mechanisms in memory formation. *Nat Rev Neurosci*. 2005; 6:108–118. [PubMed: 15654323]
- Levenson JM, Roth TL, Lubin FD, Miller CA, Huang I, Desai P, Malone LM, Sweatt JD. Evidence that DNA (cytosine-5) methyltransferase regulates synaptic plasticity in the hippocampus. *J Biol Chem*. 2006; 281:15763–15773. [PubMed: 16606618]
- Liu Y, Sun L, Jost JP. In differentiating mouse myoblasts DNA methyltransferase is posttranscriptionally and posttranslationally regulated. *Nucleic Acids Res*. 1996; 24:2718–2722. [PubMed: 8759002]
- Lucifero D, Mertineit C, Clarke HJ, Bestor TH, Trasler JM. Methylation dynamics of imprinted genes in mouse germ cells. *Genomics*. 2002; 79:530–538. [PubMed: 11944985]
- Massalini S, Pellegatta S, Pisati F, Finocchiaro G, Farace MG, Ciafrè SA. Reelin affects chain-migration and differentiation of neural precursor cells. *Mol Cell Neurosci*. 2009; 42:341–349. [PubMed: 19698788]
- Mathern GW, Pretorius JK, Mendoza D, Lozada A, Kornblum HI. Hippocampal AMPA and NMDA mRNA levels correlate with aberrant fascia dentata mossy fiber sprouting in the pilocarpine model of spontaneous limbic epilepsy. *Journal of Neuroscience Research*. 1998; 54:734–753. [PubMed: 9856858]
- Mattay VS, Callicott JH, Bertolino A, Santha AK, Tallent KA, Goldberg TE, Frank JA, Weinberger DR. Abnormal functional lateralization of the sensorimotor cortex in patients with schizophrenia. *Neuroreport*. 1997; 8:2977–2984. [PubMed: 9376542]
- Novakovic B, Wong NC, Sibson M, Ng HK, Morley R, Manuelpillai U, Down T, Rakyan VK, Beck S, Hiendleder S, Roberts CT, Craig JM, Saffery R. DNA methylation-mediated down-regulation of DNA methyltransferase-1 (DNMT1), is co-incident with, but not essential for, global hypomethylation in human placenta. *J Biol Chem*. 2010; 285:9583–9593. [PubMed: 20071334]
- Pacheco Otalora LF, Couoh J, Shigamoto R, Zarei MM, Garrido Sanabria ER. Abnormal mGluR2/3 expression in the perforant path termination zones and mossy fibers of chronically epileptic rats. *Brain Res*. 2006; 1098:170–185. [PubMed: 16793029]
- Palop JJ, Chin J, Roberson ED, Wang J, Thwin MT, Bien-Ly N, Yoo J, Ho KO, Yu GQ, Kreitzer A, Finkbeiner S, Noebs JL, Mucke L. Aberrant excitatory neuronal activity and compensatory remodeling of inhibitory hippocampal circuits in mouse models of Alzheimer's disease. *Neuron*. 2007; 55:697–711. [PubMed: 17785178]
- Patel M, Day BJ, Crapo JD, Fridovich I, McNamara O. Requirement for superoxide in excitotoxic cell death. *Neuron*. 1996; 16:345–355. [PubMed: 8789949]
- Reinhart B, Chaillet JR. Genomic imprinting: cis-acting sequences and regional control. *Int Rev Cytol*. 2005; 243:173–213. [PubMed: 15797460]
- Sen GL, Reuter JA, Webster DE, Zhu L, Khavari PA. DNMT1 maintains progenitor function in self-renewing somatic tissue. *Nature*. 2010; 463:563–567. [PubMed: 20081831]
- Singh RP, Shiue K, Schomberg D, Zhou FC. Cellular epigenetic modifications of neural stem cell differentiation. *Cell Transplant*. 2009; 18:1197–1211. [PubMed: 19660178]
- Smith AG. Embryo-derived stem cells: of mice and men. *Ann Rev Cell Dev Biol*. 2001; 17:435–462. [PubMed: 11687496]
- Tanaka KF, Ahmari SE, Leonardo ED, Richardson-Jones JW, Budreck EC, Scheiffele P, Sugio S, Inamura N, Ikenaka K, Hen R. Flexible accelerated STOP Tetracycline Operator-Knockin (FAST): A versatile and efficient new gene modulating system. *Biol Psychiatry*. 2010; 67:770–773. [PubMed: 20163789]
- Tueting P, Doueiri MS, Guidotti A, Davis JM, Costa E. Reelin down-regulation in mice and psychosis endophenotypes. *Neurosci Biobehav Rev*. 2006; 30:1065–1077. [PubMed: 16769115]

- Veldic M, Guidotti A, Maloku E, Davis JM, Costa E. In psychosis, cortical interneurons overexpress DNA-methyltransferase 1. *Proc Natl Acad Sci USA*. 2005; 102:2152–2157. [PubMed: 15684088]
- Vergun O, Han YY, Reynolds IJ. Glucosedeprivation produces a prolonged increase in sensitivity to glutamate in cultured rat cortical neurons. *Experimental Neurology*. 2003; 183:682–694. [PubMed: 14552910]
- Tucker KL. Methylated cytosine and the brain: a new base for neuroscience. *Neuron*. 2001; 30:649–652. [PubMed: 11430798]

Abbreviations

DNMT1	DNA methyltransferase 1
SZ	schizophrenia
BP	bipolar disorders
RTT	Rett syndrome
NMDAR	N-methyl D-aspartate receptor
βIII	β-III Tubulin
ES	embryonic stem
EBs	embryoid bodies
NR1	N-methyl D-aspartate receptor subunit r1

Figure 1.

DNMT1 is downregulated upon differentiation. (A) Immunoblot showing ~5-fold elevation in DNMT1 concentration in undifferentiated *Dnmt1*^{tet/tet} ES cells compared to undifferentiated R1 ES cells. This difference is lost after 24 hours of differentiation into embryoid bodies. "c/c" are homozygous mutant *Dnmt1*^{c/c} ES cells that do not express DNMT1 protein (Lei et al., 1996). (B) Immunoblots showing DNMT1 levels in undifferentiated ES cells (ES) and after differentiation of embryoid bodies into neurons. D1–D5 are days of neuronal differentiation. (C) Normalized DNMT1 expression from data in panel B and two additional experiments. (D) Relative level of *Dnmt1* transcripts in

undifferentiated ES cells and after five days of differentiation of embryoid bodies into neurons. *Dnmt1* transcript levels were determined by quantitative RT-PCR.

Figure 2. Embryoid bodies and their neuronal derivatives. (A) Morphology of embryoid bodies derived from R1 and *Dnmt1*^{tet/tet} ES cells. (B) Abnormal neuronal differentiation of *Dnmt1*^{tet/tet} ES cells. Staining of neurons and astrocytes differentiated from R1 and *Dnmt1*^{tet/tet} embryoid bodies with β-III Tubulin (green) and GFAP (red), respectively. Nuclei were counterstained with DAPI (blue). Scale bar is 500 μM in panel A and 100 μM in panel B.

Figure 3.

DNA methylation of the promoter regions of *Dnmt1*, *GAD67* and *reelin*. (A) *Dnmt1*s promoter methylation in undifferentiated R1 ES cells and after 5 days of differentiation of R1 EBs. (B) *GAD67* methylation in undifferentiated R1 and *Dnmt1^{tet/tet}* ES cells. (C) *Reelin* methylation in undifferentiated R1 and *Dnmt1^{tet/tet}* ES cells. Each horizontal line is an allele whose methylation is determined by bisulfite genomic sequencing. Filled circles represent methylated CpG dinucleotides and empty circles indicate unmethylated CpGs.

Figure 4.

Elevated levels of NR1 subunit in *Dnmt1*^{tet/tet} neurons. (A) Immunocytochemistry shows that abnormal growth of the processes and increased synaptic densities in *Dnmt1*^{tet/tet} neurons are associated with an increase of immunoreactivity for the regulatory NR1 subunit. A redistribution of NR1 was also observed in these neurons when compared to R1 neurons. (B) Quantitative comparison of NR1 immunofluorescence in R1 (n=280) and *Dnmt1*^{tet/tet} (tet/tet) (n=320) neurons. For each neuron, the NR1 immunofluorescence signal was normalized to the β -III Tubulin (β III) signal. Error bars represent standard error of the mean (SEM). The mean NR1 fluorescence in R1 neurons (109 ± 2.8) was statistically different than the mean NR1 fluorescence in *Dnmt1*^{tet/tet} neurons (219 ± 3.7) ($p < 0.0001$). (C) Immunoblots of cell lysates confirm the elevated levels of NR1 in *in vitro* day15 *Dnmt1*^{tet/tet} neurons.

Figure 5.

Dnmt1^{tet/tet} neurons show high NMDAR activity. (A) Administration of 10 μ M glutamate (Glu) in R1 neurons causes a slight increase in $[Ca^{2+}]_i$ ($340/380 = 0.32 \pm 0.05a$). (B) Glu-induced increase in $[Ca^{2+}]_i$ in R1 cells is blocked by treatment with MK801. (C) Administration of 10 μ M Glu in *Dnmt1*^{tet/tet} neurons causes a large increase in $[Ca^{2+}]_i$ ($340/380 = 0.6 \pm 0.04$). (D) Glu-induced increase in $[Ca^{2+}]_i$ in *Dnmt1*^{tet/tet} cells is blocked by treatment with MK801.