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Effect of valproic acid on mitochondrial epigenetics

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Abstract

Valproic acid (valproate), an anticonvulsant and a mood stabilizer, is a potent histone deacetylase inhibitor and a widely utilized pharmacological tool for neuroepigenetic research including DNA methylation. However, only nuclear but not mitochondrial DNA (mtDNA) has been investigated for the effects of valproate on the formation of 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC). Using mouse 3T3-L1 cells, we investigated the effects of short (1 day) and prolonged (3 days) valproate treatment on global mtDNA 5mC content, global and mtDNA sequence-specific 5hmC content, mRNA levels for ten-eleven-translocation (TET) enzymes involved in 5hmC formation, and the mitochondrial content of TET proteins. Only 5hmC but not 5mC content in mtDNA was affected (decreased) by valproate, and only after the prolonged treatment. This action of valproate was mimicked by MS-275, a class I histone deacetylase inhibitor. The prolonged but not the short valproate treatment decreased the expression of Tet1 mRNA and reduced the mitochondrial content of the TET1 protein. Hence, a likely scenario for a valproate-induced 5hmC decrease in mtDNA may involve nuclear histone deacetylase inhibition (mitochondria do not contain histones) causing the initial increase of Tet1 transcription, which is followed by a delayed compensatory decrease of Tet1 expression and a reduced presence of TET1 protein in mitochondria. Further research is needed to elucidate the functional implications of epigenetic modifications of mtDNA. The observed effects of valproate on mitochondrial epigenetics may have implications for a better understanding of both therapeutic and unwanted effects of this drug and possibly other histone deacetylase inhibitors.

Keywords

5-Methylcytosine (5mC); 5-Hydroxymethylcytosine (5hmC); Mitochondrial DNA (mtDNA); Valproic acid (valproate); Ten-eleven-translocation (TET); DNA methyltransferase 1 (DNMT1); Epigenetics

1. Introduction

Since the discovery that the anticonvulsant mood stabilizer valproic acid (VPA; valproate) is also a potent histone deacetylase (HDAC) inhibitor (Phiel et al., 2001), VPA became a widely utilized pharmacological tool for neuroepigenetic research (Detich et al., 2003; Dong et al., 2010; Guidotti et al., 2009; Jeong et al., 2003; Kundakovic et al., 2009; Tremolizzo et

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al., 2005; Zhang et al., 2004; Yildirim et al., 2003). The field of neuroepigenetics is becoming important for a better understanding of brain functioning (Riccio, 2010) and for the development of a putative epigenetic psychopharmacology (Boks et al., 2012; Marder et al., 2011).

In the nucleus, epigenetic mechanisms regulate the structure and function of chromatin, a complex of DNA and histones (Hernández-Muñoz, 2010). Two mechanisms of chromatin remodeling have attracted particular interest, a) histone acetylation and b) modifications of DNA cytosine that lead to the formation of 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC). With respect to histone acetylation, the emphasis has been on HDAC inhibitors (Chuang et al., 2009; Fischer et al., 2010; Grayson et al., 2010; Haggarty and Tsai, 2011; Kazantsev et al., 2008; Sakharkar et al., 2012). Regarding cytosine modifications, early emphasis has been placed on enzymes involved in the formation of 5mC; i.e., DNA methyltransferases (DNMTs) (Weaver et al., 2004; Levenson et al., 2006). More recently, the focus has shifted to the family of TET (ten-eleven-translocation) enzymes (TET1, TET2, and TET3), which are involved in the nuclear conversion of 5mC into 5hmC (Flax and Soloway, 2011; Iyer et al., 2009; Tahiliani et al., 2009).

The effects of VPA on DNA methylation has been proposed as a new epigenetic therapeutic mechanism for schizophrenia and bipolar disorder (Marder et al., 2011). These studies focus solely on nuclear DNA (ncDNA). However, recent research on epigenetic modifications of mitochondrial DNA (mtDNA) have led to the emergence of a new field of mitochondrial epigenetics (Chestnut et al., 2011; Dzitoyeva et al., 2012; Manev et al., 2012; Shock et al., 2011). Mitochondria contain mtDNA that encodes 13 members of the oxidative phosphorylation complexes (NADH dehydrogenase subunits ND1-ND6 and ND4L; cytochrome c oxidases COX1-COX3; ATP synthase 6 - ATP6; ATP synthase 8 - ATP8; and cytochrome b - CYTB), 2 ribosomal RNAs, and 22 transfer RNAs (Falkenberg et al., 2007). Since mitochondria do not contain histones, mtDNA is associated with different proteins into aggregates called nucleoids (Rebello et al., 2011). Hence, DNA methylation/hydroxymethylation rather than histone modifications appear to be important for mitochondrial epigenetics.

To our knowledge, no studies have investigated the effects of neuropsychiatric drugs on mitochondrial epigenetics. Among antiepileptic/epigenetic pharmacological treatments, VPA is listed as a drug with the most well-known mitochondrial side effects (Finsterer et al., 2012). In this study, we analyzed for the first time the mitochondrial epigenetic characteristics of VPA-treated cells.

2. Material and methods

2.1. Cell cultures and drug treatment

Mouse fibroblast 3T3-L1 cells (American Type Culture Collection, Rockville, MD) were cultured in 10 cm diameter dishes with Dulbecco's modified Eagle's medium (DMEM, GIBCO, Grand Island, NY) containing 4.5 g/l glucose, 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), and were maintained in an incubator set to 37°C and 5% carbon dioxide. When cells reached 70% confluence, they were treated with VPA (valproic acid sodium salt, Sigma-Aldrich, St. Louis, MO; stock solution 100 mM in distilled water), an HDAC inhibitor MS-275 (Sigma-Aldrich; stock solution 10 mM in dimethyl sulfoxide, DMSO), and their respective vehicles (the final concentration of DMSO in the medium was 0.05%). For treatment, the culture medium was replaced by fresh medium containing drugs or their vehicle (control). The treatment was repeated daily for 3 days. Cells were collected after 1 day and 3 days of treatment. This treatment schedule and drug concentrations were

selected based on the previous in vitro experiments (Kundakovic et al., 2009; Zhang et al., 2004).

2.2. Isolation of mitochondria and extraction of DNA

Mitochondria were extracted according to a commercial protocol (G-Biosciences, St Louis, MO) as described recently (Dzitoyeva et al., 2012). Samples were gently lysed by 20 up-and-down passages through a 20G syringe needle in 500 µl cold cell lysis buffer and then centrifuged at 700 g for 10 min to pellet the nuclei. The supernatant was transferred to a new tube and centrifuged again at 12000 g for 15 min to pellet the mitochondria. For DNA isolation, both mitochondrial and nuclear pellets were re-suspended in a lysis buffer with proteinase K and incubated at 37°C overnight. Mitochondrial DNA (mtDNA) and nuclear DNA (ncDNA) were precipitated with isopropanol, rinsed with ethanol, and dissolved in Tris-EDTA buffer.

2.3. Analysis of mRNA content by quantitative real time PCR (qPCR)

This assay was performed as described elsewhere (Imbesi et al., 2009). Briefly, total RNA was extracted from the cell samples with TRIzol Reagent (Invitrogen, Carlsbad, CA) and treated with DNase (Ambion Inc., Austin, TX). RNA was reverse transcribed with M-MLV Reverse Transcriptase (Invitrogen). The qPCR was performed on the Stratagene Mx3005P QPCR System (Agilent Technologies, Santa Clara, CA) with Maxima SYBR Green/ROX Master Mix (Fermentas Inc., Glen Burnie, MD). The assay was performed with the following primers: Tet1 mRNA (NM_027384) = forward 5'-acgctggaacaagtggtagccata-3', reverse 5'-tgaacgtttgggtcttgagggtct-3'; Tet2 mRNA (NM_001040400) = forward 5'-gcccttgaatgaatccagcagca-3', reverse 5'-tgcctcccaagactcttcattgtt-3'; Tet3 mRNA (NM_183138) = forward 5'-aaccagaacccaaggtcagtagt-3', reverse 5'-ttgatctctctgctgctgctcagt-3'; DNA methyltransferase 1 (DNMT1; variant 1 NM_001199431 and variant 2 NM_010066), a) mitochondrial = forward 5'-actctcttgcctgtgtggtacatg-3', total forward 5'-tcagagctgttctgtcgtctgcaa-3', b) common (total) = reverse 5'-tcttccaagtctttgagccgcc-3'; internal control, cyclophilin A (NM_008907) = forward 5'-agcatcacaggtctgcatcttgt-3', reverse 5'-aaacgtccatggcttcacaatg-3'. Data were normalized against the cyclophilin internal control, calculated as a coefficient of variation (Dzitoyeva et al., 2009), and presented as percentage of the corresponding vehicle-treated control.

2.4. Enzyme-linked immunosorbent assay (ELISA) of DNA 5hmC and 5mC content

These ELISA assays were used as described in our recent publication (Dzitoyeva et al., 2012). The 5hmC content of mtDNA and ncDNA was measured by the Hydroxymethylated DNA Quantification Kit (Epigentek, Brooklyn, NY). Briefly, 100 ng of respective DNA was bound to a 96-well plate. The hydroxymethylated DNA was detected using its respective capture and detection antibodies and quantified colorimetrically by reading the absorbance at 450 nm in a microplate spectrophotometer (Bio-Rad, Model 550, Hercules, CA). For measurement of 5mC content, we used the corresponding Methylated DNA Quantification Kit from Epigentek. The results are expressed in units calculated according to the manufacturer's manual.

2.5. mtDNA sequence-specific 5hmC assay

The sequence-specific 5hmC content in selected mtDNA regions was measured by qPCR applied to samples after a glucosyltransferase step followed by an enzymatic restriction digest with two enzymes, CviAII (CATG recognition site) and CviQI (GTAC recognition site) (New England Biolabs; Ipswich, MA) (Dzitoyeva et al., 2012). Briefly, this assay is based on the following principle. First, DNA samples are glucosylated with phage β-glucosyltransferase (T4-BGT; New England Biolabs, Ipswich, MA), which specifically

transfers the glucose moiety of uridine diphosphoglucose (UDP-Glc) to the 5hmC residues but not to the 5mC residues. As a result, the 5hmC marks in T4-BGT-treated DNA samples are converted to beta-glu-5hmC (Robertson et al., 2012). Both restriction enzymes used in our assay are insensitive to cytosine methylation and hydroxymethylation (CviAII is only partially sensitive, about 25% to 5hmC) but are sensitive to beta-glu-5hmC modification. Thus, upon the treatment of samples with restriction enzymes, unmodified DNA is cleaved whereas beta-glu-5hmC modified DNA is protected, resulting in an increased signal in the qPCR (Davis and Vaisvila, 2011). mtDNA samples were digested with CviAII and CviQI in separate reactions, diluted with water and an aliquot was used in qPCR. As an input control, we used equal amounts of undigested DNA. The qPCR assay (as described above for mRNA) was performed with the following sets of primers: COX3 region: forward 5'-gctgctgacctccaacaggaattt-3', reverse 5'-tgctgatatcatgctgcggcttca-3'; ND5 region: forward 5'-gcatcggagacatcggttcatt-3', reverse 5'-tgcgtgggtacagatgtgtaggaa-3', D-loop regions: a) forward 5'-accagcacccaaagctggtattct-3' and reverse 5'-gttgttggtttcacggaggatgt-3'; b) forward 5'-agacatctcgatggtatcggtct-3' and reverse 5'-gggtttggcattaagaggaggg-3'. The D-loop region "a" was used for CviQI digestion (it contains 5 GTAC sites); region "b" was used for CviAII digestion (it contains 4 CATG sites) (see Fig. 1 for details).

2.6. Western immunoblotting

Mitochondrial and nuclear fractions were isolated as described above. These fractions were lysed in a lysis buffer supplemented with protease inhibitors for 30 min on ice. Protein concentration was measured using the BCA protein assay kit (Pierce, Rockford, IL). Thirty µg protein samples were processed on a 7.5% (w/v) Tris-HCl gel and transferred to a nitrocellulose membrane. The membranes were incubated overnight at 4°C with the following primary antibodies; goat anti-TET1 (1:500, Santa Cruz, Santa Cruz, CA); rabbit anti-TET2 (1:500, Santa Cruz); rabbit anti-VDAC1 (mitochondrial marker; 1:2000, Novus Biologicals, Littleton, CO); and mouse anti-beta actin (1:5000, Sigma, St. Louis, MO). Thereafter, the membranes were incubated with appropriate horseradish-peroxidase-linked secondary antibodies (1:1000, Amersham and Santa Cruz). An ECL plus kit (Amersham) was used for band visualization. Band densities were quantified using NIH ImageJ software. The optical density of TET bands was corrected by the optical density of the corresponding VDAC1 bands (mitochondria) or beta-actin bands (nuclei).

2.7. Statistical analysis

For statistical analysis, we used SPSS software (version 18.0). Data were analyzed by an independent sample t-test. Results are expressed as the mean ± standard error mean (S.E.M.). The $P < 0.05$ values were accepted as statistically significant.

3. Results

3.1. VPA and global 5mC and 5hmC content in mtDNA and ncDNA

Fig. 2 shows that treatment of cell cultures with a clinically relevant concentration of 1 mM VPA (Bowden et al., 1994; Cloyd et al., 2003) did not alter the global 5mC content of mtDNA regardless of treatment duration. On the other hand, prolonged treatment (daily for 3 days; Fig. 2B) but not a single 24-hour VPA treatment (Fig. 2A) significantly decreased the global 5hmC content of mtDNA. This 5hmC decrease, associated with the prolonged/repeated VPA exposure, was also significant when calculated as a percentage of the corresponding mitochondrial 5mC content (Fig. 2B). In the nucleus (Fig. 3), the global content of ncDNA 5mC and 5hmC was not significantly affected by VPA either after 1 or 3 days of treatment. However, the ncDNA 5hmC content expressed as a percentage of the corresponding ncDNA 5mC content was significantly decreased after 3 days (Fig. 3B) but not after 1 day of VPA exposure (Fig. 3A).

3.2. mtDNA sequence-specific 5hmC content

The decrease of the global mtDNA 5hmC content caused by 3 days of VPA treatment was confirmed by an assay of 5hmC content in selected sequences of mtDNA.

The content of 5hmC measured using a glucosyltransferase assay combined with CviAll enzyme was significantly decreased in the sequences of ND5 and COX3 genes and in the D-loop (Fig. 4A). In the assay using CviQI, VPA decreased the 5hmC content in the regions of ND5 and COX3 genes but not in the D-loop (Fig. 4B). Another HDAC inhibitor, MS-275 (administered in a concentration of 5 μ M) also decreased mitochondrial 5hmC (Fig. 5). Hence, prolonged treatment with MS-275 decreased the CviAll-measured content of 5hmC in the sequences of ND5 and COX3 genes and in the D-loop (Fig. 5A). The CviQI-based assay detected a significant 5hmC decrease after MS-275 exposure only in the ND5 region but not in the sequence of COX3 and D-loop (Fig. 5B).

3.3. Effect of VPA and MS-275 on Tet mRNAs

The levels of mRNAs of the known three Tet genes were affected in cells treated with both VPA and MS-275 (Fig. 6). Both short (1 day; Fig. 6A) and prolonged (3 days; Fig. 6B) VPA exposures increased the mRNA content of Tet2 and Tet3. On the other hand, whereas Tet1 mRNA content was elevated after 24 h VPA exposure (Fig. 6A), the 3-day VPA treatment, which decreased mtDNA 5hmC content, caused a significant decrease of Tet1 mRNA (Fig. 6B). Similarly, the 3-day treatment with MS-275, which also decreased mtDNA 5hmC content, decreased Tet1 mRNA content too (Fig. 6C). In contrast to VPA, MS-275 decreased the content of not only Tet1 but also the content of Tet2 mRNA (Fig. 6C).

3.4. Effect of VPA and MS-275 on DNMT1 mRNAs

Recently, an isoform of DNA methyltransferase 1 (DNMT1), mitochondrial DNMT1 (mtDNMT1), was characterized that appears to be essential for mammalian mtDNA methylation (Shock et al., 2011). We investigated the transcript amounts for both total DNMT1 and mtDNMT1 after the 3-day treatment with VPA and MS-275 (Fig. 7). These drugs produced a differential effect on DNMT1 transcripts; VPA increased (Fig. 7A) and MS-275 decreased (Fig. 7B) the content of DNMT1 mRNAs.

3.5. Effect of VPA on mitochondrial and nuclear TET protein contents

Fig. 8 shows the results of Western immunoblotting with TET1 and TET2 antibodies (the available TET3 antibodies did not recognize a specific target in our samples) to investigate mitochondrial and nuclear protein extracts for the presence of these enzymes. We found evidence for the presence of TET1 and TET2 not only in the nucleus but also in the mitochondrial fraction. Quantitative Western immunoblotting was used to assess the effect of VPA treatment on the mitochondrial (Fig. 9) and nuclear (Fig. 10) content of TET1 and TET2. A short (1 day) VPA treatment did not alter mitochondrial TET protein contents (Fig. 9A). However, a 3 day VPA treatment decreased the mitochondrial content of both TET1 and TET2 (Fig. 9B). In the nuclear fraction, only a prolonged VPA treatment affected TET protein content. It altered only the levels of TET1; i.e., VPA decreased nuclear content of TET1, but not TET2 (Fig. 10B).

4. Discussion

The present study demonstrated that VPA, used in clinically relevant concentrations (Bowden et al., 1994; Cloyd et al., 2003), is capable of affecting mitochondrial epigenetic marks as evidenced by the VPA-induced decrease in the 5hmC content of mtDNA. This action of VPA, a known HDAC inhibitor, on mtDNA 5hmC was mimicked by MS-275, a class I HDAC inhibitor (Hess-Stumpp et al., 2007). Since mitochondria do not contain

histones (a target for HDAC activity), it would appear that for HDAC inhibition to affect mtDNA 5hmC, a transcriptional HDAC-sensitive and ncDNA-encoded mediator would have to be involved. To this end, we demonstrated that the prolonged VPA treatment decreased the expression of the ncDNA-encoded Tet1 gene and reduced the mitochondrial content of the TET1 protein. Also the prolonged treatment with MS-275 reduced Tet1 expression. TET1 is the main enzyme responsible for the nuclear conversion of 5mC to 5hmC (Iyer et al., 2009; Tahiliani et al., 2009) and is a putative mediator in the formation of mitochondrial 5hmC (Dzitoyeva et al., 2012). Hence, a likely scenario for the VPA-induced 5hmC decrease in mtDNA may involve the possibility that the HDAC inhibition causing the initial increase of Tet1 transcription (observed after 24-hour VPA treatment) is followed by a delayed compensatory decrease of Tet1 expression and the reduced presence of TET1 proteins in mitochondria.

Using an assay of global mtDNA and ncDNA cytosine methylation, we did not observe significant differences in 5mC content between DNA samples from control and VPA-treated cells. Moreover, VPA and MS-275 treatments caused a differential effect on the expression of DNMT1, an enzyme involved in mtDNA methylation (Shock et al., 2011). Since epigenetic cytosine modifications that occur in a sequence-specific pattern are bidirectional (Siegmund et al., 2007), they can be easily missed by assays of global DNA methylation. Furthermore, mouse mtDNA contains relatively few CpG dinucleotides (i.e., 287; NC_005089, National Center for Biotechnology Information) and 5mC and 5hmC in mtDNA occur both in CpG and non-CpG sites. A bi-directional and site-specific occurrence of 5mC and 5hmC modifications would also explain why the VPA-induced decrease in global mtDNA 5hmC was not accompanied by a concomitant increase in global mtDNA 5mC. Nevertheless, in contrast to 5mC, the observed VPA-induced alterations of 5hmC content appear to be sufficiently robust for detection by an assay of global DNA cytosine hydroxymethylation.

Detecting and measuring sequence-specific 5hmC DNA modifications and distinguishing 5hmC from 5mC presents a technical challenge (Jin et al., 2010). To complement the assay of global mtDNA 5hmC with mtDNA sequence-specific 5hmC measurements, we used an assay employing the beta-glu-5hmC-sensitive restriction enzymes CviAII and CviQI (Dzitoyeva et al., 2012). We investigated three regions of mtDNA (COX3, ND5, and D-loop), which were selected based on their susceptibility to these enzymes, and found that VPA and MS-275, both HDAC inhibitors, decreased 5hmC content. This finding is similar to the effect of VPA on global mtDNA 5hmC and confirms that prolonged VPA treatment leads to a reduced 5hmC marking of mtDNA. A more sensitive and selective method for determining the 5hmC distribution pattern in the entire mtDNA is needed to fully characterize this action of VPA and possibly other HDAC inhibitors. The effect of VPA on 5hmC content does not appear to be restricted to mtDNA since we also observed a similar decrease of global 5hmC content after prolonged VPA treatment in ncDNA. However, this decrease in the nucleus was statistically significant only when 5hmC values were corrected to the corresponding 5mC values (Fig. 3). A possible explanation is the relatively high variability in ncDNA 5hmC values compared to mtDNA 5hmC values in cultures exposed to repeated daily vehicle and VPA treatment.

In the nucleus, the origin of 5hmC had been linked to the enzymatic activity of TET proteins, primarily TET1. As yet, no evidence has indicated the presence of a mitochondrial targeting sequence in TET proteins (Shock et al., 2011). However, various physiological mechanisms exist that are capable of translocating such proteins across mitochondrial membranes (Marom et al., 2011). For example, two DNMTs have been found in mitochondria, DNMT1 (Shock et al., 2011) and DNMT3a (Chestnut et al., 2011), but only DNMT1 contains the putative mitochondrial targeting sequence (Shock et al., 2011). The

presence of DNMT3a in mitochondria appears to be associated with neuropathological processes and is cell-type specific (Chestnut et al., 2011). Recently, the presence of TET1 and TET2 immunoreactive proteins was detected in mitochondria of primary neurons in culture (Dzitoyeva et al., 2012). We found evidence for the presence of TET1 and TET2 immunoreactive proteins in both nuclear and mitochondrial fractions prepared from 3T3-L1 cells (Fig. 8), suggesting that these enzymes might be involved in mtDNA 5hmC formation in these cells.

The initial increased expression of the ncDNA-encoded Tet1-Tet3 genes after the 24-hour VPA treatment is consistent with the previously described capability of VPA to alter the expression of a large number of ncDNA-encoded genes (Jergil et al., 2011), which in part can be attributed to VPA-triggered HDAC inhibition (Tsai et al., 2010). With prolonged VPA exposure, the initial increase in Tet1 mRNA expression (the most abundant of the three Tet mRNAs) turns into a significant reduction. This reversal may be the result of compensatory mechanisms that regulate Tet1 expression. Interestingly, no such compensation was observed in the expression of the less abundant Tet2 and Tet3 mRNAs, which were elevated after both 1-day and 3-day VPA treatment.

The early increase of Tet mRNAs was not reflected in corresponding changes of TET proteins and was not accompanied by significant changes in 5hmC content. In contrast, after 3 days of VPA treatment, the resulting decrease of Tet1 mRNA was accompanied by significantly lower levels of TET1 protein, and by a significant decrease in the 5hmC content. Interestingly, the mitochondrial but not the nuclear content of TET2 was decreased after 3 days of VPA treatment despite a concomitant increase in Tet2 mRNA levels, suggesting that the mitochondrial presence of TET proteins can be regulated by mechanisms other than the rate of gene expression. Although the inhibitory effect of prolonged VPA treatment on both Tet1 expression and the mitochondrial content of TET1 protein correlated well with the VPA-induced decreased levels of mtDNA 5hmC, further research is needed to directly prove a functional role for TET1 in the formation of mitochondrial 5hmC.

The functional significance of the VPA-induced decrease of mtDNA 5hmC is currently unclear. Even the exact nature of the association between ncDNA 5hmC levels and gene expression appears to be complex and poorly understood (for a review, see Irier and Jin, 2012). For example, in ncDNA, 5hmC density is much higher at the promoters of genes with a low rate of expression whereas 5hmC levels are very low both at the promoter and the body of a set of genes with constantly high expression levels (Xu et al., 2011). In mtDNA, an aging-associated 5hmC decrease was accompanied by the increased expression of certain mtDNA-encoded genes in the mouse cortex (Dzitoyeva et al., 2012). However, this association was not restricted to 5hmC modifications of gene promoters but was also found in modifications in the sequences of gene bodies (mitochondrial transcription produces polycistronic transcripts that are later processed into mature mRNAs, tRNAs, and rRNAs and is primarily regulated at a non-coding region known as the D-loop region). Due to significant differences between nuclear and mitochondrial epigenetic mechanisms (for example, the absence of histones in mitochondria), additional information about mitochondrial epigenetics must be obtained before 5hmC modifications of mtDNA, particularly outside of the promoter regions, could be considered important modifiers of mitochondrial gene expression. Interestingly, an additional possibility for the regulation of mitochondrial epigenetics was suggested by Smalheiser et al. (2011), who found a new family of mitochondrial small RNAs that are up-regulated in the hippocampus during learning. The functional implications of these molecules and their putative susceptibility to physiological and drug-induced mtDNA 5mC and 5hmC modifications deserve further investigation.

Our finding that VPA treatment influences mitochondrial epigenetics may have implications for a better understanding of both therapeutic and unwanted effects of this drug and possibly other HDAC inhibitors. Hence, the current emphasis on employing the VPA-mediated epigenetic mechanism in therapy for schizophrenia (Marder et al., 2011; Guidotti et al., 2009) may include epigenetic modifications of mtDNA. Although mitochondrial impairment has been implicated in the pathogenesis of schizophrenia (Ichikawa et al., 2012), as yet mitochondrial epigenetics has not been investigated in the pathobiology and/or treatment of psychiatric disorders including schizophrenia. On the other hand, the epigenetic effects of VPA may be responsible for some of the known side effects of this drug, such as the association between in utero exposure to VPA and an increased risk of impaired cognitive function in these children (Meador et al., 2009). It remains to be verified whether the mitochondrial effects of VPA observed in our in-vitro model occur in the brain in-vivo; i.e., in post-mitotic cells such as neurons and proliferation-susceptible cells such as glia. Our data suggests that further research is warranted to explore these and other possibly clinical implications of VPA-modified mitochondrial epigenetics.

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GTTAATGTAGCTTAATAACAAAGCAAAGCACTGAAAATGCTTAGATGGATAATTGTATCCCATAAACACA....
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ND5.....
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D-loop.....
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 TCAAGG**CATG**AAAGGACAGCACACAGTCTAGACGCACCTACGGTGAAGAATCATTAGTCCGCAAAACCCA
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 CCCCCACCCcctctcttaattgccaaccc.....
AATCATGTTCCGTGAACCAAACTCTAATCATACTCTATTACGCAATAAACATTAACAA

Fig. 1.

Representation of the selected mouse mitochondrial DNA regions analyzed with the beta-glu-5hmC-sensitive restriction enzymes CviAII and CviQI for 5hmC modifications. The presentation is based on the *Mus musculus* mitochondrion, complete genome 16299 residue. The first and the last sequence lines shown indicate the beginning and end of the mitochondria DNA reference sequence NC_005089.1. The three regions (COX3, ND5, and D-loop) were selected based on a previous report (Dzitoyeva et al., 2012). Bold capital letters indicate the recognition sites for the restriction enzymes CviAII (CATG) and CviQI (GTAC), lower case italic letters indicate the primer regions. The number of sites on the analyzed regions is as follows. COX3: 5 CviAII sites and 1 CviQI site; ND5: 2 CviAII sites and 2 CviQI sites; D-loop: 4 CviAII sites (region "b"), 5 CviQI sites (region "a").

Fig. 2. Effect of VPA treatment on global 5mC and 5hmC content in mitochondrial DNA (time course). Mitochondrial samples were collected after 1 day (panels in row A) and 3 days (panels in row B) of treatment with 1 mM VPA (striped bars) and its vehicle (open bars). ELISA assays were used to measure 5mC and 5hmC contents. The corresponding 5hmC/5mC ratios ($\times 100$) were calculated. Results are expressed in units (mean $\pm$ S.E.M.; $n = 5$; * $P < 0.05$ compared to the corresponding control group).

Fig. 3.

Effect of VPA treatment on global 5mC and 5hmC content in nuclear DNA. Nuclear samples were collected after 1 day (panels in row A) and 3 days (panels in row B) of treatment with 1 mM VPA (striped bars) and its vehicle (open bars). ELISA assays were used to measure 5mC and 5hmC content. The corresponding 5hmC/5mC ratios ($\times 100$) were calculated. Results are expressed in units (mean $\pm$ S.E.M.; $n = 5$; * $P < 0.05$ compared to the corresponding control group).

Fig. 4. Effect of a 3-day VPA treatment on sequence-specific 5hmC content in mitochondrial DNA. Mitochondria were isolated and their DNA extracted after 3 days of treatment with 1 mM VPA (striped bars) and its vehicle (open bars). The content of 5hmC in the sequences of ND5 and COX3 genes and in the D-loop was measured using a glucosyltransferase assay combined with CviAll (panel A) and CviQI (panel B) enzymes. Results are expressed in units (mean $\pm$ S.E.M.; $n = 5$; ** $P < 0.01$, *** $P < 0.001$ compared to the corresponding control group).

Fig. 5. Effect of a 3-day treatment with a HDAC inhibitor MS-275 on sequence-specific 5hmC content in mitochondrial DNA. Mitochondria were isolated and their DNA extracted after 3 days of treatment with 5 μ M MS-275 (striped bars) and its vehicle (open bars). The content of 5hmC in the sequences of ND5 and COX3 genes and in the D-loop was measured using a glucosyltransferase assay combined with CviAll (panel A) and CviQI (panel B) enzymes. Results are expressed in units (mean $\pm$ S.E.M.; n = 5; *P<0.05, **P<0.01 compared to the corresponding control group).

Fig. 6. Effect of VPA and MS-275 on Tet1-Tet3 mRNA content. Samples were collected after 1 day (panel A) and 3 days (panel B) of treatment with 1 mM VPA or after 3 days of treatment with 5 μ M MS-275 (panel C) (striped bars). Controls were treated with a corresponding vehicle (open bars). A qPCR assay was used to measure Tet1, Tet2, and Tet3 mRNA levels. Results are expressed as percentage of the corresponding control (mean $\pm$ S.E.M.; $n = 5$; ** $P < 0.01$, *** $P < 0.001$ compared to the corresponding control group).

Fig. 7. Effect of VPA and MS-275 on DNMT1 mRNA content. Samples were collected after 3 days of treatment with 1 mM VPA (panel A) and 5 μM MS-275 (panel B) (striped bars). Controls were treated with a corresponding vehicle (open bars). A qPCR assay was used to measure total DNMT1 and mitochondrial DNMT1 (mtDNMT1) mRNA levels. Results are expressed as percentage of the corresponding control (mean ± S.E.M.; n = 5; **P<0.01, ***P<0.001 compared to the corresponding control group).

Fig. 8.

Western blot detection of TET1 and TET2 immunoreactive proteins in a mitochondrial fraction of mouse 3T3-L1 cells. Note the presence of TET1 and TET2 immunobands in both the nuclear and mitochondrial fractions. In these cells, DNMT3a immunoreactivity was found in the nuclear fraction only (evidence for the purity of the mitochondrial preparation); a mitochondrial marker, VDAC1, was present in both mitochondrial and nuclear fractions.

Fig. 9. Effect of VPA treatment on mitochondrial content of TET1 and TET2 immunoreactive proteins. Mitochondrial protein samples were collected after 1 day (panels in row A) and 3 days (panels in row B) of treatment with 1 mM VPA (striped bars) and its vehicle (open bars). TET1 and TET2 levels were assayed by quantitative Western blot. The optical density of TET bands was corrected by the density of the corresponding VDAC1 bands. The results are expressed as a ratio (units) between TETs and their corresponding VDAC1 signals (mean $\pm$ S.E.M.; $n = 4$; * $P < 0.05$ compared to the corresponding control group).

Fig. 10.

Effect of VPA treatment on nuclear content of TET1 and TET2 immunoreactive proteins. Nuclear protein samples were collected after 1 day (panels in row A) and 3 days (panels in row B) of treatment with 1 mM VPA (striped bars) and its vehicle (open bars). TET1 and TET2 levels were assayed by quantitative Western blot. The optical density of TET bands was corrected by the density of the corresponding beta actin bands. Results are expressed in units of a ratio between TETs and their corresponding beta actin signals (mean $\pm$ S.E.M.; $n = 4$; * $P < 0.05$ compared to the corresponding control group).