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Gadd45b and N-methyl-D-aspartate induced DNA demethylation in postmitotic neurons

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Abstract

Aim—In nondividing neurons examine the role of Gadd45b in active 5-methylcytosine (5MC) and 5-hydroxymethylcytosine (5HMC) removal at a gene promoter highly implicated in mental illnesses and cognition, *Bdnf*.

Materials & methods—Mouse primary cortical neuronal cultures with and without Gadd45b siRNA transfection were treated with N-methyl-D-aspartate (NMDA). Expression changes of genes reportedly involved in DNA demethylation, *Bdnf* mRNA and protein and 5MC and 5HMC at *Bdnf* promoters were measured.

Results—Gadd45b siRNA transfection in neurons abolishes the NMDA-induced increase in *Bdnf IXa* mRNA and reductions in 5MC and 5HMC at the *Bdnf IXa* promoter.

Conclusion—These results contribute to our understanding of DNA demethylation mechanisms in neurons, and its role in regulating NMDA responsive genes implicated in mental illnesses.

Keywords

alcoholism; CpG; epigenetic; histone; schizophrenia

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Financial & competing interests disclosure

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DNA methylation in postmitotic neurons is potentially the longest lasting form of gene regulation in the body with no requirement for turnover across a lifetime [1,2]. As such it is tightly connected with maintaining neuronal identity defined by factors including subtype, brain location and functional role in memory, learning and when aberrant, pathology [3–12]. The addition of methyl groups to the 5' carbon of cytosines is catalyzed by three DNA methyltransferases, Dnmt1, 3a and 3b utilizing, S-adenosylmethionine as the methyl donor. All three Dnmts are expressed in the brain [13,14].

While the mechanisms underlying the addition of methyl groups to cytosine bases in postmitotic neurons has been well-established [14], whether and by what means the removal of these highly stable modifications occurs outside of cell division has been a source of controversy. Over the last several years there has been increasing support for an active DNA demethylation pathway that involves base excision repair (BER) processes (Figure 1A). The initial step in this pathway is the oxidation of 5-methylcytosine (5MC) to 5-hydroxymethylcytosine (5HMC) by the Tet family of enzymes [15–17]. Reductions of Tet1 in mouse embryonic stem cells reduce 5HMC and increase 5MC at discrete genomic regions. Conversely, overexpression reduces 5MC and increases 5HMC in the brain [16,18–23].

5HMC and 5MC are different cytosine modifications that likely have independent roles in regulating gene expression. This necessitates studying each of these separately. Most bisulfite sequencing or certain enzymatic methods provide a combined 5MC/5HMC measurement but cannot resolve these separately, and often only examine methylated cytosines in a CpG context. Therefore, we measured methylation changes using methylated DNA immunoprecipitation (MeDIP) and hydroxymethylated DNA immunoprecipitation (hMeDIP).

Following 5HMC production GADD45 proteins are thought to coordinate a process whereby 5HMC is removed. GADD45 proteins (GADD45a, b and g) are thought to participate in the removal of 5HMC/5MC by recruiting and acting as scaffolds for cytidine deaminases, such as APOBECs or AICDA, and DNA glycosylases, such as TDG, MBD4 or SMUG1 [15,24–31]. A direct physical interaction has been demonstrated between AICDA, TDG and GADD45a in cultured cells [28], while in zebrafish embryos a protein complex consisting of AICDA or APOBEC, MBD4 and GADD45a has been documented [29]. GADD45 proteins have also been shown to bind to nuclear hormone receptors [37] and preferentially to hyperacetylated nucleosomes [38], again suggesting that Gadd45 proteins may be involved in targeting promoters for 5HMC/5MC removal.

In vivo, Gadd45b is induced after long-term potentiation, and Gadd45b and Gadd45g increase in the mouse amygdala following fear conditioning [32–35]. In neuronal culture Gadd45b and Gadd45g have been shown to increase following depolarization [36].

One important target of this demethylation pathway is BDNF. BDNF is a growth factor that plays important roles in neurogenesis, synaptic plasticity, learning, memory and cognition [39,40]. The *BDNF* gene is comprised of multiple upstream 5' exons which splice with a common 3' protein coding exon [41]. One of these splice variants in mice and rats, *Bdnf IV*,

has been shown in neuronal culture to be robustly increased by neuronal depolarization associated with a reduction in promoter 5HMC/5MC [42,43]. *Bdnf IXa* expression and removal of promoter 5HMC/5MC have been shown to be induced in the mouse brain following electroconvulsive seizures (ECS) [15,36]. Abnormalities in DNA methylation at *BDNF* promoters have been reported in various neuropsychiatric disorders including depression, alcoholism and schizophrenia [44–46].

Prior studies regarding the BER DNA demethylation pathway have shown that *Gadd45b* knockout mice fail to remove 5HMC/5MC from the *Bdnf IXa* promoter and consequently fail to increase *Bdnf IXa* expression following ECS [36]. *Tet1* overexpression in mice leads to the removal of 5HMC/5MC from *Bdnf IXa* and *Fgf1b* promoters, and a reduction in *Tet1* expression prevents ECS-induced removal of 5HMC/5MC from *Bdnf IXa* and *Fgf1b* promoters *in vivo* [15,23].

To our knowledge, activity-dependent changes in 5HMC measured independently from 5MC in neurons has not been previously documented, nor have the proteins involved in removal following *N*-methyl-D-aspartate treatment (NMDA) agonism. Further, many of the prior studies regarding the importance of the BER pathway in demethylation were conducted *in vivo* where it is difficult to discern passive DNA demethylation that results from cell division from active DNA demethylation that occurs in nondividing cells, including most neurons [15,36]. In the current study, we attempt to determine the role of *Gadd45b* in removing 5MC and 5HMC from the *Bdnf IXa* promoter using primary cultured neurons where the potential confounds of dividing non-neuronal cells or dividing neuronal stem cells such as in the dentate gyrus of the hippocampus are obviated [42,43].

Methods & materials

Primary cortical neuronal culture

Timed pregnant C57Bl/6 mice were purchased from Charles River. Cortical neurons from E18 mouse fetuses were prepared as previously described [47,48]. All procedures were conducted in accordance with the NIH guidelines for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee. Briefly, mouse cortices were dissected into Hank's balanced salt solution (HBSS) (Life Technologies; 14170–112; NY, USA), and treated with papain (2 mg/ml in HBSS) in the presence of DNase (40 µg/ml) (Sigma-Aldrich; D5025; MO, USA) and MgCl (5 mM) (Sigma-Aldrich; M1028) for 30 min at 37°C. The tissue was centrifuged, and resuspended in Neurobasal medium (Life Technologies; 10888–022) supplemented with 1.25 µg/ml Fungizone (Life Technologies; 15290–018), 100 µg/ml gentamicin (Life Technologies; 15750–078) and 10 mM D-(+)-glucose. Tissue was further dissociated by repeated passages through a Pasteur pipette, and cells were filtered through a nylon mesh of 40 µm pore size. Cells were then spun down and resuspended in Neurobasal complete media which included 2 mM GlutaMAX (Life Technologies; 35050-061) and 1× B27 supplement (Life Technologies; 17504-044). Cells were plated on 100 µg/ml poly-D-lysine (Sigma-Aldrich; P1149) coated plates at $1\text{--}2 \times 10^6$ cells/ml. At 3 days *in vitro* (DIV) cells were treated with cytosine arabinoside (5 µM) (Sigma-Aldrich; C6645). NMDA (Sigma-Aldrich; M3262) and MK-801 (Sigma-Aldrich; M107) were administered directly into the culture-well at 7–8 DIV with

the appropriate vehicle control administered in parallel (water or DMSO). In preliminary dose and time experiments, we found that 2-h treatment with 45 μ M of NMDA induces a robust increase in *Gadd45b* expression. Prior reports using a similar dose of NMDA indicate that *Bdnf IV* mRNA and protein levels begin to increase after 1 h with peak *Bdnf IV* mRNA expression at 6 h [49]. Based on our data and these prior reports we chose to conduct the rest of the experiments using NMDA 45 μ M at 2 h. All experiments were conducted using at least four replicates per condition.

RNA interference of *Gadd45b* in postmitotic neuronal cell culture

Small interfering RNAs (siRNAs, 25 nt) were designed against *Gadd45b* mRNA (GenBank NM_008655.1). The 25 nt targeting sequence for the siRNA is as follows (5'–3'): CACTTCACCCTGATCCAGTCGTTCT (Life Technologies; 1320001). Cells were transfected with 30 nM siRNA using Dharmafect3 transfection reagent (Thermo Fisher; T-2003-03; MA, USA) at 6 DIV, media changed after 24 h and cells harvested at 8 DIV.

Quantitative reverse transcriptase-PCR

Total RNA was isolated using TRIZOL reagent (Life Technologies; 15596-026). DNA was removed using DNA-freeTM DNA Removal Kit (Life Technologies; AM1906). Target gene expression was normalized to *Hprt1* (see Table 1 for primer sequences). For qRT-PCR, cDNA samples were analyzed using PikoReal Real-Time PCR (Thermo Fisher) and Maxima[®] SYBR Green/ROX qPCR Master Mix (Thermo Fisher; K0222). To confirm amplification specificity, the PCR products were subject to a melting curve analysis, in which only one peak was observed. Crossing point values were measured with the PikoReal analysis software. Primers were designed to span at least one intron–exon boundary, with the exception of *Bdnf IXa* which lacks an intervening intron. The following cycling conditions were used: 10 min at 95°C, then 40 cycles at 95°C for 30 s, 60°C for 1 min and 72°C for 30 s.

Methylated DNA immunoprecipitation or hydroxymethylated MeDIP

Primary cortical neuronal culture samples were sonicated to 200–500 bp segments in SDS lysis buffer. DNA was then extracted using proteinase K at 37°C overnight followed by phenol-chloroform extraction. One microgram of DNA was used for each IP and 0.3 μ g for input. IP samples were diluted in 250 μ l ChIP dilution buffer. Samples were boiled for 10 min and then immediately placed on ice. Samples were incubated with either 4 μ l of 5MC monoclonal mouse (Diagenode; C15200081; NJ, USA) or 2 μ l of rabbit polyclonal 5HMC antibodies (Active Motif; 39769; CA, USA) overnight on a rotator at 4°C. Subsequently, antibody-DNA complexes were precipitated using Protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology; sc-2003; TX, USA) for 3 h on a rotator at 4°C. Following low salt, high salt, lithium chloride and TE washes, complexes were eluted from beads at 67°C for 2 h using elution buffer. DNA was then extracted using proteinase K at 37°C overnight followed by phenol–chloroform extraction and resuspended in water. Samples were measured using qPCR. IP samples were normalized to input.

We purchased 338 base pair oligonucleotides corresponding to a promoter region of the human adenomatous polyposis coli (*APC*) gene in which all the cytosines are replaced with

either 5HMC, 5MC or unmethylated cytosine (UM; Active Motif; 55008). We performed the hMeDIP procedure as detailed above. We find that this procedure precipitated 99.8% less 5MC oligo, and 89.1% less UM oligo compared with 5HMC oligo, indicating the specificity of this procedure for the 5HMC base.

Bdnf immunoblotting

Primary cortical neuronal culture samples were lysed in buffer containing protease inhibitors. Bdnf quantification was carried out by SDS-PAGE. Nitrocellulose membranes were incubated overnight in 1% blocking solution with rabbit anti-Bdnf (Santa Cruz Biotechnology; sc-546) at a 1:200 dilution. Antirabbit secondary antibody was then used at a 1:2500 dilution in 1% blocking solution. β -actin was detected using mouse anti- β -actin antibody (Sigma-Aldrich; A5316) at a 1:2500 dilution in 5% blocking solution for 30 min followed by anti-mouse secondary antibody at a 1:2500 dilution in 5% blocking solution for 2 h. ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences; RPN2232; NJ, USA) was detected using a Kodak Image Station 4000R Pro and quantified using ImageJ software.

Statistics

Data were analyzed with Student's t-test or one-way analysis of variance (ANOVA) with *post hoc* Bonferroni tests as appropriate. All statistical analyses were performed using GraphPad Prism (version 15.0 for Windows). All statistical tests were two sided.

Results

In mouse primary neuronal cultures NMDA increases DNA demethylating gene expression & decreases Dnmts

In NMDA-treated neurons we measured mRNA expression of genes responsible for DNA methylation and demethylation. We find that treatment with NMDA 45 μ M for 2 h significantly decreases *Dnmt3a* ($t_{18} = 2.211$; $p = 0.04$), *3b* ($t_{18} = 6.012$; $p < 0.0001$) and *Tet1* ($t_{17} = 3.065$; $p = 0.007$) with a trend for a decrease in *Dnmt1* ($t_{18} = 1.969$; $p = 0.06$) (Figure 2A & B). Additionally, NMDA induced *Gadd45b* mRNA expression ($t_{16} = 3.578$; $p = 0.003$), but not other BER pathway genes measured including *Apobec1*, thymidine and uracil glycosylases, and *Parp1* (Figure 2C). A similarly robust induction of *Gadd45b* is produced by depolarization with veratridine 2 mM (Data not shown) and in prior reports by KCl [36].

NMDA significantly induces *Gadd45b* ($F_{3,31} = 6.940$; Bonferroni *post hoc* test; $p < 0.001$) and *g* ($F_{3,24} = 4.822$; Bonferroni *post hoc* test; $p < 0.05$), which is prevented by treatment with NMDA antagonist MK-801 at a dose of 50 μ M. MK-801 50 μ M alone has no effect on *Gadd45* expression (Figure 2D).

NMDA induces both the expression of Bdnf mRNA & protein & removal of 5HMC & 5MC at Bdnf IV & IXa promoters

We find that treatment with NMDA 45 μ M for 2 h significantly induces the expression of *Bdnf IV* ($t_{21} = 3.852$; $p = 0.0009$) and *IXa* ($t_{13} = 4.272$; $p = 0.0009$) mRNA and Bdnf protein ($t_{17} = 2.235$; $p = 0.04$) (Figures 3B & C). We find significant reductions in 5HMC at both

Bdnf IV ($t_8 = 2.570$; $p = 0.03$) and *IXa* ($t_8 = 2.412$; $p = 0.04$) promoters, and a reduction in 5MC at the *Bdnf IXa* ($t_7 = 2.301$; $p = 0.05$), but not the *IV* promoter in primary neuronal cultures treated with NMDA 45 μ M for 2 h (Figures 3D & E).

Gadd45b siRNA knockdown (siGadd45b) in primary cortical neuronal cultures

Prior studies have suggested that Gadd45b plays a role in activity-dependent DNA demethylation [36]. However, these studies were conducted *in vivo* and in the dentate gyrus of the hippocampus [36]. Therefore, it is difficult to discern whether the demethylation event was primarily in neurons or glia, and whether it was truly independent of cell division as glia can divide and dividing neuronal stem cells are found in the dentate gyrus. We transfected siGadd45b into primary mouse cortical neuronal cultures at 6 DIV and harvested cells at 8 DIV. Successful knockdown was assessed based on a significant reduction in *Gadd45b* mRNA ($t_{22} = 5.884$; $p < 0.0001$) (Figure 4A), without impacting mRNA expression of the other *Gadd45* genes. This reduction in *Gadd45b* affected baseline levels of *Bdnf IV* ($t_{16} = 6.950$; $p < 0.0001$) but not *Bdnf IXa* mRNA expression, Bdnf protein levels, nor 5MC or 5HMC at either *Bdnf IV* or *IXa* promoters. Gadd45b knockdown also does not affect *Tet1* mRNA levels (Figure 4B). However, it did reduce expression of *Dnmt3a* ($t_{16} = 2.259$; $p = 0.04$) and *3b* ($t_{16} = 2.575$; $p = 0.02$).

Impaired NMDA-induced changes in gene expression in neurons with reduced Gadd45b levels

We treated neuronal cultures that had been transfected with siGadd45b 48 h earlier with NMDA 45 μ M for 2 h. Cells transfected with scrambled RNA (siScr) behaved similarly to nontransfected cells (Figure 3B & C), in that *Bdnf IV* ($t_{13} = 12.30$; $p < 0.0001$) and *IXa* mRNA ($t_{16} = 4.077$; $p = 0.0009$) and Bdnf protein expression ($t_{18} = 3.138$; $p = 0.005$) are induced by NMDA (Figure 5A & B). On the other hand, in siGadd45b transfected neurons there is an inability to induce *Bdnf IXa* mRNA expression, while induction of *Bdnf IV* is not attenuated (Figure 5A). Similarly, NMDA fails to induce Bdnf protein expression in siGadd45b transfected neurons (Figure 5B). Additionally, *Tet1*, which decreases in nontransfected and siScr transfected neurons treated with NMDA ($t_{17} = 3.013$; $p = 0.008$), does not in siGadd45b transfected cells (Figure 5C). siGadd45b transfection reduced *Dnmt3a* and *3b* expression with no further significant reduction following NMDA treatment.

Impaired NMDA-dependent DNA demethylation in neurons with reduced Gadd45b levels

We measured NMDA-induced *Bdnf IXa* and *Bdnf IV* promoter demethylation in siGadd45b transfected neurons. Similar to nontransfected cells (Figure 3D & E), we find that in siScr transfected cells NMDA induces a significant reduction in both 5MC ($t_{25} = 2.407$; $p = 0.02$) and 5HMC ($t_{23} = 3.762$; $p = 0.001$) at the *Bdnf IXa* promoter and a reduction in 5HMC at the *Bdnf IV* promoter ($t_{23} = 2.717$; $p = 0.01$) (Figure 6A & B). However, in siGadd45b transfected neurons, NMDA fails to reduce 5MC at the *Bdnf IXa* promoter (Figure 6A). Moreover, in siGadd45b transfected neurons, NMDA does not merely fail to reduce 5HMC at the *Bdnf IXa* and *Bdnf IV* promoters but actually leads to significant increases at *Bdnf IV* ($t_{14} = 2.682$; $p = 0.02$) and *IXa* ($t_{15} = 2.981$; $p = 0.009$) (Figure 6B).

Discussion

The role of Gadd45b as a key coordinator of active DNA demethylation has been largely based on *in vivo* experiments where it is difficult to distinguish active DNA demethylation that nondividing neurons use exclusively from passive demethylation that can occur in dividing cells such as glia. To avoid these confounding factors we used a homogeneous population of postmitotic cells, mouse E18 primary cortical neuronal cultures. We find that NMDA agonism induces *Gadd45b*, *Bdnf IV* and *IXa* mRNA expression, while reducing *Tet1*, *Dnmt3a* and *Dnmt3b* mRNA expression. In addition, NMDA treatment leads to the removal of 5HMC but not 5MC at the *Bdnf IV* promoter, and the removal of both 5MC and 5HMC at the *Bdnf IXa* promoter.

When primary neuronal cultures were transfected with Gadd45b siRNA (siGadd45b) and treated with NMDA, *Bdnf IV* mRNA expression increases as it does in siScr and nontransfected cells, however there is a lack of *Bdnf IXa* mRNA or Bdnf protein induction. We find that siGadd45b leads to an increase in 5HMC at *Bdnf IV* and *IXa* promoters following NMDA treatment and a lack of removal of 5MC from the *Bdnf IXa* promoter. These changes are associated with a lack of induction of *Bdnf IXa*, but they do not appear to affect *Bdnf IV* induction. In the brain 5HMC accounts for 10–40% of modified cytosines, and exclusively occurs in a CpG context unlike 5MC [50,51]. When located in intragenic regions 5HMC positively correlates with gene transcript levels in the brain [50,52–54], and is abundant in euchromatin in neuronal tissue [20,53,55]. On the other hand, 5HMC at transcription start sites (TSS) often correspond to decreases in gene expression [51,53,56–58]. The fact that an increase in promoter 5HMC was associated with a lack of *Bdnf IXa* increased expression and did not affect the induction of *Bdnf IV* may indicate that 5MC is more important in terms of regulating activity-dependent gene expression than 5HMC. Similar to prior reports [36], we did not find neuronal activity to induce the removal of 5MC at the *Bdnf IV* promoter after an acute treatment and using MeDIP procedures. Overall, the reliable interaction between Gadd45b and *Bdnf IXa*, in which Gadd45b appears to serve as a factor in the removal of *Bdnf IXa* promoter 5MC and 5HMC, is a consistent finding from this study, while the regulation of *Bdnf IV* may involve different mechanisms.

The current study was limited by the fact that we examined two of the nine *Bdnf* transcripts, one time point was selected, and we did not examine CpGs with single base pair resolution. *Bdnf IV* was selected in the current study because prior reports indicated that *Bdnf IV* is demethylated in primary neuronal cultures by depolarization [42,43], and *Bdnf IXa* was selected because *in vivo* studies demonstrated that Gadd45b has a role in demethylating its promoter [36]. Other *Bdnf* transcripts have also been shown to be induced by excitatory neuronal activity [59], but less is known about the dynamics of their promoters' DNA methylation statuses or whether Gadd45b is involved in their regulation. The lack of base-specific resolution of our methylation assays is also a limitation of the current study. However, there are few established assays capable of independently measuring 5HMC and 5MC outside of a CpG context. While MeDIP is unable to provide base-specific resolution, it is able to measure 5HMC and 5MC separately and without regard to whether the modified cytosine is followed by a guanine.

In the current study, we find that Tet1 expression paralleled changes in 5HMC at the *Bdnf IV* and IXa promoters. NMDA led to decreases in 5HMC at both promoters and, in agreement with data from the Sweatt lab in which neurons were treated with KCl, decreases in *Tet1* mRNA expression [23]. On the other hand, in siGadd45b transfected cells *Tet1* expression was not suppressed following NMDA treatment which was associated with an increase in 5HMC at both promoters. Prior studies indicate that *Tet1* overexpression is associated with globally increased 5HMC [23]. Basal Bdnf expression in neurons has been shown to be unaffected by Tet1 knockdown and increased by Tet1 overexpression [15,23]. However, the effects of nonsuppression of Tet1 during excitatory neuronal activity on Bdnf expression to our knowledge have not been documented previously. Future studies utilizing immunoprecipitation under control and NMDA conditions are required to identify whether Gadd45b directly binds to the *Tet1* promoter. However, it is also possible that under NMDA stimulation Gadd45b affects the expression of other transcription factors or epigenetic enzymes that decrease Tet1.

Conclusion

In conclusion, our results indicate the importance of Gadd45b in NMDA-induced demethylation. Based on these results we propose a mechanism whereby neuronal excitatory activity initiates the suppression of Dnmt expression and in part through Gadd45b leads to a reduction in Tet1 enzyme expression, thereby reducing the production of 5MC and 5HMC (Figure 1A). On the other hand, when Gadd45b is reduced via siRNA there is an elimination of Tet1 suppression. This leads to greater production of 5HMC from 5MC. Moreover, the Gadd45b deficit directly affects the removal of 5HMC in that it is no longer able to coordinate the BER machinery [15,28,29]. Thus, Gadd45b knockdown does not merely prevent the removal of 5HMC, but in fact leads to an increase. Ultimately, this may increase 5MC as the result of a backup of the demethylation pathway or it may be possible that Gadd45b removes 5MC at times independently of 5HMC (Figure 1B). Furthermore, these results suggest a nonlinear relationship between 5HMC in gene promoter regions and gene expression. By elucidating the roles of these modifications and the proteins that mediate their removal independently in neurons it may become possible to develop targeted therapeutics that enhance expression of genes implicated in mental illnesses such as Bdnf.

Future perspective

Without the existence of active DNA demethylation, neurons once misprogrammed could never be reprogrammed as the DNA methyl mark can persist across a lifetime as well as be passed to subsequent generations [60–63]. The potential to pharmacologically alter this code would then be minimal due to the stability of the covalent bonds that link methyl groups to cytosines. In other words, if not for the DNA demethylation pathway chronic mental illnesses characterized by dysfunctional neuronal networks in part encoded by aberrant DNA methyl marks would be irreversible because of the stability of the methyl-cytosine bond. By elucidating the key players in the DNA demethylation pathway it may become possible to pharmacologically target this pathway to rewrite neuronal identity, thereby forming new more beneficial networks. This would allow for not merely treating an otherwise chronic mental illness, but actually reversing its underlying pathology.

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Executive summary

- The role of Gadd45b as a key coordinator of active DNA demethylation has been largely based on *in vivo* experiments where it is difficult to distinguish active DNA demethylation that nondividing neurons use exclusively from passive demethylation that can occur in dividing cells such as glia.
- To avoid these confounding factors we used a homogeneous population of postmitotic cells, mouse E18 primary cortical neuronal cultures.
- We find that *N*-methyl-D-aspartate (NMDA) agonism induces *Gadd45b*, *Bdnf IV* and *IXa* mRNA expression, while reducing *Tet1*, *Dnmt3a* and *Dnmt3b* mRNA expression.
- NMDA induces a significant reduction in 5-hydroxymethylcytosine (5HMC) at both *Bdnf IV* and *IXa* promoters, and a reduction in 5-methylcytosine (5MC) at the *Bdnf IXa* but not the *IV* promoter in primary neuronal cultures.
- In siGadd45b siRNA transfected neurons the increase in *Bdnf IXa* mRNA and decrease in *Tet1* mRNA expression is abolished following NMDA treatment.
- In Gadd45b siRNA transfected neurons, NMDA fails to reduce 5MC at the *Bdnf IXa* promoter, and in fact leads to significant increases of 5HMC at *Bdnf IV* and *IXa* promoters.
- Our results indicate that Gadd45b might be involved in the removal of 5MC and 5HMC at *Bdnf* promoters.
- These results contribute to our understanding of DNA demethylation mechanisms in neurons, and their roles in regulating NMDA regulated genes implicated in mental illnesses.

Figure 1. Hypothetical pathway of activity-dependent *Bdnf* DNA demethylation

(A) Effects of neuronal depolarization on DNA demethylation pathway. (1) Neuronal activity decreases DNA methyltransferase (DNMT) expression leading to less production of 5-methylcytosine (5MC). This decreases the amount of substrate used to produce 5-hydroxymethylcytosine (5HMC). (2) Tet1-catalyzed conversion of 5MC to 5HMC at *Bdnf IXa* may maintain the promoter in a state primed for demethylation. However, once the neuron is depolarized Tet1 expression decreases lead to reduced 5HMC production. (3) Neuronal activity increases Gadd45b expression. Gadd45b participates in *Bdnf IXa* promoter demethylation by facilitating the removal of 5HMC directly, and as a direct or indirect negative regulator on Tet1 expression. Both these actions serve to decrease 5HMC. **(B)** Effects of Gadd45b knockdown on activity-dependent *Bdnf* demethylation. (1) In the current study, we knocked down Gadd45b expression using siRNA. This may prevent the Gadd45b-coordinated *N*-methyl-D-aspartate (NMDA)-induced removal of 5HMC. (2) Gadd45b knockdown may also prevent it from suppressing Tet1 expression following NMDA treatment. Inhibition of both actions of Gadd45b would lead to an accumulation of 5HMC at *Bdnf* promoters. This may explain why Gadd45b knockdown did not merely prevent the removal 5HMC from *Bdnf* promoters, but it actually led to an increase following NMDA treatment. (3) By preventing the removal of 5HMC, the pathway is blocked, leading to a failure in NMDA-dependent 5MC removal.

5HMC: 5-hydroxymethylcytosine; 5MC: 5-methylcytosine.

Figure 2. N-methyl-D-aspartate induced changes in DNA methylating and demethylating factors in mouse primary cortical neuronal cultures

(A) NMDA 45 μ M at 2 h significantly reduces *Dnmt3a* and *3b* mRNA expression with a trend for a decrease in *Dnmt1*. (B) NMDA induces significant reductions in the expression of *Tet1* mRNA. (C) *Gadd45b* mRNA is significantly induced by NMDA, but expression of no other base excision repair pathway genes measured are affected. (D) NMDA significantly induces *Gadd45b* and g, which is prevented by co-treatment with NMDA antagonist MK-801 50 μ M. MK-801 50 μ M alone has no effect on *Gadd45* expression. Error bars represent standard error of mean.

*p < 0.05, **p < 0.01, ***p < 0.001.

CTL: Control; NMDA: N-methyl-D-aspartate.

Figure 3. N-methyl-D-aspartate induces *Bdnf* DNA demethylation

(A) Map of the *Bdnf* promoter regions examined in this study. The top figure represents the promoter region of *Bdnf IV*. Each CpG site is represented by a red vertical line. MeDIP and hydroxyl-MeDIP primers encompass a region from -156 to +31 relative to the transcription start site (black horizontal line). This region includes nine CpG sites. For *Bdnf IXa*, MeDIP and hMeDIP primers encompass a region from -171 to +32 (blue line) relative to the transcription start site. This region includes seven CpG sites. (B) NMDA significantly induces the expression of *Bdnf IV* and *IXa* mRNA. (C) NMDA increases Bdnf protein expression after 2 h. Below bar graph is a representative western blot with control (C) and NMDA (N) conditions. (D) NMDA treatment induces a reduction in 5HMC at *Bdnf IV* and *IXa* promoters. (E) NMDA treatment leads to a reduction in at *Bdnf IXa*, while having no effect on *Bdnf IV* 5MC levels. Error bars represent standard error of mean.

*p < 0.05, ***p < 0.001.

5HMC: 5-hydroxymethylcytosine; 5MC: 5-methylcytosine; CTL: Control; MeDIP:

Methylated DNA immunoprecipitation; NMDA: N-methyl-D-aspartate.

For color images please see online at: www.futuremedicine.com/doi/full/10.2217/EPI.15.12

Figure 4. Effect of Gadd45b knockdown via siRNA in mouse primary cortical neuron culture
(A) Neurons were transfected with either a scrambled nontargeting siRNA (siScr) or a Gadd45b specific siRNA (siGadd45b) at the sixth day *in vitro* (DIV). Successful knockdown of Gadd45b was assessed based on reduced mRNA expression. siGadd45b produces no significant changes to, *Gadd45a* or *g*, the other two Gadd45 isoforms. **(B)** siGadd45b transfection also has no effect on *Tet1* mRNA expression. Error bars represent standard error of mean.

*** $p < 0.001$.

Figure 5. Gadd45b is necessary for N-methyl-D-aspartate induction of Bdnf expression

(A) Neurons were transfected with a scrambled nontargeting siRNA (siScr) or a Gadd45b specific siRNA (siGadd45b) at the sixth day *in vitro*, then treated with NMDA 45 μM for 2 h on day *in vitro* 8. Results and significance levels are expressed as fold change in NMDA treated cells relative to no NMDA control condition (dotted line). NMDA induces the expression of *Bdnf IV* and *Bdnf IXa* in cells transfected with siScr compared with control condition. siGadd45b transfection does not affect NMDA-induced increase in *Bdnf IV*, but does prevent the induction of *Bdnf IXa* mRNA expression relative to no NMDA control. (B) Similarly, NMDA induces Bdnf protein expression based on western blot in siScr transfected cells, but fails to in siGadd45b transfected cells. (C) NMDA induces a reduction in *Tet1* mRNA expression in siScr neurons relative to control, but siGadd45b transfected cells showed no decrease in expression. Error bars represent standard error of mean.

** $p < 0.01$, *** $p < 0.001$.

NMDA: N-methyl-D-aspartate; NS: Non significant.

Figure 6. Gadd45b is necessary for *N*-methyl-D-aspartate induced demethylation of *Bdnf*
(A) Neurons were transfected with a scrambled nontargeting siRNA (siScr) or a Gadd45b-specific siRNA (siGadd45b) at day 6 *in vitro* (DIV), then treated with NMDA 45 μ M for 2 h on day 8 *in vitro*. Results and significance levels are expressed as fold change in NMDA treated cells relative to no NMDA control condition (dotted line). In siScr transfected cells, NMDA treatment significantly reduces *Bdnf IXa* promoter 5MC, but does not alter 5MC at the *Bdnf IV* promoter. siGadd45b eliminates the NMDA-induced reduction in 5MC at the *Bdnf IXa* promoter. **(B)** In siScr transfected cells, NMDA treatment significantly reduces *Bdnf IV* and *IXa* promoter 5HMC. siGadd45b does not merely prevent the reduction in 5HMC, but in fact causes increases in 5HMC at *Bdnf IV* and *IXa* promoters. Error bars represent SEMs.

* $p < 0.05$, ** $p < 0.01$.

5HMC: 5-hydroxymethylcytosine; 5MC: 5-methylcytosine; NMDA: *N*-methyl-D-aspartate.

Table 1

Primer sequences used in this study.

Genes	5' primer	3' primer
mRNA expression primers		
<i>Apobec1</i>	GCTCCTCCCTTGAAATCAGA	GTCCTGGCTCATGATGTCCT
<i>BdnfIV</i>	TCGCTGAAGGCGTGCAGTA	GCACACCTGGGTAGGCCAAG
<i>BdnfIXa</i>	GCAGCTGGAGTGGATCAGTAA	TGGTCATCACTCTTCTCACCTG
<i>Dnmt1</i>	AACCATCACCGTGCAGACA	AGGGTGCTGAATTGCCTGGA
<i>Dnmt3a</i>	AGGAAGACCCCTGGAACTGCT	ACGTCCCCGACGTACATGATCT
<i>Dnmt3b</i>	GCGTCAGTACCCCATCAGTT	CACGAGGTACCTATTCCAAA
<i>Gadd45a</i>	TGCGAGAACGACATCAACAT	TCCCGGCAAAAACAAATAAG
<i>Gadd45b</i>	GTTCTGCTGCGACAATGACA	TTGGCTTTTCCAGGAATCTG
<i>Gadd45g</i>	ATGACTCTGGAAGAAGTCCGT	CAGGGTCCACATTCAGGACT
<i>Hprt1</i>	GCCGAGGATTTGGAAAAAGT	ACAGAGGGCCACAATGTGAT
<i>Mbd4</i>	CAAACCTTGGGGACAACAGA	TCACCAGGTCCTTTCCATCT
<i>Parg</i>	GGTCCGAAACCTTTTCCAA	TACTCCCTGCAGTTCGCTCA
<i>Parp1</i>	AACCTTGCTGGCATCCTGTC	TGCACTTTTGGACACCATGT
<i>Smug1</i>	AACCCGGTGGATTATGCTTG	GTCCCGGACCACATTCACTT
<i>Tdg</i>	TTCCTAACATGGCAGTCACG	GCTCTGGGTTTCCTTTTCCT
<i>Tet1</i>	TGTGGGGAGTGACCTACTG	TTGGGGCCATTTACTGGTTT
Methylated DNA immunoprecipitation primers		
<i>Bdnf IV</i> –156 to +31	CCCTGGAACGGAATTCTTCT	AGTCCTCTCCTCGGTGAATG
<i>Bdnf IXa</i> –171 to +32	CATGAGACCGGGCAAGTC	CCTTGGGAGGAATGTGTGAT