Behavioral/Cognitive

Dnmt3a in the Medial Prefrontal Cortex Regulates Anxiety-Like Behavior in Adult Mice

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Recently, it has been suggested that alterations in DNA methylation mediate the molecular changes and psychopathologies that can occur following trauma. Despite the abundance of DNA methyltransferases (Dnmts) in the brain, which are responsible for catalyzing DNA methylation, their roles in behavioral regulation and in response to stressful challenges remain poorly understood. Here, we demonstrate that adult mice which underwent chronic social defeat stress (CSDS) displayed elevated anxiety-like behavior that was accompanied by a reduction in medial prefrontal cortex (mPFC)-DNA methyltransferase 3a (Dnmt3a) mRNA levels and a subsequent decrease in mPFC-global DNA methylation. To explore the role of mPFC-Dnmt3a in mediating the behavioral responses to stressful challenges we established lentiviral-based mouse models that express lower (knockdown) or higher (overexpression) levels of Dnmt3a specifically within the mPFC. Nonstressed mice injected with knockdown Dnmt3a lentiviruses specifically into the mPFC displayed the same anxiogenic phenotype as the CSDS mice, whereas overexpression of Dnmt3a induced an opposite, anxiolytic, effect in wild-type mice. In addition, overexpression of Dnmt3a in the mPFC of CSDS mice attenuated stress-induced anxiety. Our results indicate a central role for mPFC-Dnmt3a as a mediator of stress-induced anxiety.

Key words: anxiety; DNA methyltransferases; stress

Significance Statement

DNA methylation is suggested to mediate the molecular mechanisms linking environmental challenges, such as chronic stress or trauma, to increased susceptibility to psychopathologies. Here, we show that chronic stress-induced increase in anxiety-like behavior is accompanied by a reduction in DNA methyltransferase 3a (Dnmt3a) mRNA levels and global DNA methylation in the medial prefrontal cortex (mPFC). Overexpression or knockdown of mPFC-Dnmt3a levels induces decrease or increase in anxiety-like behavior, respectively. In addition, overexpression of Dnmt3a in the mPFC of chronic stressed mice attenuated stress-induced anxiety. We suggest that mPFC-Dnmt3a levels mediates anxiety-like behavior, which may be a primary molecular link between chronic stress and the development of anxiety disorders, including post-traumatic stress disorder.

Introduction

Epigenetic modifications, particularly DNA methylation, have been implicated as key molecular drivers of stress-induced psy-

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considering that there are already pharmacological tools available that inhibit Dnmt activity. Initial studies have found that fear conditioning induces an increase in the expression of Dnmt3a in the hippocampus and that pharmacological inhibition of Dnmts in that same brain area can impair short-term learning (Miller and Sweatt, 2007), whereas inhibition of Dnmts in the frontal cortex can erase remote memories of fear (Miller et al., 2010). Transgenic studies of mice lacking both Dnmt1 and Dnmt3a in forebrain excitatory neurons exhibit an attenuated hippocampal-dependent learning (Feng et al., 2010), and Dnmt3a expression in the nucleus accumbens (NAc) promotes depression-like behavior in the social defeat procedure (LaPlant et al., 2010). Therefore, the endogenous role of Dnmts in psychological behavior is both brain region specific and variable among the different Dnmts.

The brains' limbic system is central in orchestrating the systemic and behavioral response to stress. While activation of the amygdala can promote an enhanced stress response, prefrontal cortex (PFC) innervations of the amygdala can attenuate this response (Herman et al., 2005). Multiple lines of evidence have shown that the PFC is highly susceptible to stressful challenges and can undergo morphological changes following chronic stress (Arnsten, 2009). Both murine and human imaging studies have shown that dysregulation of the amygdala and PFC (decreased activity) are present in individuals with anxiety disorders and post-traumatic stress disorder (PTSD) (for review, see Yehuda and LeDoux, 2007). Therefore, molecular mechanisms that are dysregulated in the PFC are likely to be involved in stress-induced anxiety and PTSD.

In this study, using the chronic social defeat stress (CSDS) procedure, well established for the induction of prolonged anxiety-like behavior in rodents (Krishnan et al., 2007), we sought to examine the role of limbic Dnmts in anxiety-like behavior. Toward this end, we identified which specific Dnmts were dysregulated in various brain regions following CSDS. Here, we show that the elevation in anxious behavior following CSDS accompanies a robust reduction in Dnmt3a mRNA levels and global DNA methylation in the medial PFC (mPFC). Next, we genetically manipulated the Dnmt3a expression levels in naive mice and tested the behavioral phenotype. We also examined the potential of Dnmt3a as an anxiolytic agent by overexpressing Dnmt3a in the mPFC of chronic-stressed mice. Our data suggest that expression of Dnmt3a in the mPFC mediates anxiety-like behavior, which may be a primary molecular link between chronic stress and the development of anxiety behaviors and disorders, including PTSD.

Materials and Methods

Animals

Adult (9-week old) C57BL/6j mice (Harlan) were maintained in a pathogen-free temperature-controlled (22 ± 1°C) mouse facility on a reverse 12 h light-dark cycle at the Weizmann Institute of Science, according to institutional guidelines. Food (Harlan) and water were given ad libitum. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the Weizmann Institute of Science.

Chronic social defeat stress

Nine-week-old C57BL/6j mice were subjected to a CSDS protocol as previously described (Krishnan et al., 2007). Briefly, the mice were placed in the home cage of an aggressive ICR (CD1) outbred mouse (Harlan) and allowed to physically interact for 5 min. During this time, the ICR mouse attacked the intruder mouse and the intruder displayed subordinate posturing. A perforated clear Plexiglas divider was then placed between the animals, and the mice remained in the same cage for 24 h allowing sensory contact. The procedure was then repeated with an unfamiliar ICR mouse for each of the 10 consecutive days. Control mice were housed in the same room as the social defeat mice but were taken out of the room during the 5 min interaction with the ICR. Control mice were handled daily and housed two per cage separated by a perforated clear Plexiglas divider.

Behavioral assessments

All behavioral assessments were performed during the dark phase following a 2 h habituation to the test room before each test. Behavioral tests were conducted in the following order, from the least stressful procedure to the most stressful, ending with locomotor testing: open field, dark-light transfer (DLT), elevated plus maze (EPM), and home cage locomotion.

Open-field test. The open-field test was performed in a 50 × 50 × 22 cm white box, lit to 120 lux. The mice were placed in the box for 10 min. Locomotion in the box was quantified using a video tracking system (VideoMot2; TSE Systems).

DLT test. The DLT test apparatus consists of a polyvinyl chloride box divided into a black dark compartment (14 × 27 × 26 cm) and a connected white 1200 lux illuminated light compartment (30 × 27 × 26 cm). During the 5 min test, the time spent in the light compartment, the distance traveled in light area, and the number of light-dark transitions were quantified with a video tracking system (VideoMot2; TSE Systems).

EPM. The apparatus in the EPM test is a plus sign shape containing 2 barrier walls and 2 open arms. During the 5 min test, which is performed in relative darkness (6 lux), the number of entries, the distance traveled, and the time spent in the open arms are automatically scored using a video tracking system (VideoMot2; TSE Systems).

Home cage locomotion. Home cage locomotion was assessed using the InfraMot system (TSE Systems). Mice were housed individually for 72 h, of which the first 24 h were considered habituation to the individual housing conditions. Measurements of general locomotion consisted of two light and two dark cycles in the last 48 h, collected at 10 min intervals.

Microdissection and preparation of RNA

Immediately after decapitation, the brain was removed and placed into a 1 mm metal matrix (catalog #51380; Stoelting). The brain was sliced using standard razor blades (GEM, 62-0165) into 1 or 2 mm slices that were quickly frozen on dry ice. Blunted syringes of different diameters were used to extract the brain regions from slices removed from the matrix, which were then stored at −80°C. RNA extraction was performed using Nucleospin RNA XS kit (Machery-Nagel). RNA was reverse transcribed to cDNA using the High Capacity RNA to cDNA kit (Applied Biosystems). The cDNA was then analyzed by qRT-PCR.

Genomic DNA purification and global DNA methylation analysis

The 14-gauge punches from mouse mPFC were incubated overnight at 50°C in 400 μl lysis buffer containing Proteinase K. Tissue was then fully suspended in solution by vigorous pipetting. Solution was cleared of debris by a 5 min centrifugation at 16,000 × g, followed by DNA precipitation with 500 μl isopropanol and 15 min centrifugation at 4°C. DNA was washed with 70% ethanol, centrifuged at 16,000 × g for an additional 5 min, and then resuspended in 30 μl deionized water; 100 ng of genomic DNA was used for DNA global methylation analysis performed with the MethylFlash Methylated DNA Quantification Kit (Colorimetric; Epigentek Group) according to the manufacturer’s instructions. Raw values were quantified, and methylation levels were estimated using a standard curve of methylated DNA standard provided by the manufacturer. Values are presented as methylation percent relative.

qRT-PCR

We used qRT-PCR to detect the levels of mRNA both in the in vitro and in vivo experiments. The expression of HPRT mRNA served as the internal control. The RT-PCR was performed using a 7500 RT-PCR system (Applied Biosystems) using fluorescent SYBR Green technology (ABgene). The PCR conditions were as follows: cDNA equivalent to 10 ng of total RNA was amplified by PCR for 45 cycles at an annealing temperature of 60°C. Each qPCR contained 10 μl 2 × SYBR Green Mastermix, and a final primer concentration of 250 nM. The specificity of the amplification products was verified by melting curve analysis. Primer sequences used were as follows: Dnmt1 sense, 5′-CCTAGTTCCGTGGC
TACGAGGAGAA3; Dnmt1 antisense, 5'TCTCTCTCCTTGCACTGCGA
CGACTCA3; Dnmt3a1 sense, 5'CCTAGTCGGGCTACGAGG
AGAA3; Dnmt3a1 antisense, 5'TCTCTCTCCTTGCACTGCGAC
GCTCA3; Dnmt3a2 sense, 5'CCAGATTTACAGAGGTCCTC
AGGTAGATGCCCAAA3; Dnmt3b sense, antisense, 5'TGAGAA
GCTGCCACCCAAATXG3; HPRT antisense, 5'GGCTCCTTTTACG
GAGA3.

**Lentiviral vectors, infection, and expression**

**DNMT3a knockdown.** Lentiviral vectors were constructed to produce lentiviruses expressing short hairpin RNA (shRNA) against Dnmt3a transcripts or scrambled control. Five different target sequences (see Fig. 2A) from the open reading frame of a Dnmt3a mouse cloned to the plKO.1 plasmid following a U6 promoter were obtained (Sigma-
Aldrich), and high titer lentiviruses were produced (see Fig. 2C) as described previously (Torscini et al., 2006). Briefly, recombinant lentiviruses were produced by transient transfection in HEK293T cells. Infectious particles were harvested at 48 and 72 h after transfection, filtered through 0.45-μm-pore cellulose acetate filters, concentrated by ultracentrifugation, resuspended in sterile HBSS, aliquoted, and stored at −80°C. The most efficient shRNA-shDnmt3a#1 (CCGCAGAGATG
TCTTGCGACTTACGAGCTTGTTTGTGAGT) that targets both Dnmt3a isoforms -1 and -2, was selected for the in vivo experiments (see Fig. 2C).

**Dnmt3a1 overexpression.** Lentiviral vectors were designed and constructed to produce lentiviruses expressing mouse Dnmt3a1. Dnmt3a1, isolated from mouse brain cDNA using RT-PCR, was subcloned into BamHI restriction enzyme site between the CMV promoter and the IRES-GFP sequence of the lentiviral expression vector, pCSC-SP-PW-CMV-IRES/GFP (kindly provided by Dr Inder Verma, Salk Institute for Biological Studies, La Jolla, CA). Lentiviruses were produced as described above.

**In vitro validation of Dnmt3aKD lentiviral vectors**

The ability of the shDnmt3a vectors to knock down Dnmt3a mRNA and protein expressions was assessed using RT-PCR and Western blot analysis, respectively.

**RT-PCR.** N2a cells were infected with the five different shDnmt3a lentiviruses. At 72 h after infection, cells were lysed and the RNA extracted using Tri reagent (Beit Haemek Biological Industries) according to the manufacturer’s protocol. RNA was reverse transcribed to cDNA using the High Capacity RNA to cDNA kit (Applied Biosystems). The cDNA was then analyzed using qRT-PCR.

**Western blot.** N2a cells were infected with the shDnmt3a#1 lentiviruses. At 72 h after infection, cells were harvested in lysis buffer [25 mM Tris-HCl, pH 7.4, 150 mM KCl, 1.5 mM MgCl2, 1% (w/v) glycerol, 1% (w/v) NP40] containing protease inhibitors. Cell lysates were subjected to gel electrophoresis on 10% SDS-polyacrylamide gel, and separated using the High Capacity RNA to cDNA kit (Applied Biosystems). The cDNA was then analyzed using qRT-PCR.

**In vivo validation of lentiviral vectors**

Brain samples were taken from Dnmt3aKD or Dnmt3a1 overexpressing (OE) and control injected mice 14 d after the injection from the mPFC. After removing the brain and placing it on an acrylic 1 mm brain matrix (catalog #51380; Stoelting), 2 mm slices were taken based on designated anatomical markers using standard razor blades (GEM, 62-0165). Blunted syringes of different diameters were used to extract the brain regions from slices removed from the matrix. RNA extraction and RNA reverse transcription were performed, and the relative quantities were assessed using RT-PCR.

**Stereotactic intracranial injections**

Injections were performed as previously described (Sztainberg et al., 2010). Briefly, adult (9-week-old) C57BL/6 male mice (Harlan) received bilateral stereotactic injections of lentivirus into the mPFC (1 μl of lentivirus per hemisphere, 0.1 μl/min). Mice were anesthetized with isoflu-
quantified the mRNA levels of all known Dnmts using RT-PCR in the forebrain centers known to be related to anxiety at week 3 (i.e., after the last day of CSDS) (Fig. 1G–I). We found that Dnmt3a was selectively downregulated in the mPFC (n = 6 or 7, $T_{(11)} = -3.485, p = 0.005$; Fig. 1G) and upregulated in the central amygdala (CeA) (n = 5 or 6, $T_{(9)} = 2.339, p = 0.044$; Fig. 1H). In contrast, CeA-Dnmt3b was significantly downregulated (n = 4–7, $T_{(9)} = -3.970, p = 0.003$; Fig. 1H). Interestingly, no
significant differences in any of the Dnmts’ mRNA levels were observed in the CA1 region of the hippocampus (Fig. 1F) or in the bed nucleus of the stria terminalis (data not shown). We further examined the relative mRNA expression of the different Dnmts in the mPFC. Our results are in line with the literature and indicate significant differences between the groups, where Dnmt1 is the most abundant in this area with a 1.6-fold change relative to Dnmt3a (n = 13, T_{24} = −5.830, p = 0.000, one-way ANOVA followed by Student’s t test; Fig. 1J) and 72-fold change relative to Dnmt3b (n = 13, T_{24} = −44.724, p = 0.000, one-way ANOVA followed by Student’s t test; Fig. 1J). Dnmt3a is the second most abundant with mRNA expression levels that are 45-fold higher relative to Dnmt3b (n = 13, T_{24} = −33.759, p = 0.000, one-way ANOVA followed by Student’s t test; Fig. 1J).

Given the fact that we observed a robust reduction of Dnmt3a within the mPFC of stressed mice, we focused our following experiments on this brain region. We first examined whether the downregulation of mPFC-Dnmt3a observed after CSDS had an effect on the global methylation levels in this area. To that end, we checked global DNA methylation levels in the mPFC of mice, 4 weeks after CSDS. There was a significant reduction in the methylation levels in the mPFC of the CSDS mice (n = 8, T_{14} = −2.140, p = 0.05; Fig. 1K). Dnmt3a has two different splice variants, Dnmt3a1 and Dnmt3a2; we designed primers to distinguish them and checked their baseline relative mRNA expression levels in the mPFC. The results indicated ~1000-fold difference in their expression levels, with Dnmt3a1 being much more abundant in this area (n = 7, T_{12} = −42.011, p = 0.000; Fig. 1M). Next, we checked their mRNA levels in the mPFC following CSDS compared with the unstressed control group. The mPFC-Dnmt3a1 mRNA levels were significantly reduced following CSDS (n = 5 or 6, T_{19} = −3.732, p = 0.005; Fig. 1L), whereas no significant change was detected in the levels of Dnmt3a2.

To explore possible mechanisms by which CSDS regulates Dnmt3a expression, we checked for putative glucocorticoid binding sites in the promoter region of Dnmt3a1. We found a negative glucocorticoid response element sequence 150bp up-stream of the DNMT3A1 transcription start site, in the nucleotide sequence TGCCTCCGGGGCGCT. This negative regulatory site has a very specific matrix, therefore decreasing the possibility of nonspecific binding, and specifically binds NR3C1.

Establishment and validation of shRNA viral constructs for Dnmt3a knockdown
Because we observed robust changes in mPFC-Dnmt3a levels following CSDS, we sought to manipulate the endogenous levels of Dnmt3a specifically in the mPFC of adult mice and check its effects on anxiety-like behavior. To this end, we established lentiviruses expressing shDnmt3a specifically in the mPFC of adult mice and check its effects on anxiety-like behavior. To this end, we established len-tiviruses targeting the Dnmt3a transcript (Fig. 2A, B). To evaluate the efficiency of 5 different shDnmt3a constructs in reducing Dnmt3a mRNA expression, the mouse neuroblastoma cell line N2a was infected with the different shDnmt3a viral constructs or scrambled control. We compared the Dnmt3a mRNA expression level of each shDnmt3a-infected N2a cell to control infected cells. Four constructs significantly decreased the mRNA expression levels of Dnmt3a (Fig. 2C), whereas shRNA#1 (n = 4, T_{18} = 5.962, p = 0.009, one-way ANOVA followed by Student’s t test) induced a 45% reduction in Dnmt3a mRNA expression levels. We further analyzed the efficiency of shDnmt3a#1 to knock down the levels of Dnmt3a by comparing Dnmt3a protein levels in N2a cells infected with shDnmt3a#1 to cells infected with scrambled control viruses (Fig. 2D). Western blot analysis revealed a significant reduction in Dnmt3a protein levels in the shDnmt3a#1-infected cells by ~50% (n = 4, T_{6} = 3.496, p = 0.013; Fig. 2E).

Knocking down Dnmt3a in the mPFC increases anxiety-like behavior
After validation of the virus, adult mice were injected bilaterally with either shDnmt3a virus (KD group) or control viruses into the mPFC. To test the effects of Dnmt3a knockdown on anxiety-like behavior, we examined performance in the EPM. Mice that received injections with shRNA-Dnmt3a in the mPFC showed a significant reduction in the time spent in the open arms (n = 10 or 11, T_{19} = 2.524, p = 0.021; Fig. 3A) and in the total distance traveled in the open arms (n = 10 or 11, U = 18.00, p = 0.009, Mann–Whitney U test; Fig. 3B). We also checked anxiety levels in another established anxiety test, the DLT test. In accordance with the EPM results, there was a significant reduction in the duration of time spent in the light (n = 20 or 21, T_{39} = 2.201, p = 0.034; Fig. 3E), total distance traveled in the light (n = 20 or 21, T_{39} = 2.631, p = 0.012; Fig. 3F), and in the number of visits to the light area (n = 20 or 21, T_{39} = 2.868, p = 0.033; Fig. 3G) relative to control virus-injected mice. To exclude behavioral changes associated with locomotor deficits, locomotor activity was evaluated across the circadian cycle. Comparing the Dnmt3a KD with the control group (using one-way ANOVA with repeated measures) revealed no significant difference between the groups (Fig. 3H, I). The results of the behavioral tests showed that knocking down Dnmt3a in the mPFC is sufficient to mimic the increase in anxiety-like behavior following CSDS, which suggests that Dnmt3a in the mPFC plays a key role in mediating anxiety-like behavior.

mPFC-Dnmt3a1 overexpression reduces baseline anxiety in adult mice
As a complementary experiment, we used genetic tools to selectively overexpress Dnmt3a1 in the mPFC and investigate its effect on baseline anxiety levels of adult mice. To establish a Dnmt3a1 OE lentivirus, the mouse Dnmt3a1 sequence was amplified and cloned into a GFP containing lentiviral vector, and lentiviruses were produced (Fig. 4A). To determine the efficiency of these viruses to overexpress Dnmt3a1, we compared the relative mRNA expression of Dnmt3a in the mPFC of mice injected with Dnmt3a OE lentivirus to mice injected with the control virus. Micro-punching of mPFC was performed 3 weeks after injection, and mRNA levels were determined using qRT-PCR. This revealed a significant increase in mPFC-Dnmt3a
mRNA expression in mice injected with Dnmt3a OE lentivirus

LaPlant et al., 2010,
Oliveira et al., 2012)

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Restoring the levels of Dnmt3a in the mPFC reverses the increase in anxiety-like behavior in CSDS mice

The potential of modulating Dnmts to “rescue” behavioral abnormalities has been previously proposed (LaPlant et al., 2010, Oliveira et al., 2012). We now report that there is a reduction in mPFC-Dnmt3a1 levels following CSDS. Additionally, we show that genetic manipulation of mPFC-Dnmt3a levels has a robust effect on anxiety levels of adult mice. Considering these data, we
hypothesized that restoring the mPFC-Dnmt3a1 levels of CSDS mice may prevent the increase in their anxiety-like behavior. Therefore, we designed an experiment where mice were subjected to 10 d of CSDS and then were bilaterally injected with either Dnmt3a1 OE viruses or control viruses (n = 12 per group) specifically into the mPFC. Following 2 weeks of recovery, the mice were tested for anxiety-like behavior (Fig. 5A). The results of the behavioral tests were quite conclusive, showing a clear reduction in the anxiety-like behavior of the CSDS mice that were injected with Dnmt3a1 OE virus. In the EPM test, the CSDS-Dnmt3a1 OE group showed reduced anxiety-like behavior exhibited by a significant increase in the time spent in the open arms (n = 11, U =

Figure 3. Increase in anxiety-like behavior in mice injected with shDnmt3a (Dnmt3a KD) construct in the mPFC. Dnmt3a KD mice showed significant reduction in the (A) total time spent in and (B) total distance traveled in but (C) made a similar number of entries into the open arms of the EPM compared with control mice. D, Representative activity traces from control and Dnmt3a KD in the EPM. C, Closed arm; O, open arm. n = 10 or 11 (Mann–Whitney U test). Dnmt3a KD mice showed a significant reduction in the (D) time spent in and (E) distance traveled in and (G) made fewer entries to the light compartment in the DLT test. n = 20 or 21 (Mann–Whitney U test). H, Dnmt3a KD mice exhibit similar locomotor activity levels as control throughout the entire circadian cycle. I, Total activity during dark and light cycles. n = 9 (one-way ANOVA with repeated measures). *p < 0.05, Dnmt3a KD mice versus controls. **p < 0.01, Dnmt3a KD mice versus controls. Data are mean ± SEM.
25.00, p = 0.035; Fig. 5B) and in the total distance traveled in the open arms (n = 11, U = 24.50, p = 0.032, Mann–Whitney U test; Fig. 5C). Similarly, the DLT test found significant differences between the CSDS-Dnmt3a1 OE and the CSDS-control group in the duration of time spent in the light (n = 11, T_{20} = -2.726, p = 0.013; Fig. 5E) and in the total distance traveled in the light (n = 11, T_{20} = -2.708, p = 0.014; Fig. 5F). No changes were observed in the motor activity of mice injected with Dnmt3a1 OE or control viruses (Fig. 5H, I). These behavioral results infer that restoring the levels of mPFC-Dnmt3a1 in stressed mice has an anxiolytic effect.

**Discussion**

Our results suggest that Dnmt3a in the mPFC has a functional role in anxiety-like behavior and that the regulation of this gene may serve as a possible molecular link between chronic stress and the development of anxious behavior. In our initial experiments, we demonstrated that CSDS induced a persistent reduction in the expression of mPFC-Dnmt3a1 that coincides with a reduction in the global methylation levels and with prolonged elevated anxious behavior. Considering the anatomical complexity of both the amygdala and PFC, it is highly possible that DNMTs are
differentially regulated in subregions of these areas, therefore complicating our efforts to detect changes when sampling the whole region. Therefore, we specifically probed the CeA, which is a major output nucleus of the amygdala, and the mPFC, which has a high level of connectivity to the amygdala. Examining expression levels of different Dnmts across the limbic system showed that the effect of chronic stress on Dnmts’ expression is brain region specific and variable between the different Dnmts. Glucocorticoid secretion is a prominent part of the stress response. One possible mechanism by which stress might regulate Dnmt3a expression may involve the modulation of the binding transcription factor, glucocorticoid receptor (NR3C1), to a negative glucocorticoid response element found 150 bp upstream of the Dnmt3a transcription start site (Surjit et al., 2011). However, additional promoter analysis studies are needed to confirm the importance of this negative glucocorticoid response element for Dnmt3a expression levels.

In our functional experiments, we showed that knocking down mPFC-Dnmt3a in naive mice induced anxiety-like behavior, similarly to the effect of CSDS. Conversely, overexpressing this gene in the PFC had an anxiolytic effect. Our observations support an increasing line of evidence that dysregulation of Dnmts is causative of stress-induced behaviors and cognitive deficits. In a previous study, mice subjected to the CSDS procedure showed increased Dnmt3a expression levels in the NAc that was accompanied by increased depressive-like behavior (LaPlant et al., 2010). Additionally, impaired hippocampal-related memory tasks in aged mice were linked to reduced hippocampal Dnmt3a2 levels (Oliveira et al., 2012). Moreover, the importance of Dnmts was demonstrated in memory formation by using Dnmt1 and Dnmt3a double KO mice (Feng et al., 2010) and Dnmt inhibitors (Miller et al., 2010). Human studies have also suggested a role for Dnmts in psychopathologies. A postmortem study showed significant alterations in Dnmts levels in the frontopolar cortex of depressed suicide victims (Poulter et al., 2008). Expression of Dnmts was also found in the peripheral blood of patients suffering from major depressive disorder, which was not apparent when the same patients were in a remissive state (Higuchi et al., 2011).

Brain imaging and postmortem studies have reported lower PFC volumes in subjects with stress-related psychopathologies. Studies using animal models of chronic stress have shown a re-
duction in the number of dendritic spines and function of neurons in the PFC (Radley et al., 2006; Liu and Aghajanian, 2008). Thus, chronic stress decreases neuronal activity in the PFC, weakening the structures that provide negative feedback in the stress response. In our results, stress induced a decrease in Dnmt3a levels in the mPFC, whereas overexpression of Dnmt3a1 precipitated an increased ability to withstand stress-induced anxiety. Therefore, Dnmt3a appears to positively affect the ability of the mPFC to fulfill its role in the context of stress-induced behaviors. This finding is supported by previous reports of Dnmt3a expression appearing to positively correlate with increased activity in that brain region. For example, fear conditioning increases Dnmt3a expression in the hippocampus, and inhibition of Dnmts inhibits hippocampal-dependent fear learning in mice. In another notable study, an increase of Dnmt3a expression in the NAc was observed following chronic social stress (LaPlant et al., 2010). The same research group previously showed that the NAc is overactivated following CSDS (Berton et al., 2006). At the cellular level, Dnmt3a overexpression increased spinal density in the NAc, and inhibition of Dnmts in cell cultures inhibited long-term potentiation. Contrary to the PFC, the amygdala increases in volume under chronic stress and studies in rodents have shown that prolonged stress causes dendritic hypertrophy in the amygdala (Vyas et al., 2002), which leads to hyperactivity in this brain region. In support of our hypothesis, we observed increased expression of Dnmt3a in the CeA following CSDS.

While previous evidence indicates that Dnmt3a regulates spinal density, long-term potentiation, and trauma-induced behaviors, we still do not appreciate how Dnmt3a performs these functions at the level of regulation of gene transcription. Several lines of evidence have suggested a possible link between modification in DNA methylation patterns of particular genes to neuropsychiatric disorders in humans and in animal models for depression and anxiety disorders (Weaver et al., 2004; Oberlander and Weinberg, 2008; McGowan et al., 2009; Murgatroyd et al., 2009; Elliott et al., 2010; Keller et al., 2010; Uchida et al., 2011). However, most of these studies have concentrated on DNA methylation of genes that are known to be central to stress-related behaviors, and not particularly involved in synaptic or electrophysiological properties. Therefore, it is not clear how Dnmt3a may help to promote the function of specific brain regions or increase synaptic function. Dnmt3a regulation of gene expression is rather complex; and although binding to the promoter region can lead to gene silencing, Dnmt3a binding to non-promoter regions of a gene promotes gene expression (Wu et al., 2010). We speculate that the effect of prolonged stress on synaptic plasticity, as demonstrated by hypotrophy in the mPFC (Radley et al., 2004) and hypertrophy in the NAc (LaPlant et al., 2010), is partly mediated through Dnmt3a modification on the expression of a subset of synaptic genes. Therefore, it is possible that the Dnmt3a-induced resiliency to stress-induced anxiety is mediated through the increased expression of synaptic genes in the PFC; however, this hypothesis needs experimental investigation in future studies.

Our study suggests that the effects of stress-induced anxiety can be mediated through the modulation of Dnmt3a expression. Because Dnmt3a overexpression in the mPFC was able to attenuate stress-induced anxiety, we propose that Dnmt3a is a primary enzyme involved in the stress response, and continued experimentation into Dnmt inhibition and overexpression in various brain regions is highly warranted.

References


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