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Histone Lysine Demethylases of JMJD2 or KDM4 Family are Important Epigenetic Regulators in Reward Circuitry in the Etiopathology of Depression

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Major depressive disorder (MDD) is debilitating mental illness and is one of the leading contributors to global burden of disease, but unfortunately newer and better drugs are not forthcoming. The reason is lack of complete understanding of molecular mechanisms underlying the development of this disorder. Recent research shows dysregulation in epigenetic regulatory mechanisms, particularly the transcriptionally repressive di- and tri-methylation of histone 3 lysine 9 (H3K9me2/me3) in nucleus accumbens (NAc), a critical region of the reward pathway involved in the development of anhedonia, the hallmark of depression. However, the role of histone lysine demethylases, which can remove methylation from H3K9, in particular Jumonji domain containing demethylases 2 or Jmjd2 family, has not been studied. Using social defeat stress-induced mouse model of depression, this study uncovered that transcripts of most of the Jmjd2 members were unchanged after 5 days of defeat during the onset of depression, but were downregulated after 10 days of defeat in full-blown depression. Blocking the Jumonji domain containing demethylases by chronic administration of inhibitors dimethyloxalylglycine (DMOG) and ML324 resulted in depression-like phenotype even in absence of stress exposure, which was associated with an increase in transcriptionally repressive epigenetic marks H3K9me2/me3 in NAc, causing altered neuroplastic changes as reported in NAc in depression models. Thus, we report for the first time that Jmjd2 class demethylases are critical epigenetic regulators involved in etiopathology of depression and related disorders and activation of these demethylases can be a good strategy in the treatment of MDD and related psychiatric disorders. *Neuropsychopharmacology* (2017) **42,** 854–863; doi:10.1038/npp.2016.231; published online 26 October 2016

INTRODUCTION

Major depressive disorder is known to be one of the most widespread illnesses that are likely to be foremost in the global burden of diseases by 2020 (WHO, 2002). Depression and related mood disorders take huge toll on society both in terms of economic and societal parameters (Holtzheimer and Mayberg, 2011). The depressive disorder comprises of multiple subtypes and is considered to be heterogeneous syndrome characterized by diverse symptoms such as anhedonia (reduced ability to experience pleasure from natural rewards); alterations in sleep patterns, appetite, weight, and libido; feelings of despair, hopelessness, worthlessness, and guilt; inability to think or concentrate; decreased energy and fatigue; and recurrent thoughts of death and suicide (Kessler *et al*, 2005; Krishnan and Nestler, 2008; Nestler *et al*, 2002). Despite intense molecular research in past several decades in this field there is dearth of newer and better medication and the reason appears to be incomplete understanding of the molecular mechanisms in etiopathology of depression and related mood disorders (Massart *et al*, 2012). Recently role of epigenetic regulatory mechanisms, in particular histone methylation has been implicated in the etiology of depression in studies using mouse models and post-mortem human brain (Akbarian and Huang, 2009; Sun *et al*, 2012a; Tsankova *et al*, 2007; Vialou *et al*, 2013). Studies on mouse models of stress-induced depression (Tsankova *et al*, 2006; Wilkinson *et al*, 2009) and drug-induced stress vulnerability (Covington *et al*, 2011) have shown that changes in histone methylation may occur globally or at few critical genes resulting in the disease phenotype (Covington *et al*, 2011; Tsankova *et al*, 2006; Wilkinson *et al*, 2009). Aggressive confrontations during social defeat stress result in profound changes in the neural activity via epigenomic and genomic actions in the ventral striatum or nucleus accumbens (NAc) (Ikemoto and Panksepp, 1999; Nestler and Carlezon, 2006; Wise, 2004), the critical neural substrate of the reward pathway which mediates anhedonia, anxiety and reduced motivations, which are the key symptoms known to occur in patients of depression and related disorders (Carlezon Jr and Thomas, 2009; Newton *et al*, 2002; Wise, 1982). It has been shown that manipulation of NAc through cellular and molecular methods

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exerts various effects in mouse models of depression such as social defeat (Berton *et al*, 2006; Wilkinson *et al*, 2009). Social defeat induces lasting neural adaptations that can be seen even after months of the termination of stress events and are caused by dysregulation of few epigenetic regulators in NAc (Krishnan *et al*, 2007). It has been shown recently that deleting a methyltransferase Kmt2a in neurons of NAc increases anxiety of mice (Shen *et al*, 2016). Environmental factors such as stress appears to recruit epigenetic and transcription regulatory machineries via various inter/intracellular signaling pathways in neural cells leading to differential expression of such critical regulators of gene expression.

A change in transcriptionally repressive methylation, histone H3 dimethylation at K9 and K27 (H3K9me2 and H3K27me2) was found on many gene promoters in the NAc of susceptible mouse that underwent social defeat for 10 days (Wilkinson *et al*, 2009). Interestingly, chronic treatment with an antidepressant imipramine resulted in reversal of depression-like phenotype which also correlated with the restoration of this altered repressive methylation level on most of the gene promoters (Wilkinson *et al*, 2009). Chronic social defeat stress for 10 days has also been shown to downregulate the expression level of several histone lysine methyl transferases (KMTs) such as G9a, Glp, and Suv39h1 that act on H3K9me2/3 and also of a co-repressor CoREST in NAc of defeated mice, whereas the transcript level of these KMTs were found upregulated in resilient mice (Covington *et al*, 2011). Interestingly, somewhat similar results were also observed in the NAc of depressed human subjects (Covington *et al*, 2011). Thus, it is evident that these transcriptionally repressive histone methylations (H3K9me2/me3) have important role in the etiopathology of depression. This lysine H3K9 is also under the control of another class of epigenetic regulators, known as erasers that act to remove the repressive methyl marks. This study was undertaken to uncover the role of Jumonji domain containing histone lysine demethylases 2 family or Jmjd2 family (also known as Kdm4 family), in reward circuitry in depression and related affective disorders, which has not been investigated so far. Most of the earlier studies that used social defeat model explored the epigenetic changes in reward circuit only in the advanced stage of depressive disorder, ie, at the end of day 10 of repeated defeat episodes. Here, for the first time we investigated the role of Jmjd2 family during the onset of depression and development of its etiopathology using a brief 5 days social defeat paradigm, in addition to the most prevalent stress protocol of 10 days. Once we uncovered that most of the members of this Jmjd2 class are dysregulated in NAc, we subjected the mice repeatedly to chronic systemic administration of dimethyloxalylglycine (DMOG), a cell permeable competitive inhibitor of Fe (II), 2-oxoglutarate (2OG)-dependent histone lysine demethylases (Ponnaluri *et al*, 2011). The mice upon DMOG treatment developed social aversion and showed high immobility in forced swim test (FST), measures of depression-like phenotype in rodents. This was associated with an increase in transcriptionally repressive H3K9me2 and H3K9me3 in NAc. We also validated this finding by administrating another inhibitor of Jmjd2 family, ML324, and found an increase in the repressive histone methylation in NAc and the development of depressive phenotype. So, our study for the first time shows the critical role few of JMJD2 or KDM4 family of histone lysine demethylases play in the reward circuit in the development as well as maintenance of depression-like condition in mice.

MATERIALS AND METHODS

Animals

C57bl/6 mice (males, 8–10 weeks old), originally purchased from Charles River Laboratories and bred in the institutional animal house were used in this study. Retired breeder male CD1 mice were used as aggressors for the social defeat paradigm. All animal procedures were carried out in 'accordance' with the approved guidelines of the Institutional Animal Ethics Committee.

Social Defeat

C57bl/6 mice were subjected to a chronic social defeat paradigm as described in earlier reports (Krishnan *et al*, 2007; Veeraiah *et al*, 2014) with minor modification such as 5 min of encounter, instead of 10 min, daily. In brief, an experimental mouse (C57bl/6) was allowed to face social defeat for 5 min daily and then kept in the other half of the resident home cage with only sensory contact but without physical contact for next 24 h. The same procedure was repeated daily for 10 consecutive days with a different aggressor mouse each time, to minimize inter-aggressor variability. Control mice were also housed on either side of the similar divided cages and were allowed to interact with each other daily for 5 min, upto 10 days. Unlike earlier studies where mice are subjected to 10 days defeat stress, we also included a 5 days defeat stress paradigm in this study. After 24 h of the last defeat, the mice were subjected to behavior tests such as social interaction and elevated plus maze test before brain tissues were collected half an hour after the last behavior test.

Social Interaction (SI) Test

In this behavioral test, we measured the interaction/avoidance of the subject mouse with an unfamiliar aggressor according to previously reported protocols (Krishnan *et al*, 2007; Veeraiah *et al*, 2014), which is known to give us the measure of reduced motivation, a hallmark of depression. Briefly, the time spent by the subject C57bl/6 mouse in the interaction zone was measured in the presence and absence of the target CD1 mouse using Ethovision 3.1 (Noldus, The Netherlands) and the SI ratio was calculated.

Sucrose Preference Test

Sucrose preference test was performed as reported earlier (Veeraiah *et al*, 2014). Briefly, in a two-bottle choice between 2% sucrose solution and water the sucrose preference was analyzed by measuring average sucrose consumed by the animal over the last 3 days of testing. The attenuation in sucrose preference gives the measure of anhedonia, a hallmark characteristic of depression. After both these tests the data were analyzed and the mice subjected to social defeat showing an SI ratio <100 and a decrease in the sucrose preference by $<65\%$ were considered depressed

(termed 'susceptible' now onwards), and the rest were considered as 'resilient'.

Elevated Plus Maze Test

The mice were tested for their anxiety levels in an elevated plus maze as reported earlier (Krishnan *et al*, 2007). Briefly, the mouse was placed at the center of an elevated plus maze and the time spent in closed and open arms as well as the number of explorations of open and closed arms were determined by video tracking software (Ethovision 3.1 software, Noldus, The Netherlands).

Forced Swim Test

The FST was performed according to previously published protocols (LaPlant *et al*, 2010). Mice were tested in a 10-L Pyrex glass beaker, filled upto ~23 cm with normal water having a temperature of 25 ± 2 °C, for 5 min. The entire swimming test session was recorded with a video camera and then was scored manually for the time spent immobile. Total immobility was measured as the time spent without noticeable movement, except for single-limb paddling to maintain flotation.

Brain Tissue Collection

Twenty-four hours after the last stress event, animals were rapidly decapitated, and brains were removed and placed on chilled brain matrix submerged in cold, sterile PBS. Dissections of NAc and caudate putamen (bilateral) were taken with a 12-gauge needle punch from 1 mm slices with the help of brain matrix (ZIVIC Instruments, USA) and quickly frozen in liquid nitrogen and stored at − 80 °C until RNA/protein was extracted. From appropriate slices, four punches of both the area were used.

RNA Isolation and Gene Expression

RNA was isolated using Purelink RNA mini kit (Ambion) according to manufacturer's instructions. Equal amounts of RNA from each sample were processed for cDNA synthesis using Superscript reverse transcriptase III (Invitrogen). Quantitative real-time PCR was performed in triplicate by using SYBR Green PCR Master Mix Detection System (Applied Biosystems). The mRNA level of genes studied in each sample was normalized by the level of mRNA expression of the housekeeping gene Gapdh. Relative gene expression analysis (ΔΔCt method) was performed for genes that code for Jmjd (2a, 2b, 2c and 2d). The sequence of primers used in the study is in Supplementary Material.

Drug Treatment

Dimethyloxalylglycine (cat #D1070, Frontier Scientific Inc.) was purchased, dissolved in saline, and injected intraperitoneally in mice at a dosage of 40 mg/kg body weight, once daily in forenoon for 5 consecutive days. The mice were subjected to behavior tests on the sixth day. ML324 (cat # 17472) and Jib-04 (cat # 15338) were purchased from Cayman Chemical. Both these drugs were dissolved initially in DMSO (10 mg/ml), then diluted in saline and injected intraperitoneally in mice at a dosage of 30 mg/kg body weight, once daily in forenoon for 5 consecutive days. The animals were subjected to behavior tests on the sixth day and brain regions were dissected out on the seventh day.

Golgi-Cox Staining and Structural Analysis of Medium Spiny Neurons of NAc

Golgi-Cox staining was done as described by us in recently published report (Chakravarty *et al*, 2015). The cryoprotected brain was sectioned (70 μm) using vibratome (OTS--4500, Harvard Apparatus, USA) through striatal region and processed for silver staining and mounted on slide. For analyses, 15 dendritic segments (50 μm each) were randomly selected from each group and measured for spine density using Image J software.

Immunoblotting

Brain tissue punches (four punches per mouse) were lysed, sonicated, and centrifuged in lysis buffer, following which 40 μg of supernatant protein was electrophoresed on 12% SDS gel. Following transfer, PVDF membranes were blocked in 5% w/v BSA, incubated with appropriate antibodies (anti-Jmjd2a (Abcam mouse monoclonal, 1:250); anti-Jmjd2c (Abcam, rabbit polyclonal, 1:250); anti-H3K9me2 (Abcam mouse monoclonal, 1:500); anti-H3K36me2 (Abcam rabbit polyclonal, 1:500); anti-H3K9me3 (Abcam rabbit polyclonal, 1:500); anti-H3K36me3 (Abcam rabbit polyclonal, 1:500); anti-β-actin (Sigma mouse monoclonal, (1:10,000); and anti-Hif-1α (Abcam mouse monoclonal, 1:1000)) overnight and afterwards probed with peroxidase-labeled secondary antibody (1:5000); the bands were visualized by enhanced chemiluminescence (Vilber Lourmat) and the densitometry performed using ImageJ software.

Statistics

All data obtained were tested for levels of significance by the student's *t*-test. When significant differences were observed, *P*-values for pairwise comparisons (control *vs* susceptible/control *vs* resilient/vehicle *vs* drug) were calculated using the two-tailed *t*-test. P-value < 0.05 was considered significant. The hypothesis was tested at a level of significance of $p < 0.05$* or $p < 0.01$**. All values included in the figure legends represent mean ± SEM (*$P < 0.05$; **$P < 0.01$).

RESULTS

Both 5 and 10 Days of Social Defeat Led to an Attenuation in Social Interaction and Induction of Anhedonia, the Hallmarks of Depression, in Susceptible Mice

We subjected C57bl/6 mice to bouts of defeat daily for 5 min either for 5 or 10 days and analyzed their social avoidance behavior, sucrose consumption, and anxiety levels on the sixth and eleventh day, respectively. In 5 days defeat paradigm, ~70% of defeated mice showed interaction ratios similar to that of non-defeated controls (resilient); however, 30% of defeated mice showed a reduction in interaction ratio (⩽upto 35%), termed as susceptible (Figure 1a and Supplementary Figure 1). On the other hand, in 10 days

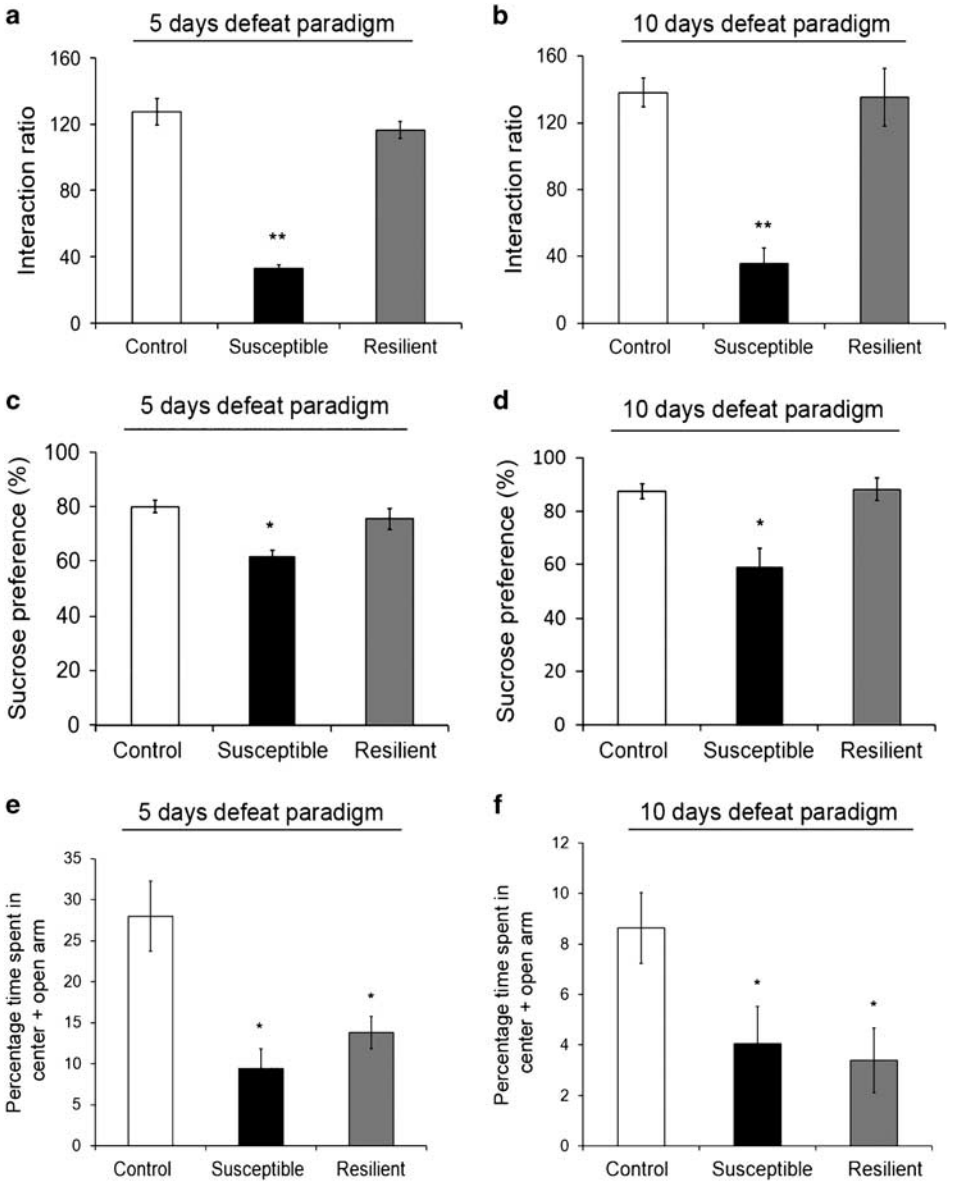

Figure 1 Chronic social defeat paradigms of 5 and 10 days led to induction of anhedonia and reduction in social interaction in susceptible mice. Average interaction ratio after 5 days defeat (a) and 10 days defeat (b), calculated from the time spent in interaction zone in the social interaction test on days 6 and 11, respectively. Only susceptible mice displayed decreased preference for 2% sucrose after 5 days defeat (c) and 10 days defeat (d), while both susceptible and resilient mice displayed increased anxiety, as evident by less time spent in the center plus open area of the elevated pus maze after 5 days defeat (e) and 10 days defeat (f). Bars represent mean ± SE with $n = 6–12$, * indicates significant *post hoc* differences with respect to non-defeated control mice; * ⩽ 0.05, ** ⩽ 0.01 by Student's *t*-test.

defeat paradigm ~40% of defeated mice were found resilient, while 60% of them were found susceptible (Figure 1b). On the basis of the results of social interaction, we segregated the defeated mice into susceptible and resilient groups. Similar results were found in sucrose preference test wherein the sucrose preference in both 5- and 10-days defeat paradigm decreased for susceptible group, while it remain unchanged for resilient group (Figure 1c and d). Although the resilient mice did not show behavioral phenotype of depression, they did show signs of exposure to chronic stress in terms of increase in anxiety in both the 5- and 10-days defeat paradigms in the elevated plus maze test (Figure 1e and f). Earlier studies have shown that 10 day of defeat episodes could successfully induce depression and related mood disorder such as anxiety in C57bl/6 mice (Krishnan *et al*, 2007; Tsankova *et al*, 2006). We report here for the first time that defeat stress induces anhedonia and social aversion as early as sixth day of the defeat paradigm, ie, after 5 days of chronic stress exposures. Social defeat led to the development of anxiety in both 5- and 10-days paradigms in all the defeated (susceptible and resilient) mice.

The Induction of Depression-like Phenotype was Associated with Transcriptional Dysregulation of a Number of Jmjd2 Members as Well as Few KMTs in NAc

Chronic aversive stimulus such as defeat stress leads to dysregulation of many genes in NAc by epigenomic actions

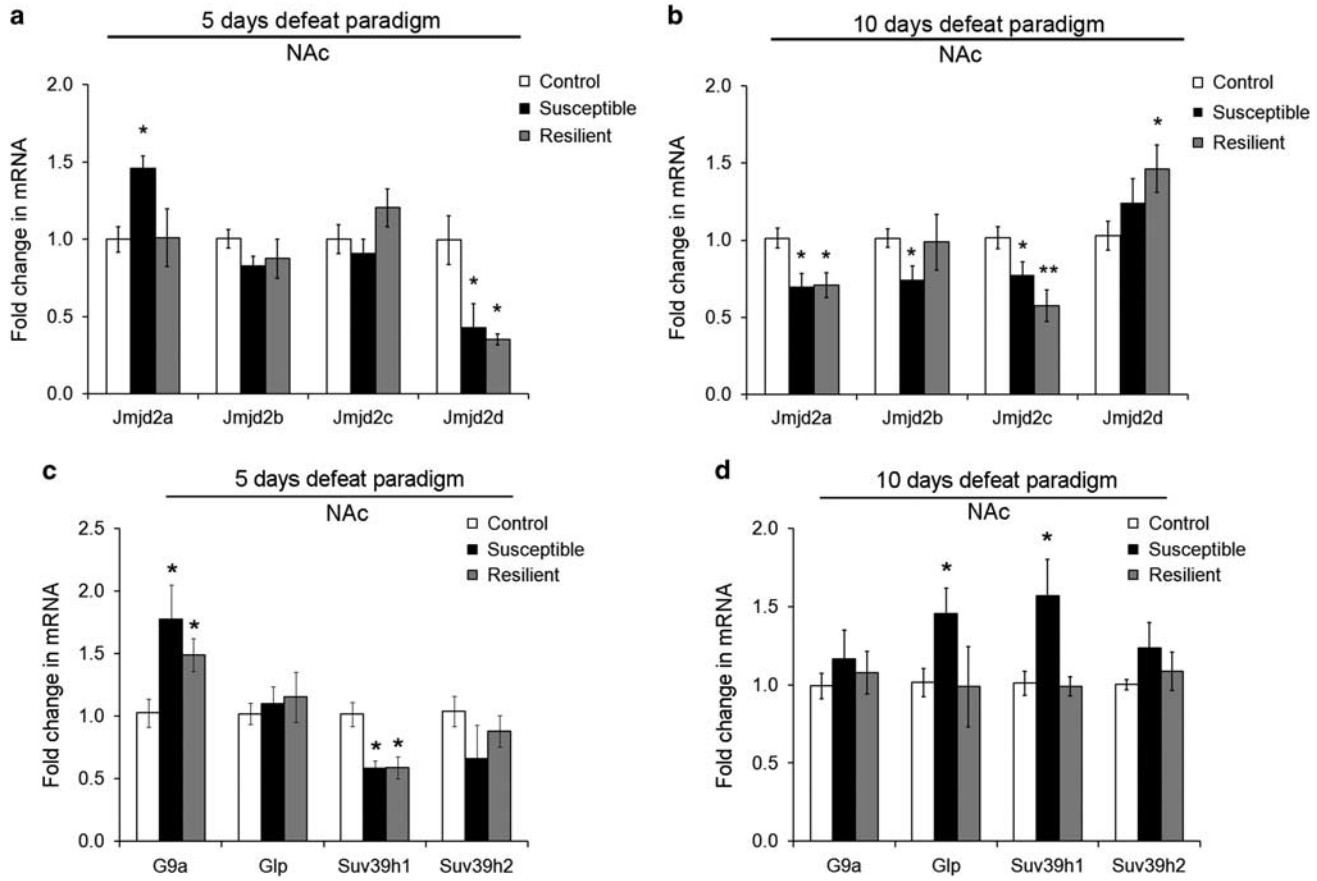

Figure 2 Chronic social defeat for 5 and 10 days led to dysregulation of Jmjd2 family of demethylases and lysine methyl transferases in NAc. mRNA expression profiles of Jmjd2 family of histone lysine demethylases in NAc after 5 days (a) and 10 days of defeat (b) show that most of the demethylases were unchanged in case of 5 days while they were downregulated after 10 days of defeat. mRNA expression of methyl transferases in NAc that act on the transcriptionally repressive mark H3K9, critically implicated in depression and mood disorders, were checked after the defeat episodes in both paradigms, 5 days (c) and 10 days (d). Bars represent mean ± SE with $n = 6–12$, * indicates significant *post hoc* differences with respect to non-defeated control mice; * ⩽ 0.05, ** ⩽ 0.01 by Student's *t*-test.

such as regulation of H3K9me2 (Chakravarty *et al*, 2014; Wilkinson *et al*, 2009). So, we looked into the mRNA expression levels of histone demethylases of the Jmjd2 family that are responsible for the demethylation of H3K9, using real-time quantitative PCR in NAc. The analysis of expression profiles showed that few demethylases were dysregulated after 5 days of defeat, while most others were downregulated following 10 days of defeat (Figure 2a and b). Jmjd2a was differentially regulated of susceptible and resilient mice on the sixth day, upregulated in susceptible mice, while down-regulated in both the groups on the eleventh day (Figure 2a and b). Jmjd2b and Jmjd2c did not change on sixth day but were downregulated on the eleventh day in susceptible populations. Jmjd2d was downregulated initially after 5 days of defeat in both the groups but returned to its normal expression level in susceptible mice following 10 days of defeat, while it increased further in the resilient ones. The most striking finding of the study is that except Jmjd2d, the expression of all other known members of the family Jmjd2a, 2b and 2c were downregulated in depressed mice in full-blown depression-like conditions, ie, on day 11 (Figure 2a and b).

However, this dysregulation of expression levels of Jmjd2 demethylases was specific for NAc. The expression profile in the dorsal striatum or caudate putamen was quite different (see Supplementary Figure 2).

Next we looked for the change in expression of KMTs that regulate H3K9 methylation. The transcript level of G9a, one of the KMTs was significantly upregulated in susceptible as well as resilient mice after 5 days of defeat stress (Figure 2c); however, the G9a level remained unchanged in both the defeated groups on day 11 after 10 days of stress (Figure 2d). There was no change in the expression level of other KMTs that act on H3K9, like Glp and Suv39h2, during the onset of depression and anxiety, ie, after 5 days of defeat (Figure 2c). Suv39h2 does not appear to be involved in chronic stress response in the NAc as it remains unchanged both in 5- and 10-days stress paradigms. In contrast, the mRNA level of Suv39h1, another KMT for H3K9 was the only methylase, which was found to be downregulated in both susceptible and resilient animals after 5 days of defeat episodes (Figure 2c). Interestingly, Suv39h1 was found upregulated only in susceptible mice after 10 days of defeat stress (Figure 2d). Another KMT, Glp that was unchanged in 5 days stress was found upregulated in 10-days paradigm (Figure 2d). So, it appears that the role of these methyl transferases is very different in 10-days and 5-days stress

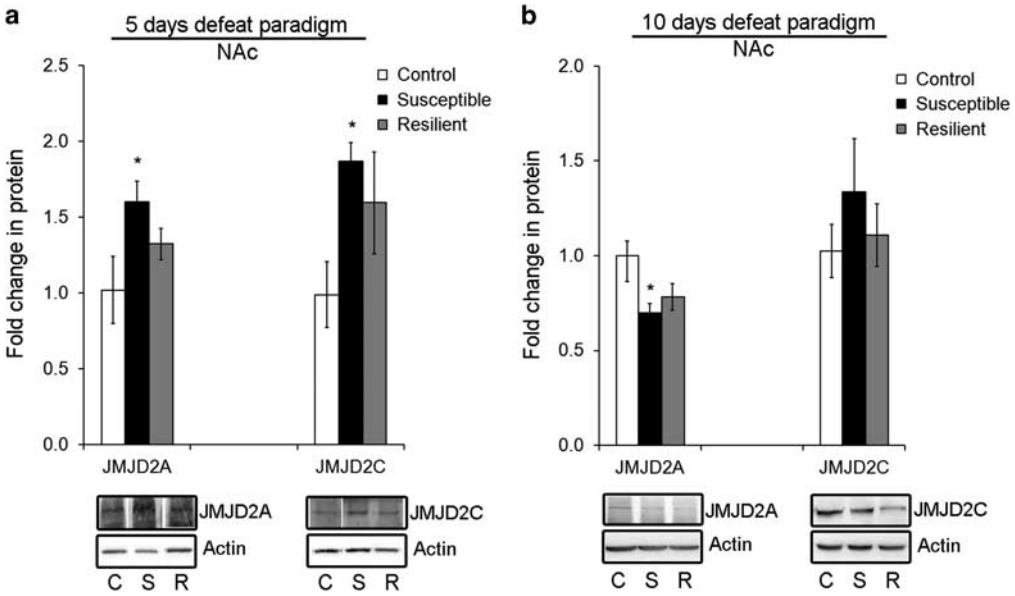

Figure 3 Jmjd2a was differentially regulated in NAc after social defeat at protein level. Jmj2a and Jmjd2c protein levels were assessed by western blot in 5 days defeat (a) and 10 days defeat accumbens samples (b). Bars represent mean ± SE with $n = 4–6$, calculated after densitometry of blots. Representative, cropped blots are displayed below each bar. * indicates significant *post hoc* differences with respect to non-defeated control mice; * $\leqslant 0.05$ by Student's *t*-test.

paradigms. Most of the methylases (G9a in 5-day while Glp and Suv39h1 in 10-day paradigm) that act on H3K9 methylation/s appear upregulated after defeat stress in susceptible or depressed mice and interestingly there is upward trend for the remaining ones (G9a and Suv39h2 in 10-day paradigm) in susceptible ones (Figure 2a and b).

Jmjd2a was Differentially Regulated in Susceptible Population at Protein Level

When differential expression of demethylases was observed at the mRNA level in response to chronic defeat stress, we next checked whether few of these demethylases such as Jmjd2a and Jmjd2c also got dysregulated in NAc at the protein level. The analysis of our western blotting results suggests that the change in JMJD2A protein level in both 5- and 10-days paradigms was similar to that observed at mRNA level, ie, upregulated in 5 days but downregulated in 10 days in susceptible animals (Figure 3a and b). However, Jmjd2c was found differentially regulated at mRNA and protein level in 5-day paradigm (Figure 3a and b). Jmjd2c mRNA was unchanged in 5-day paradigm (Figure 2a) in defeated mice, while JMJD2C protein was upregulated in susceptible animals (Figure 3a). Unlike what we found about JMJD2A protein level in 5-day paradigm (upregulated), in 10 days defeat its level was found attenuated in susceptible mice (Figure 3b). This was similar to what was observed at the mRNA level in 10 days defeat (Figure 2b). In contrast, JMJD2C protein was unchanged after 10 days of repeated defeat stress (Figure 3b), although the Jmjd2c mRNA level was significantly down (Figure 2b). This suggests that some post-transcriptional regulation might also be involved in maintaining the levels of these demethylases in NAc.

The Overall Repressive Histone Lysine Methylation Level in NAc Does not Appear to be Affected in Susceptible Mice After 10 Days of Defeat Unlike that in 5 Days of Defeat

After we found a dysregulation of KDMs and KMTs in response to chronic stress, we measured changes in the global levels of methylation marks H3K9me2, H3K9me3, H3K36me2 and H3K36me3, which are the known substrates of Jmjd2 family. There were significantly high levels of H3K9me2, H3K36me2 and H3K36me3 but not H3K9me3 in susceptible mice, after 5 days of defeat stress (Figure 4a), which correlates with higher level of gene expression of G9a and lower level of expression of Jmjd2d. However, the unchanged global levels of H3K9me2, H3K9me3, H3K36me2 and H3K36me3 of susceptible mice after 10 days of defeat (Figure 4b) do not correlate with the mRNA level change in demethylases (where the transcripts of most Jmjd2 class members were down in susceptible ones) and also with the change in JMJD2A protein level (downregulated) (Figure 3b). So, the discrepancy in the correlation of mRNA level of demethylases and their target protein level in 10-days paradigm could be due to the fact that few of the KMTs that work on H3K9 and H3K36 also appear affected in NAc after social defeat (like Glp and Suv39h1 was significantly up in susceptible ones) and might be involved in restoring the global levels of methylation. Interestingly, H3K9me3 and H3K36me3 level was significantly high in NAc of resilient animals (Figure 4b).

Administration of Inhibitors of Jmjd Class Members Induced Depression-like Phenotype in Mice and Led to Increasing Levels of H3K9me2/me3

Once our study showed that members of Jmjd2 class are downregulated in NAc, we tested the hypothesis that

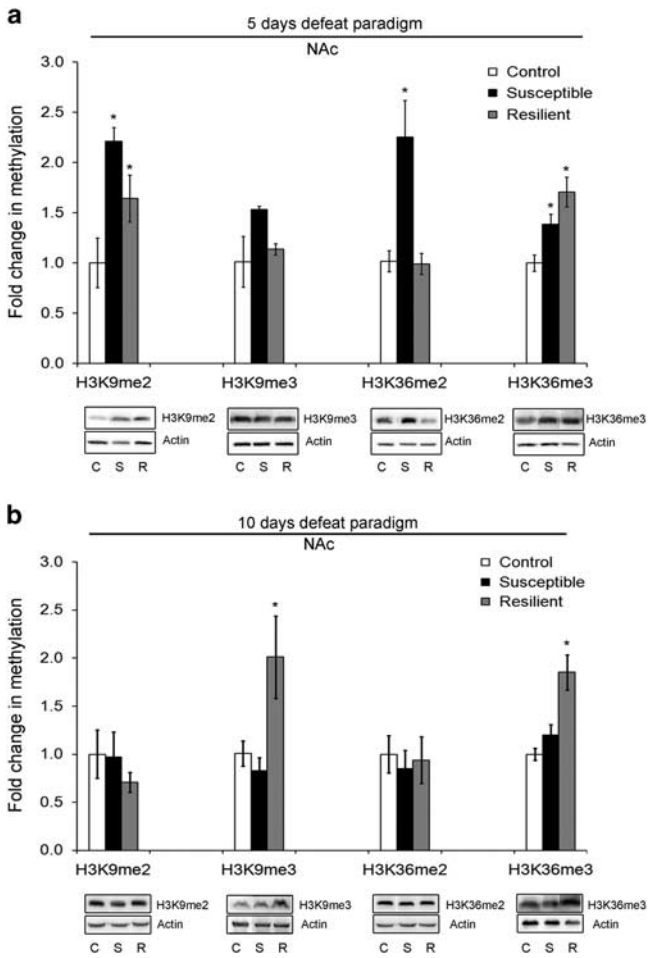

Figure 4 Change in the global levels of transcriptionally repressive histone H3 methylation in NAc of susceptible mice after 5 and 10 days of social defeat stress. H3K9me3/me2 and H3K36me3/me2 protein levels were assessed by western blot in 5 days (a) and 10 days defeat accumbens samples (b). Bars represent mean ± SE with $n = 4–6$, calculated after densitometry of blots. Representative, cropped blots are displayed below each bar. * indicates significant *post hoc* differences with respect to non-defeated control mice; * $\leqslant 0.05$ by Student's *t*-test.

blocking the activity of Jumonji domain containing histone demethylases using DMOG might affect the circuitry negatively. DMOG is a cell permeable competitive inhibitor of Fe (II), 2-oxoglutarate (2OG)-dependent histone lysine demethylases (Ponnaluri *et al*, 2011). Treatment of mice with the inhibitor for 5 days but not just for 1 day, led to the development of depression-like phenotype, as shown by the decrease in social interaction (Figure 5a) and increase in the total immobility time in FST (Figure 5b). So, blocking the activity of Jmjd2 demethylases by the inhibitor led to social aversion as well as behavioral despair in mice, the hallmarks of depression. We checked the overall histone methylation levels in NAc and found that chronic administration of DMOG resulted in significant increase in the repressive epigenetic marks H3K9me2 and H3K9me3 while the activating marks H3K36me2 or H3K36me3 remained unaffected (Figure 5c).

DMOG is a broad competitive inhibitor of prolyl hydroxylases, which includes not only JMJDs but also other important molecules, such as HIF-α prolyl hydroxylase responsible for the stabilization of HIF-1α. Therefore, the Hif-1α protein levels after the DMOG treatment was evaluated in NAc. There was no change in Hif-1α level after the DMOG treatment in NAc (Supplementary Figure 3B). Then, it was also tested whether social defeat stress lead to any change in Hif-1α level. The analysis of immunoblotting results did not show any change in Hif-1α levels after 10 days of stress in susceptible or resilient populations (Supplementary Figure 3A).

It is well known that chronic stress leads to morphological alterations in medium spiny neurons of NAc, which results in imbalanced connectivity between brain regions and development of depression-like phenotype (Christoffel *et al*, 2011b). The depression-like phenotype induced by systemic administration of DMOG for 5 days was similar to that induced by chronic defeat stress in our study. Therefore, we decided to look into the morphology of medium spiny neurons in NAc following chronic administration of DMOG. The brains of DMOG and saline treated mice were processed for the Golgi-Cox staining and the images were quantitatively and qualitatively analyzed. There were significant changes in the morphology of medium spiny neurons in mice that showed depression-like phenotype after the treatment with DMOG (Supplementary Figure 4A). We found visible changes in the morphology of NAc neurons and significantly higher number of spines in DMOG-treated mice (Supplementary Figure 4B and C), compared with that treated with saline, similar to what has been reported in NAc of chronic stress-induced depressed mice (Bessa *et al*, 2013; Christoffel *et al*, 2011a; Taylor *et al*, 2014).

ML324 and Jib-04 are other known inhibitors of Jmjd2 family that do not act on Hif-1α (Rai *et al*, 2013; Wang *et al*, 2013). We have used these inhibitors to ascertain the fact that depressive phenotype induced by DMOG is a methylation-specific effect and not acting through Hif-1α. ML324, but not Jib-04, treatment led to an increase in immobility in the FST (Figure 5d), suggesting induction of despair, the depression-like phenotype. Interestingly, only ML324 led to an increase in the transcriptionally repressive H3K9me2 mark but not Jib-04 (Figure 5e).

DISCUSSION

Exposure to chronic psychosocial stress leads to depression in some individuals but not in all, which is characterized by inhibition to social interaction or social aversion and anhedonia. In social defeat paradigm, when mice are subjected to psychosocial stress by defeat for ~10 days some of them develop depression-like disorder (susceptible ones), while others do not (resilient ones). The development of depression-like phenotype is not clearly understood at molecular level, particularly during the development of the depression-like condition. So, in addition to the typical 10 days of social defeat paradigm prevalent in most of the laboratories we also utilized a brief 5 days sub-maximal defeat stress paradigm, which might be the right window to capture the molecular and epigenetic changes in the reward circuitry of the onset of depression-like phenotype. Unlike 10 days of defeat paradigm where almost 60% of mice develop anhedonia and social aversion, our 5-day paradigm

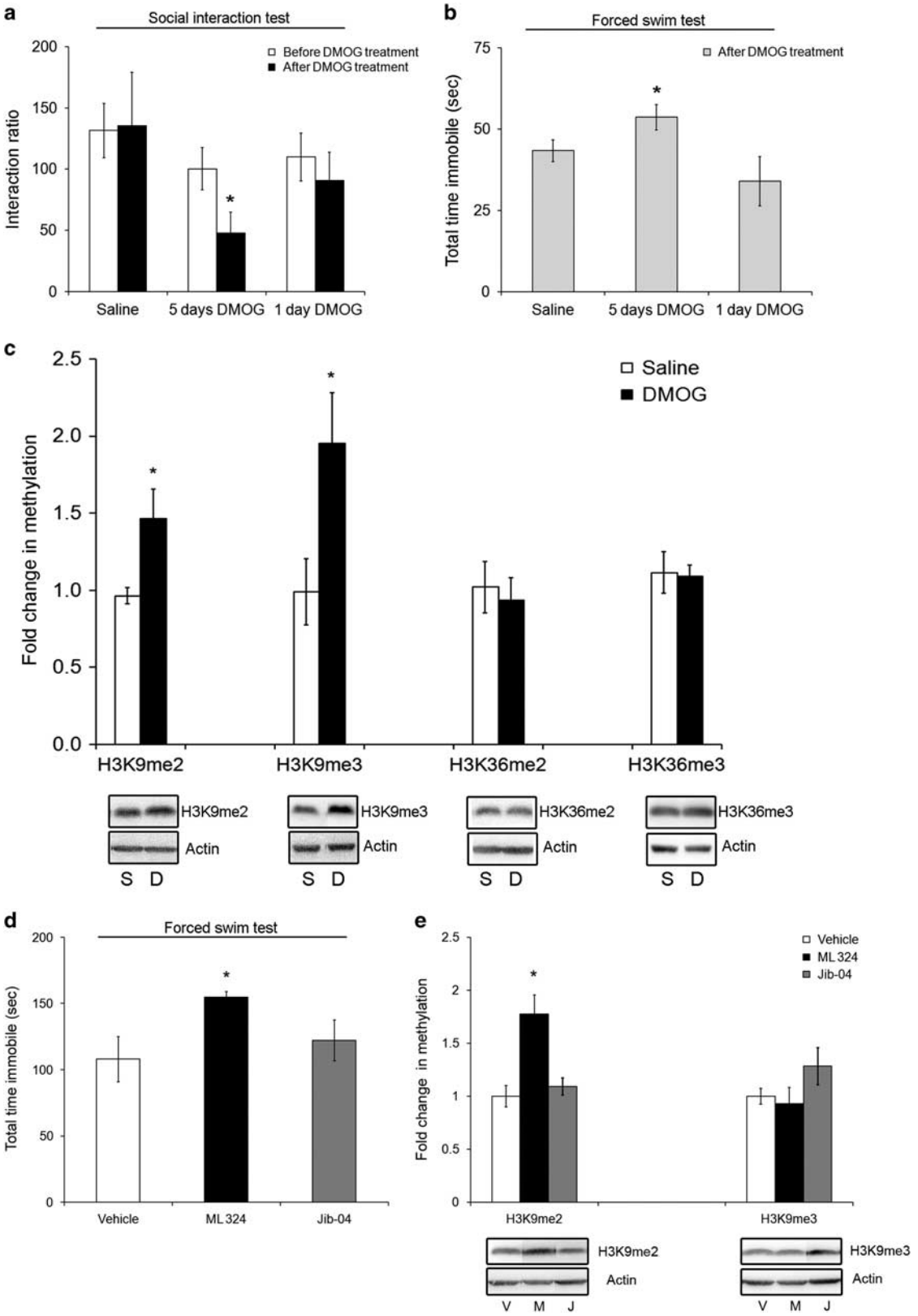

Figure 5 Inhibiting the activity of histone demethylases by pharmacological inhibitors DMOG and ML324 led to depression-like conditions in mice without even subjecting the animals to stress. Mice administered with DMOG displayed significantly less interaction in the social interaction test (a) and increased immobility time (b) in the FST, both signs of depressive behavior. Inhibiting histone demethylases by DMOG led to increase in repressive marks H3K9me3/2 levels in NAc while no change was observed in activating marks H3K36me3/2 (c). Mice administered with ML324, but not Jib-04, displayed significantly increased immobility time (d) in the FST. Inhibiting histone demethylases by ML324, but not Jib-04, led to increase in repressive mark H3K9me2 in NAc (e). Representative, cropped blots are displayed below each bar. Bars represent mean ± SE with $n = 6$ (a–c), $n = 3$ (d, e); * indicates significant *post hoc* differences with respect to saline / vehicle treated mice, * $\leqslant 0.05$ by Student's *t*-test.

results in induction of anhedonia and social aversion, the hallmarks of depression, only in ~40% of defeated mice.

Chronic stressful situations appear to cause molecular changes in various brain circuitries, which in turn induce persistent cellular and morphological changes leading to depression, anxiety and related mood disorders (Christoffel *et al*, 2011b; Krishnan and Nestler, 2008; Nestler *et al*, 2002). Wilkinson *et al*. (2009) showed in social defeat and social isolation induced depression mouse models that repeated stressful events affects the promoter H3K9me2 and H3K27me2 methylation status of many genes in NAc. Here, for the first time we show the evidence of involvement of a class of epigenetic regulators, Jmjd2 family of lysine demethylases, erasers of the methylation marks of H3K9 and H3K36, in the etiopathology of depression-like disorder using social defeat mouse model. We show how the demethylases that act on H3K9 and H3K36 methylation are dysregulated in NAc. Another highlight of this study is the critical involvement of few demethylases such as Jmjd2a and Jmjd2c in the development of anhedonia, the first indication of the depressive disorder phenotype we see in our brief 5-days defeat stress paradigm, thus implicating these epigenetic regulators in the development of depression-like phenotype.

Alteration in transcriptionally repressive histone H3 methylation and changes in few of many lysine methyl transferases (KMTs) in NAc have been shown in addiction (Covington *et al*, 2011; Maze *et al*, 2010; Sun *et al*, 2012b) as well as 10 days of social defeat-induced depression models (Covington *et al*, 2011). We show here for the first time not only the alteration in gene expression of Jmjd2 family of demethylases but also in histone H3 lysine methylation in NAc that underlies at the onset of depression phenotype (ie, in 5-day paradigm) and after the phenotype sets in completely (ie, in 10-day paradigm).

It is also clear from our study that a significant reduction in the expression of lysine demethylases in NAc appears critical in inducing depression-like phenotype, rather than an increase in the expression of methylases (KMTs). This is also supported by the fact that systemic administration of JMJD inhibitor (DMOG) was sufficient to induce depression-like symptoms in mice. Similar outcome (ie, depression) was achieved in animals upon the treatment with ML324 (Rai *et al*, 2013), another known inhibitor of Jmjd2 demethylases. The administration of a pan JMJD inhibitor Jib-04 (Wang *et al*, 2013), however, failed to induce the depressive phenotype. Interestingly, the administration of ML324 but not Jib-04, led to an increase in the transcriptionally repressive epigenetic mark H3K9me2. The failure of Jib-04 to induce the phenotype could be due to its activity on other demethylases apart from Jmjd2 family such as Jarid1A and Jmjd3 (as it is a nonspecific pan JMJD inhibitor). Thus, just the inhibition of demethylase activity in brain over a short period of time (5 days in our study), appears sufficient to induce the development of depression-like phenotype, even without stress exposure. So, ours is the first such study implicating lysine demethylases of JMJD2 or KDM4 family in the etiopathology of depression and related affective disorders. Most of the epigenetics based studies so far have only implicated the role of few of the lysine methylases in depression and related conditions. Thus, our study shows that Jmjd2 family of histone demethylases has a critical role in brain reward circuitry and their dysregulation results in depression-like phenotype.

Behavior of an organism is regulated by synaptic plasticity, which is a mechanism of storage of information in neuronal circuits, neurons, and synapses (Borrelli *et al*, 2008). Synaptic plasticity can be modulated by external stimuli as an individual has to adapt to its constantly changing environment; however, all adaptations are not beneficial. Prolonged psychosocial stress leads to dysregulation in these mechanisms and thus the connectivity between various brain regions is altered resulting in pathological behaviors (Christoffel *et al*, 2011b). There are reports of chronic social defeat stress leading to increased stubby spines in medium spiny neurons of NAc (Christoffel *et al*, 2011a), chronic mild stress leading to hypertrophy of dendritic trees, increased dendritic lengths and increased spine densities in NAc core (Bessa *et al*, 2013), and chronic variable stress resulting in increased dendritic complexity in NAc core (Taylor *et al*, 2014). However, there have not been many reports of direct correlation of histone methylation and dendritic complexity till now, except for this one study where a KMT, G9a when downregulated in NAc leads to decrease in histone methylation and increase in dendritic complexity and enhanced preference to cocaine (Maze *et al*, 2010). Recently it was shown that when a histone demethylase Jmjd2b is deleted specifically in neurons an increase in number of total spines but decrease in the number of mature spines is observed in CA1 region of hippocampus (Fujiwara *et al*, 2016). We report here for the first time that the inhibition of JMJD by DMOG leads to increased repressive histone methylation (H3K9me2 and H3K9me3) in NAc leading to increased spine density in medium spiny neurons. This increase in spine density appears to be pathological as it leads to the depression-like phenotype observed after the inhibition of demethylases. However, there is no direct correlation of increase in methylation levels with increased spine density and development of depressive phenotype. Future studies will be required to address this problem. The insight we got from this study can be exploited for the treatment of depression by boosting Jmjd2 demethylases and thus reversing the transcriptionally repressive H3K9 methylations on genes critically involved in the functioning of the reward circuitry.

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The authors declare no conflict of interest.

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