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Dnmt3a regulates emotional behavior and spine plasticity in the nucleus accumbens

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Despite abundant expression of DNA methyltransferases (Dnmts) in brain, the regulation and behavioral role of DNA methylation remain poorly understood. We found that Dnmt3a expression was regulated in mouse nucleus accumbens (NAc) by chronic cocaine use and chronic social defeat stress. Moreover, NAc-specific manipulations that block DNA methylation potentiated cocaine reward and exerted antidepressant-like effects, whereas NAc-specific Dnmt3a overexpression attenuated cocaine reward and was pro-depressant. On a cellular level, we found that chronic cocaine use selectively increased thin dendritic spines on NAc neurons and that DNA methylation was both necessary and sufficient to mediate these effects. These data establish the importance of Dnmt3a in the NAc in regulating cellular and behavioral plasticity to emotional stimuli.

Chronic cocaine use and chronic social defeat stress alter gene expression, neuronal plasticity and, ultimately, behavior, and chromatin remodeling has been shown to be important for regulating these events in the NAc, an important brain reward region[1–9]. However, previous studies have focused primarily on more labile epigenetic modifications such as histone acetylation and methylation. Given the persistent nature of addiction, one possibility is whether more stable epigenetic modifications, such as DNA methylation, can more persistently influence gene expression in NAc to maintain this behavior.

Despite abundant neuronal expression of Dnmts[10], little is known about the function of DNA methylation in brain. Behavioral studies suggest that DNA methylation is required for hippocampal-dependent memory formation[11,12]. Pharmacological inhibition of DNA methylation blocks long-term potentiation in hippocampus[13], and this effect depends on the activity of both Dnmt1 and Dnmt3a[11]. Moreover, Dnmt inhibition markedly reduces functional synapses formed by cultured hippocampal neurons, as measured by a reduction in mEPSC frequency[14]. Together, these studies suggest that DNA methylation is crucial for memory formation in the hippocampus and associated effects at the synapse.

We examined the NAc to determine whether these concepts regarding DNA methylation extend to drug addiction and depression models. We analyzed the role of DNA methylation in the context of both chronic cocaine (a rewarding stimulus with persistent effects) and chronic social defeat stress (an aversive stimulus with persistent effects) to determine the influence of this lasting epigenetic modification in NAc across a spectrum of complex behaviors in which this brain region is involved. Toward this goal, we identified which specific Dnmts (Dnmt1, Dnmt3a or Dnmt3b) were regulated in chronic cocaine and chronic stress procedures, whether manipulating DNA methylation in NAc affected addictive- and depressive-like behavior, and whether DNA methylation affected synaptic plasticity in NAc.

RESULTS

Transcriptional regulation of Dnmt3a in NAc by cocaine

As a first step in determining the role of DNA methylation in cocaine action, we performed quantitative PCR (qPCR) on NAc tissue of mice treated acutely or chronically (seven daily injections) with cocaine for all known Dnmts and methyl-binding domain proteins (**Fig. 1**). Among these genes, we found that *Dnmt3a* was selectively upregulated at 4 h and downregulated after 24 h following both acute and chronic cocaine administration and we found only limited changes for the other genes that we analyzed (**Fig. 1a** and **Supplementary Fig. 1**). Upregulation of *Dnmt3a* during early withdrawal time points was supported by equivalent findings from a recently published microarray study performed 4 h after 15 chronic cocaine injections[15]. Downregulation of *Dnmt3a* at 24 h was not persistent, as mRNA analysis at 48 h after the last chronic injection showed no change from control values (data not shown). Together, these data support the notion that *Dnmt3a* expression in NAc is biphasically regulated in response to each cocaine

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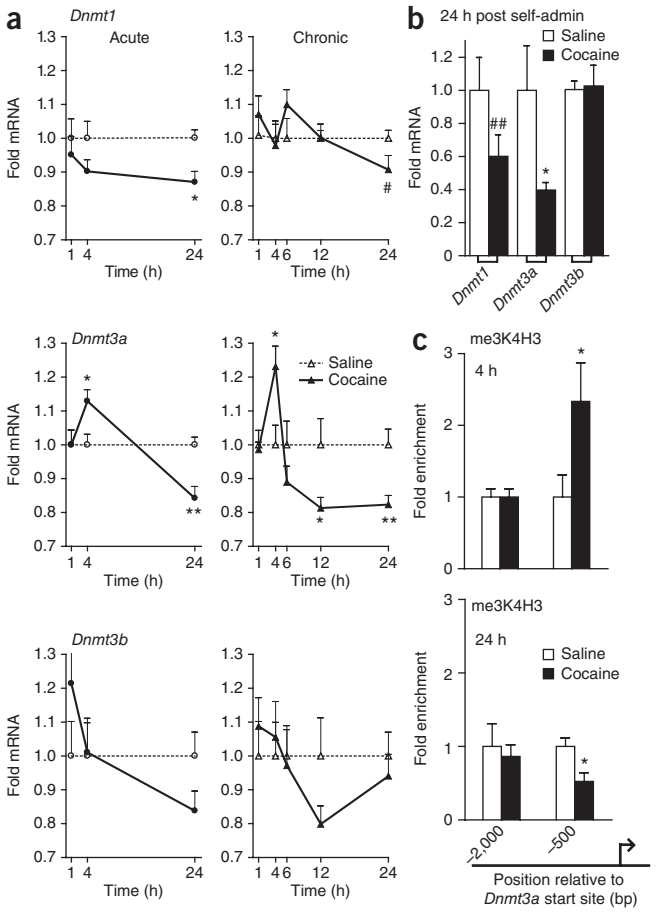

Figure 1 Transcriptional regulation of Dnmt3a by chronic cocaine. (**a**) qPCR analysis of NAc of acute and chronic (7 d) cocaine–treated mice (20 mg per kg of body weight per d, intraperitoneal) revealed a transient increase in *Dnmt3a* mRNA levels 4 h after the final injection ($n = 14$ acute, $n = 7$ chronic) and a decrease after 24 h ($n = 6$ acute, $n = 16$ chronic). Dnmt1 ($^{\#}P = 0.08$) and Dnmt3b transcripts were not altered significantly by chronic cocaine; however, acute cocaine significantly reduced Dnmt1 expression at 24 h ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.005$, $^{\#}P = 0.08$. (**b**) *Dnmt3a* mRNA was significantly reduced in the NAc of chronic (13 d) self-administering rats examined 24 h after the last drug dose ($n = 6$ control, $n = 7$ self-administration). $^{\#\#}P = 0.12$. (**c**) ChIP analysis revealed a significant cocaine-induced increase (4 h) and decrease (24 h) in me3K4H3 binding –500 bp upstream of the *Dnmt3a* promoter ($n = 4$, 5 mice pooled per sample), with no regulation seen –2,000 bp upstream from the promoter. Data are mean ± s.e.m.

injection. This biphasic regulation of *Dnmt3a* appears to occur via transcriptional mechanisms, as chromatin immunoprecipitation (ChIP) analysis of a well-known marker of gene activation, histone 3 tri-methyl-Lys4 (me3K4H3), indicated that the *Dnmt3a* gene, and not the *Dnmt1* or *Dnmt3b* genes, exhibited transiently enhanced (at 4 h) and suppressed (at 24 h) me3K4H3 binding at its promoter (**Fig. 1c**). To provide further relevance to addiction, we analyzed tissue from rats that chronically (13 d) self-administered cocaine and found a significant downregulation in *Dnmt3a* expression in NAc at 24 h ($P < 0.05$) after the last cocaine infusion (**Fig. 1b**). Notably, Dnmt1 was significantly downregulated by acute cocaine ($P < 0.05$); however, this effect was not significant with chronic cocaine or self-administered cocaine ($P > 0.05$; **Fig. 1a**,**b**). *Dnmt3b* was not regulated under any condition analyzed. Moreover, analysis of relative levels of each Dnmt in mouse and rat NAc revealed that Dnmt3a was by far the most predominant Dnmt expressed in the NAc (**Supplementary Fig. 2**). Given the dynamic regulation of Dnmt3a by cocaine and its enrichment in NAc, we focused our studies on this enzyme subtype.

Although the molecular events that occur during the transition to the addicted state, such as the aforementioned biphasic regulation of Dnmt3a, are an important component underlying the pathophysiology of addiction, a second major area of research focuses on the mechanisms that maintain the addicted state. More long-term regulation of Dnmts is of particular interest given the theoretical, persisting influence that these enzymes may have on downstream gene targets and behavior. Therefore, we injected mice with cocaine for a more prolonged time period (28 d) and analyzed Dnmt mRNA levels after an additional 28 d of withdrawal. This injection procedure causes robust and long-lasting molecular and cellular changes, such as increased dendritic spine density on NAc neurons[16–18]. Under these conditions, we found that *Dnmt3a* mRNA expression in NAc was selectively increased (**Fig. 2a**). We also found that *Dnmt3a* mRNA levels were similarly increased in NAc of rats that underwent 3 weeks of cocaine self-administration followed by 28 d of withdrawal (**Fig. 2b**). Together, these data indicate that *Dnmt3a* is induced in a sustained manner after relatively long periods of cocaine withdrawal and may be important, not only for the transition to addiction, but also for the maintenance of the addicted state.

DNA methylation modulates behavioral responses to cocaine

To understand the behavioral importance of the dynamic regulation of Dnmt3a in the NAc by cocaine, we used complementary pharmacological and genetic tools to manipulate DNA methylation in this brain region in the context of cocaine conditioned place preference (CPP), which provides an indirect measure of cocaine reward. We first administered methionine (a methyl donor) systemically with an injection regimen that hypermethylates specific gene promoters in rodent cortex and striatum[19], which caused a robust decrease in cocaine CPP (**Fig. 3a**). An important limitation of this experiment is its lack of anatomical and biochemical specificity. Therefore, we next tested whether intra-NAc delivery of RG108, a potent, non-nucleoside inhibitor of DNA methylation[20,21], influences reward behavior. At a dose that decreased global DNA methylation, the continuous intra-NAc infusion of RG108 markedly enhanced cocaine CPP and the induction of locomotor sensitization to the drug (**Fig. 3b–d**).

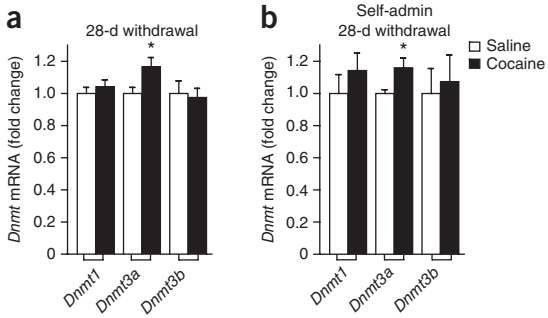

Figure 2 Prolonged induction of Dnmt3a by chronic cocaine after 28 d of withdrawal. (**a**) qPCR analysis of NAc of chronic (28 d) cocaine–treated mice (20 mg per kg of body weight per d, intraperitoneal) that have undergone 28 d of drug withdrawal revealed an increase in *Dnmt3a* mRNA levels ($n = 11$). $^{*}P < 0.05$. (**b**) Dnmt3a mRNA expression was significantly increased after 28 d of withdrawal in the NAc of chronic (3 week) self-administering rats ($n = 6$ control, $n = 7$ self-administration). Data are mean ± s.e.m.

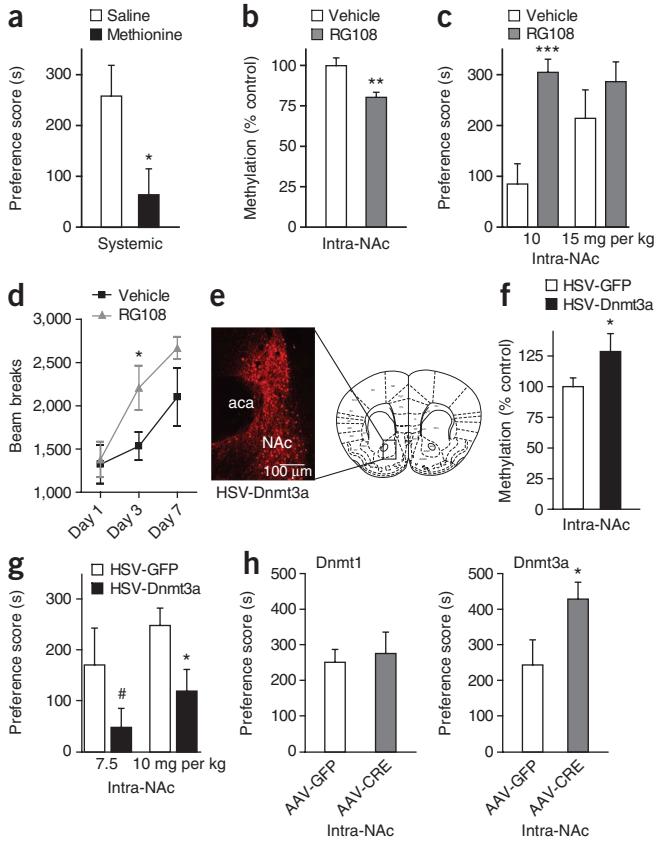

Figure 3 DNA methylation regulates cocaine reward. (**a**) Chronic (7 d) methionine (0.78 g per kg of body weight, twice a day subcutaneously) diminished the rewarding effects of cocaine in the CPP procedure ($n = 9$). $^{*}P < 0.05$. (**b**–**d**) Continuous intra-NAc infusion over 7 d of the Dnmt inhibitor RG108 (100 µm) decreased global DNA methylation levels in NAc ($n = 6$ vehicle, $n = 7$ RG108, **b**), increased cocaine CPP at 10 mg per kg (intraperitoneal, $n = 9$, **c**) and enhanced the induction of locomotor sensitization to chronic cocaine (20 mg per kg, intraperitoneal; $n = 8$). $^{**}P < 0.005$, $^{***}P < 0.0005$. (**e**) Verification of anatomical placement and viral infection in NAc after HSV–Dnmt3a-GFP injection; immunostaining for GFP is shown. Cartoon shows the location of the injection site at 1.54 mm bregma. aca, anterior commissure area. (**f**) HSV-Dnmt3a increased global DNA methylation levels ($n = 8$ HSV-GFP, $n = 5$ HSV-Dnmt3a). (**g**) Intra-NAc HSV-Dnmt3a significantly attenuated cocaine reward at 10 mg per kg cocaine ($n = 10$), with a trend seen at a lower dose ($^{\#}P = 0.13$, $n = 10$). (**h**) Intra-NAc AAV-Cre injected into *Dnmt3a*$^{loxP/loxP}$ mice significantly increased cocaine CPP at 7.5 mg per kg ($n = 14$ AAV-CRE, $n = 18$ AAV-GFP), with no effect seen in *Dnmt1*$^{loxP/loxP}$ mice. All raw CPP data are provided in **Supplementary Table 1**. Data are mean ± s.e.m.

These data suggest that DNA methylation in NAc, possibly via Dnmt3a as the predominant Dnmt in this brain region, negatively regulates cocaine reward. To further test this possibility, we developed a herpes simplex virus (HSV) vector to temporally and specifically overexpress Dnmt3a in the NAc (**Fig. 3e**). Dnmt3a overexpression increased global DNA methylation in this brain region (**Fig. 3f**) and, consistent with methionine administration, attenuated cocaine CPP (**Fig. 3g**). To obtain the converse information, we administered an adeno-associated virus (AAV) vector that expresses Cre recombinase into the NAc of mice homozygous for a *loxP*-flanked *Dnmt3a* gene. Such NAc-specific knockout of *Dnmt3a* potentiated cocaine CPP, an effect that was not seen for a similar local knockout of *Dnmt1* (**Fig. 3h**). Notably, none of these manipulations of DNA methylation in NAc altered baseline locomotor behavior and Dnmt3a overexpression did not impair general tests of learning and memory (**Supplementary Fig. 3**). These behavioral data, coupled with the dynamic regulation of Dnmt3a expression, suggest that increased Dnmt3a expression in NAc negatively regulates cocaine reward, whereas decreased Dnmt3a enhances cocaine reward.

DNA methylation regulates dendritic spine density in NAc

Among the most persistent drug-induced neuroadaptations known is cocaine's ability to increase dendritic spine density of NAc neurons[18]. However, the contribution of spinogenesis to the addicted state remains unclear[22] (see Discussion). We analyzed NAc neuron spine density in mice that received intra-NAc HSV-Dnmt3a or a control virus after chronic cocaine or saline treatment. Because of the limited time course of HSV overexpression (which wanes within 6 d of injection), we used a five-injection chronic cocaine schedule that has been shown to increase spine density 4 h after the last injection[5,23]. As expected, cocaine increased NAc spine density and Dnmt3a overexpression alone was sufficient to increase spine density to cocaine-comparable levels (**Fig. 4a**).

We next performed a more detailed spine analysis under identical conditions in which we found Dnmt3a expression to be cocaine regulated at 4 and 24 h following the last of seven daily cocaine injections (**Fig. 1a**). We imaged Lucifer yellow–filled NAc medium spiny neurons and analyzed dendrites using NeuronStudio software; these techniques are amenable to spine density and spine type analysis[24]. We found an increased spine density at both 4 h ($P < 0.05$) and 24 h ($P < 0.01$) after withdrawal from chronic cocaine (**Fig. 4b**). Moreover, spine type analysis revealed that these effects at both time points were the result of selective increases in thin spines, with no effect seen in mushroom or stubby spines (**Fig. 4c**). The shared increase in thin spine density suggests a mechanistic link for these two time points, which might reflect a lasting consequence of the transient induction of Dnmt3a seen at 4 h. To test this hypothesis, we followed the same chronic cocaine dosing regimen while continuously delivering the Dnmt inhibitor RG108 into the NAc and analyzed spine density and spine type 24 h after the last cocaine dose. We found that RG108 completely blocked cocaine-induced spinogenesis (**Fig. 4c**). Moreover, spine type analysis revealed that this effect was a result of a specific effect on thin spines (**Fig. 4c**).

DNA methylation in NAc regulates depression-like behavior

In addition to being important for addictive behavior, the NAc is known to be critically involved in depression and, as with cocaine models, labile histone modifications such as acetylation and methylation in NAc have been implicated in rodent models of depression[1,7,25]. However, the role of DNA methylation in depressive behavior remains unexplored. We analyzed mRNA levels for Dnmts and methyl-binding domain proteins in NAc at 1 and 10 d after chronic (10 d) social defeat stress, an ethologically relevant model of depression that induces several depressive-like behaviors including prolonged social avoidance[26]. We found increased Dnmt3a levels in the NAc at both time points after chronic defeat stress (**Fig. 5a**). In contrast, no regulation was seen for any of the other proteins that we studied (**Supplementary Fig. 4**). In addition, no significant regulation of *Dnmt3a* mRNA was observed at 90 min after the last defeat or at 2 or 24 h after an acute defeat ($P > 0.1$; data not shown).

We next tested the influence of Dnmt3a on susceptibility to social defeat by subjecting mice that received HSV-GFP (green fluorescent protein) or HSV-Dnmt3a injections into the NAc to submaximal

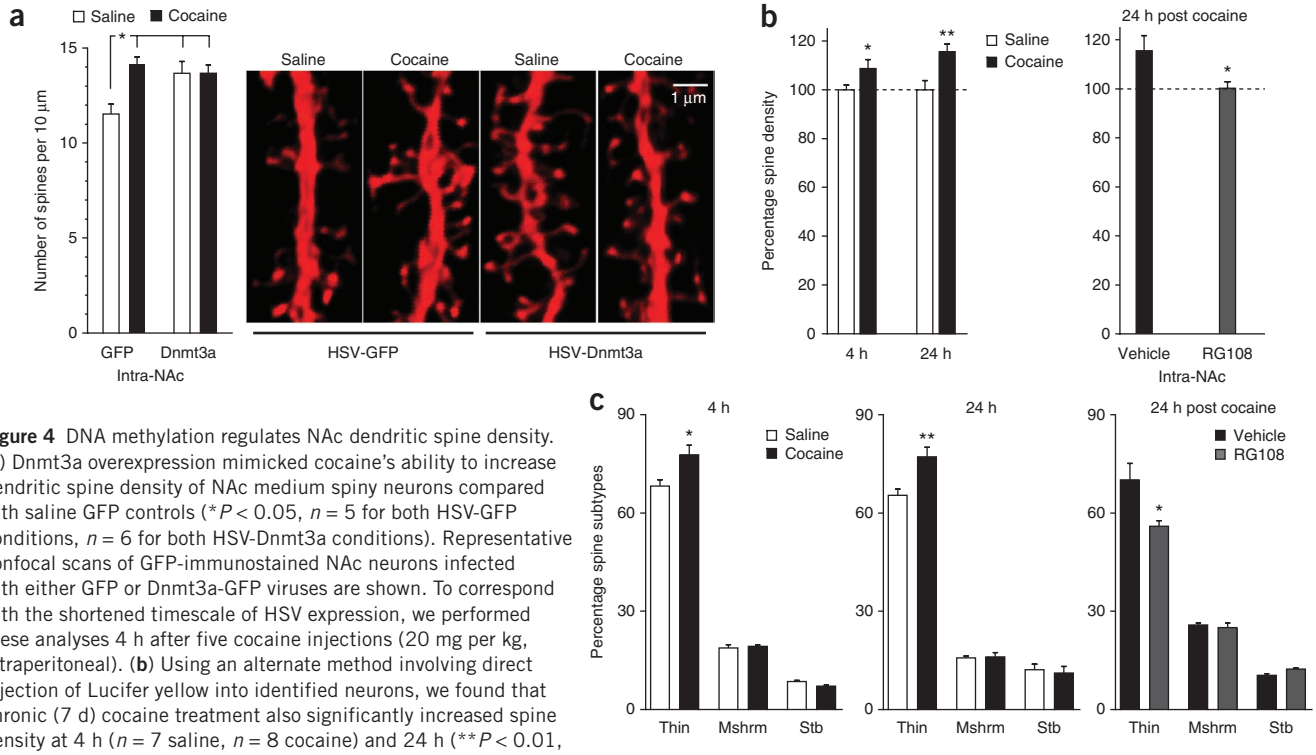

Figure 4 DNA methylation regulates NAc dendritic spine density. (**a**) Dnmt3a overexpression mimicked cocaine's ability to increase dendritic spine density of NAc medium spiny neurons compared with saline GFP controls (*$P < 0.05$, $n = 5$ for both HSV-GFP conditions, $n = 6$ for both HSV-Dnmt3a conditions). Representative confocal scans of GFP-immunostained NAc neurons infected with either GFP or Dnmt3a-GFP viruses are shown. To correspond with the shortened timescale of HSV expression, we performed these analyses 4 h after five cocaine injections (20 mg per kg, intraperitoneal). (**b**) Using an alternate method involving direct injection of Lucifer yellow into identified neurons, we found that chronic (7 d) cocaine treatment also significantly increased spine density at 4 h ($n = 7$ saline, $n = 8$ cocaine) and 24 h (**$P < 0.01$, $n = 8$ saline, $n = 7$ cocaine) after the last cocaine injection. Continuous (7 d) intra-NAc RG108 infusion significantly reduced spine density 24 h after the last chronic cocaine injection ($n = 4$ vehicle infused, $n = 5$ RG108 infused). Data are expressed as percentage change in spine density relative to saline controls. (**c**) NeuronStudio spine type analysis of the same images from **b** revealed that chronic cocaine selectively increased thin spines both 4 and 24 h after the last cocaine injection. Similarly, intra-NAc RG108 selectively reduced thin spines of chronic cocaine injected animals. None of these conditions significantly affected the number of mushroom (mshrm) or stubby (stb) spines. Data are mean ± s.e.m.

defeat stress. In this procedure, animals undergo just 1 d of defeat, which is not sufficient to induce social avoidance; in fact, normal mice display increased interaction with a social target under these conditions[26,27]. As expected, HSV-GFP mice exhibited this significantly increased interaction time ($P < 0.05$). In contrast, Dnmt3a overexpression in NAc attenuated social interaction, consistent with a pro-depressive–like phenotype (**Fig. 5b**). To complement these data, we assessed a second model of depressive-like behavior[28] and found that Dnmt3a overexpression significantly reduced the latency to immobility ($P < 0.001$) in the rat forced swim test (**Fig. 5c**), which is also a pro-depression–like effect. These data suggest that the prolonged induction of Dnmt3a in NAc by chronic social defeat stress promotes depressive behavior. To further test this hypothesis, we continuously infused RG108 into the NAc between 1 and 10 d after defeat stress. We found that this local RG108 infusion reversed social avoidance in defeated mice, an similar effect to that observed using the standard antidepressant fluoxetine (**Fig. 5d**), indicating that Dnmt inhibition in this brain region exerts antidepressant-like effects.

DISCUSSION

Our results suggest that Dnmt3a expression is subject to dynamic regulation in NAc by two types of chronic emotional stimuli. Chronic social defeat stress induced a persistent upregulation of Dnmt3a in this brain region. In contrast, cocaine biphasically regulated Dnmt3a expression on a short timescale, whereas longer-term withdrawal resulted in persisting upregulation of Dnmt3a. The results of our functional experiments indicate that the cocaine-induced downregulation of Dnmt3a enhanced cocaine reward, whereas upregulation of Dnmt3a exerted the opposite effect. Similarly, we found prolonged upregulation of Dnmt3a in NAc that drove depressive-like behavior in the chronic defeat stress and forced swim procedures. Taken together, these data suggest that an appropriate balance of DNA methylation in NAc crucially gates behavioral responses to emotional stimuli; a hypermethylated state dampens responses to rewarding stimuli and heightens responses to aversive stimuli, whereas a hypomethylated state heightens responses to rewarding stimuli and dampens responses to aversive stimuli.

Our analysis of dendritic spine density on NAc neurons indicates that DNA methylation is an essential mediator of cocaine-induced spinogenesis; Dnmt3a overexpression in NAc mimicked the cocaine-induced increase in spines, whereas RG108 infusion into this region blocked cocaine's action. An important question is how such a role for Dnmt3a in NAc spine regulation relates to the highly dynamic regulation seen for Dnmt3a expression. Because Dnmt3a overexpression alone is sufficient to regulate spine density, and as DNA demethylation is an enzymatically unfavorable chemical reaction and debate still exists regarding the enzymatic basis of active DNA demethylation, we speculate that the transient reduction in Dnmt3a expression, such as what we observed 24 h after chronic cocaine, is likely not of a sufficient timescale to downregulate spines[29]. Instead, we speculate that the transient increase in Dnmt3a expression seen 4 h after each cocaine exposure leads to the progressive accumulation of DNA methylation and is therefore responsible for the overall induction of NAc dendritic spines. The fact that a highly persistent increase in Dnmt3a expression was seen 4 weeks after withdrawal, a time point at which NAc spine density is robustly induced[16–18], is consistent with our hypothesis. The mechanisms responsible for the complex time course of Dnmt3a regulation in NAc, early induction,

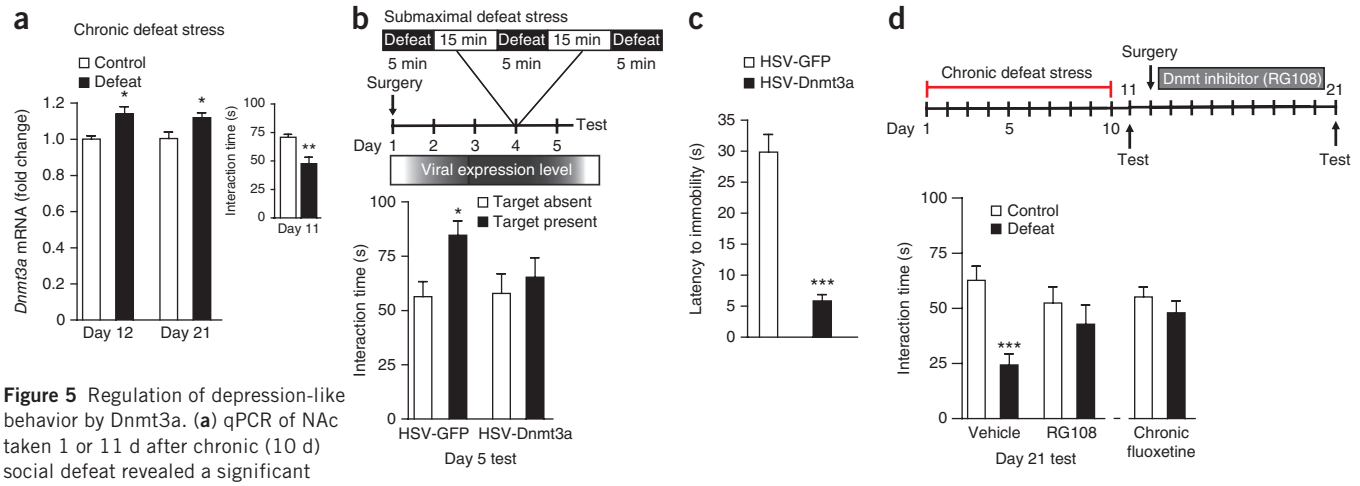

Figure 5 Regulation of depression-like behavior by Dnmt3a. (**a**) qPCR of NAc taken 1 or 11 d after chronic (10 d) social defeat revealed a significant increase in *Dnmt3a* levels at both time points (*$P < 0.05$). Inset, social avoidance, a depressive-like behavior, was observed in these mice (**$P < 0.005$, $n = 16$ control, 26 defeated). (**b**) Naive mice infused intra-NAc with HSV-GFP or HSV-Dnmt3a were subjected to a submaximal protocol of social defeat. HSV-GFP mice showed a significant increase in interaction with a social target ($F_{1,42} = 5.219$), as would be expected from control mice[25], whereas HSV-Dnmt3a mice lacked this phenotype ($P > 0.05$), a pro-depressive–like response. (**c**) Intra-NAc injection of HSV-Dnmt3a significantly decreased latency to immobility on day 2 of the rat forced-swim test, also a pro-depressive–like response (***$P < 0.001$, $n = 7$). (**d**) Conversely, intra-NAc infusion (10 d) of RG108, initiated 1 d after the last defeat episode, completely reversed the chronic social defeat–induced social avoidance exhibited by vehicle-infused mice ($F_{1,44} = 12.876$), an effect that was equivalent to that seen for chronic (20 mg per kg per d, intraperitoneal, 14 d) fluoxetine administration ($P > 0.05$). Data are mean ± s.e.m.

quickly followed by suppression and then a slowly developing, but very sustained, induction, are unknown and require future exploration (see below). Similarly, the persistent induction of Dnmt3a in NAc after chronic social defeat stress raises the possibility that NAc spine density may be regulated under these conditions as well, something that has been observed in preliminary investigations[30].

Our finding that Dnmt3a induction increased NAc spine density while attenuating cocaine reward highlights an important question in the addiction field. What is the behavioral relevance of cocaine's induction of dendritic spines on NAc neurons? Several conflicting reports exist on this subject[22]. One study positively correlated spine induction with increased locomotor sensitization[31]. Three studies that directly manipulated genes (*Fosb*, *Nfkb1* or *Ehmt2*, also known as *G9a*) in NAc that are known to regulate spines found that manipulations that block spine induction also block cocaine's behavioral responses[5,23]. However, two other studies yielded conflicting results; blocking CDK5 or activating MEF2 blocks cocaine-induced spinogenesis and enhances cocaine reward[17,32]. The pattern seen for *Dnmt3a* matches these latter findings. The basis for these paradoxical results is unknown. One possibility is that regulation of different types of spines might exert very different functional effects on NAc neurons and, consequently, on behavior. A recent study reported highly complex regulation of various spine types over a course of chronic cocaine exposure and withdrawal[33] and we found a selective effect of DNA methylation on the regulation of thin spins. The observation that Dnmt3a induced thin spines, but blunted cocaine's behavioral effects, raises the possibility that the induction of thin spines actually represents a homeostatic adaptation that serves to oppose the behavioral effects of cocaine. Clearly, this question requires further investigation.

The electrophysiological function of cocaine-induced, DNA methylation–dependent thin spines remains speculative at this point; however, electrophysiological correlates have been reported under similar cocaine injection regimens. In association with the increase in thin spines, chronic cocaine causes a reduction in firing rate in the NAc shell[34], synaptic depression (decreased AMPA/NMDA ratio) during early (24 h) withdrawal[35] and synaptic potentiation (increased AMPA/NMDA ratio) during late (10–14 d) withdrawal[35]. First, some evidence exists for spines driving neuronal firing rate, as it was found recently that the spine density reductions that are associated with dopamine depletion induce a homeostatic response of increased firing of medium spiny neurons[36]. Second, synaptic depression, as indicated by decreased AMPA/NMDA ratio, may represent an increased pool of AMPA receptor–lacking synapses, which are also known to be regulated by cocaine[37]. As thin spines are thought to represent highly plastic, newly formed spines that lack AMPA receptors, we speculate that cocaine-induced DNA methylation generates such less-responsive thin spines[38]. Furthermore, it is also possible that, as these spines mature, they may incorporate AMPA receptor and thereby contribute to the increased AMPA/NMDA ratio as well as to the behavioral sensitization found during later withdrawal. Finally, the influence of DNA methylation on synaptic plasticity is heavily supported by previous findings. In cultured hippocampal neurons, consistent with our finding that RG108 reduces cocaine-induced spine density of NAc neurons, Dnmt inhibition causes a marked reduction in functional synapses in an activity-dependent manner, as measured by a reduction in mEPSC frequency[14]. Moreover, both Dnmt inhibition and conditional knockout of *Dnmt1* and *Dnmt3a* block long-term potentiation, a process that is thought to involve the formation and/or consolidation of dendritic spines[11,12]. These studies point to the importance of understanding the electrophysiological changes brought about by DNA methylation in the NAc. Such studies will provide insight into whether cocaine-induced, DNA methylation-dependent changes in thin spines are a cause or a consequence of changes in firing rate and/or synaptic depression.

As noted earlier, the mechanisms responsible for cocaine's short-term biphasic regulation of *Dnmt3a* mRNA expression are unknown. In hippocampus, Dnmt3a was found to be rapidly upregulated by fear conditioning, but the expression pattern following this upregulation over longer time points has not been assessed. As Dnmt inhibition in

hippocampus blocks memory formation, we presumed that Dnmt3a's transient increase initiates downstream methylation of target genes that ultimately influence fear memory[12]. This is analogous to our proposal that the transient increases in Dnmt3a that occur with each cocaine injection cause more lasting regulation of cellular and behavioral plasticity. The fact that me3K4H3 binding to the *Dnmt3a* promoter was commensurately associated with *Dnmt3a* mRNA regulation at both the 4 and 24 h time points suggests that a histone 3, Lys4 methyltransferase, such as KMT2A (also known as MLL1), may regulate Dnmt3a expression. In addition, me3K4H3 is well known to inversely correlate with DNA methylation at particular gene promoters, raising the possibility that Dnmt3a may feed back and regulate its own mRNA expression[39]. On a general level, one hypothesis that may explain Dnmt3a's complex regulation could be a transcriptional response to the rapid fluctuations in levels of dopamine or BDNF that are seen with cocaine exposures[40]. This is a possibility as BDNF protein, similar to *Dnmt3a* mRNA, also accumulates with prolonged cocaine withdrawal[41], and chronic social defeat stress induces lasting enhancement of BDNF signaling in NAc[26] and caused lasting increases in Dnmt3a expression in this brain region.

Finally, these data, in conjunction with studies of histone acetylation and methylation[1,5–7,25], support an emerging model that epigenetic alterations in NAc have profound effects on the regulation of emotional behavior in models of both drug addiction and depression. The regulation is complex, with different epigenetic mechanisms exerting distinct effects on behavioral endpoints. A promising line of future research would be to assess the behavioral function of additional enzymes that mediate still other forms of epigenetic modifications. In parallel, the gene targets (and their degree of overlap) for these various enzymes remain largely unknown. The elaboration of these targets should help us to identify the many ways in which epigenetic mechanisms, including Dnmt3a-mediated DNA methylation, regulate NAc neuronal function to mediate the complex behavioral phenotypes of addiction and depression. It will also be important to explore how Dnmt3a regulation and other epigenetic modifications in NAc contribute to the complex interactions that are known to exist between cocaine and stress vulnerabilities.

We found that both chronic cocaine and stress differentially regulated the *de novo* Dnmt Dnmt3a in NAc to control dendritic spine plasticity and behavioral responses to these stimuli. We found that Dnmt3a activity in NAc *in vivo* was necessary for cocaine-induced increases in dendritic spine density selectively for the thin type of spines. Our findings also suggest that the rewarding responses to cocaine negatively correlated with thin spines and also raise the possibility that depressive responses to chronic stress may positively correlate with increased spine density. Taken together, these observations implicate an epigenetic modification, DNA methylation, in the molecular mechanisms controlling cocaine- and stress-induced structural and behavioral plasticity and could ultimately lead to the development of improved treatments for drug addiction and depression.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureneuroscience/.

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

Q.L. and E.J.N. were responsible for the overall study design. Q.L., J.F., I.M. and J.W.K. designed and conducted the RNA and ChIP experiments and analyzed the results. Q.L., V.V., H.E.C., I.M., B.W. and R.S.O. designed and performed the CPP, locomotor sensitization, and learning and memory behavioral experiments. Q.L., V.V., R.S.O., B.W., S.D.I. and C.A.B. designed and conducted the submaximal defeat, forced swim and social defeat experiments. Q.L., D.D., D.M.D., J.H.M., B.W. and E.L.W. designed and conducted the dendritic spine analysis. Q.L. carried out confocal imaging of dendritic spines, D.D. performed single-cell filling, V.V., H.E.C., Q.L. and B.W. performed stereotaxic surgeries, W.R. and Q.L. performed HSV cloning of Dnmt3a, F.H., H.W., M.A.N., Y.R., A.J.E., M.K. and Y.L.H. designed and performed the self-administration experiments, R.L.N. prepared new HSV vectors and performed quality-control experiments on all of the HSV vectors, E.M. prepared new AAV vectors and performed quality-control experiments on all of the AAV vectors, Q.X. performed ChIP-chip statistical analyses, G.F. provided *loxP*-flanked Dnmt mice and Q.L. and E.J.N. wrote the paper with the help of the other authors.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Animals. We used adult male C57BL/6J mice (Jackson) and Long-Evans rats (Charles River). Animals were habituated in our facility 1 week before experimentation and housed on a 12-h light-dark cycle with access to food and water *ad libitum*. All animal experiments were approved by the Institutional Animal Care and Use Committees of Mount Sinai and University of Texas Southwestern.

Drugs. For chronic cocaine (Sigma) experiments, we used a standard cocaine injection procedure[2,5–7]: seven daily intraperitoneal injections of 20 mg per kg cocaine. For acute experiments, mice were injected for 6 d with saline before a cocaine injection on day 7. Controls were injected with saline. For spine analysis, we used an established injection schedule that is known to increase spine density, consisting of five injections (20 mg per kg). This protocol was shortened to coincide with the duration of HSV-mediated overexpression, which wanes 6 d post-surgery[23,42].

For methionine (MP Biochemicals) experiments, animals were injected subcutaneously with 0.78 g per kg L-methionine twice a day for 10 d. This was timed to ensure that the minimum dose and injection duration previously shown to increase DNA methylation in the striatum[19] would coincide with the beginning of CPP training. During training, animals were injected with methionine 2–3 h before behavioral experiments.

For RG108 (Sigma) experiments, we used 7–10 d of continuous (0.25 µl h^{-1}) intra-NAc delivery of 100 µM RG108 dissolved in 5% hydroxypropyl β-cyclodextrin vehicle (wt/vol, Trappsol). This dose of RG108 was found to decrease DNA methylation *in vivo* and showed no detectable neurotoxicity as assessed by activated caspase-3 staining. RG108 was chosen over other Dnmt inhibitors, such as 5-aza-cytidine, because it inhibits methylation without incorporation into DNA, which has been shown to be linked to cytotoxicity[43], and it is chemically stable with a mean 37 °C half-life of 20 d, whereas the 37 °C half-life of 5-aza-cytidine is on the order of hours[20]. Osmotic delivery was performed as previously described[1,5,7].

For fluoxetine (Tocris) experiments, we injected mice with 20 mg per kg fluoxetine intraperitoneally for 14 d, as described previously[1].

Cocaine self-administration. Self-administration was performed as previously described[44,45]. Animals had 3 h daily access to cocaine (0.75 mg per kg per infusion) under a fixed ratio-1 (FR1) reinforcement schedule. For the 24-h withdrawal experiment, rats had 13 d of cocaine intake; for the 28-d withdrawal experiment, rats had 3 weeks of intake.

Herpes simplex and adeno-associated virus injections. We used the bi-cistronic p1005+ HSV vector, which expressed GFP alone or GFP with Dnmt3a. In this system, HSV infection occurs selectively in neurons. GFP expression is driven under the human immediate early cytomegalovirus (CMV) promoter, whereas the gene of interest, Dnmt3a, is driven by the IE4/5 promoter[46]. We used a previously cloned mouse Dnmt3a-1 plasmid[47]. AAV-GFP or AAV-CreGFP was used as described[5]. Stereotaxic surgery was performed as described previously[5,7].

Conditioned place preference. We used a standard, unbiased CPP procedure[6,7,23]. In brief, before experimental manipulation, animals were pretested for 20 min in a photo-beam monitored box with free access to environmentally distinct chambers. The mice were then arranged into control and experimental groups with equivalent pretest scores. After experimental manipulation, mice underwent four 30-min training sessions (alternating cocaine and saline pairing). On the test day, mice had 20 min of unrestricted access to all chambers and a CPP score was ultimately assigned by subtracting the time spent in the cocaine-paired chamber from the time spent in the saline-paired chamber.

Cocaine-induced locomotor sensitization. We used a standard locomotor sensitization procedure[17]. In brief, mice (saline habituated) were injected daily with cocaine (intraperitoneally) 30 min after being placed in standard plastic cages similar to their home cages. Total locomotor activity was measured via photobeam breaks for 30 min following their injection (20 mg per kg).

Social defeat stress. Experimental C57BL/6J mice were subjected to 10 d of social defeat stress as previously described[26,27]. In summary, mice were exposed daily to an unfamiliar aggressive CD1 mouse for 5 min. For the remaining 24 h, the defeated mouse was housed in a 'protected' compartment of the same cage in which it endured chronic stress from the CD1. On the 11th day, mice underwent a social interaction test to verify defeat-induced social avoidance[26,48]. As described previously, control mice spent more time interacting with a social target as compared with no target, whereas chronically defeated mice spent substantially less time interacting with the target mouse compared with no target[26,48].

For RG108 and fluoxetine experiments, we selected mice displaying robust social avoidance: showing day 11 interaction times of less than 40 s. Using baseline interaction times, we divided animals into three similar groups (controls: intra-NAc vehicle (75.1 ± 3.1 s), intra-NAc RG108 (75.4 ± 4.1 s) or systemic fluoxetine (75.5 ± 6.1 s); defeats: intra-NAc vehicle (20.5 ± 3.3 s), intra-NAc RG108 (20.4 ± 3.6 s), fluoxetine (20.1 ± 3.1 s)).

To test for a pro-depressive phenotype, we performed a submaximal defeat experiment as previously described[26,27]. Naïve mice were subjected to three consecutive 5-min defeat episodes interspersed by 15-min rest periods 3 d after HSV surgery. Mice underwent the social interaction test the following day. In this procedure, these stressors are not sufficient to cause social avoidance in HSV-GFP mice, as indicated by a significant increase in target interaction time versus no target interaction time[26,27].

Forced swim test. Immobility measures were obtained using a 2-d forced-swim test procedure commonly used in rats[27,28]. Sprague-Dawley rats were forced to swim for 15 min in plastic cylinders (20 × 45 cm) 3 d after surgery. The following day, rats were tested under identical conditions for 5 min. All sessions were videotaped and scored by a blinded observer. Latency to immobility was defined as the time that the rat first initiated a stationary posture that did not reflect attempts to escape.

Novel object recognition and object habituation tests. Tests were performed as described previously with minor modifications[49]. Briefly, for the novel object recognition test, mice were placed in an open field area to habituate (15 min). After 3 min of rest, they were exposed to three objects for 5 min in the same arena. Each object was placed in opposite corners of the open field arena. Mice were re-exposed two additional times to the same objects in the same locations and, on the final trial, one object was switched with a novel object. A normal mouse with intact memory is expected to spend more time with this novel object as compared with the familiar objects. For habituation to a novel object, we exposed mice to a single object for 150 s and the re-exposure time was extended to 1 h. As mice were re-exposed to this object, they were expected to become disinterested in the object, as measured by number of object investigations.

RNA isolation, reverse transcription, quantitative PCR and primers. Bilateral NAc punches were obtained and the tissue homogenized in Trizol (Invitrogen). RNA was purified with RNeasy Micro columns (Qiagen). Reverse transcription of RNA was carried out using iScript (Biorad). qPCR was run using approximately 5 ng of cDNA per reaction. Each reaction was run in triplicate and quantified using the ΔΔCt method as previously described[48]. A complete list of primers is given in **Supplementary Table 2**.

Global DNA methylation analysis. We used Epigentek's Methylamp Global DNA Methylation Quantification Ultra Kit and followed the manufacturer's instructions. This method is ideally suited for our experiments on NAc tissue because of its ability to use small amounts of input DNA and for its high degree of sensitivity. Raw values were colorimetrically quantified and total methylation levels estimated by generating a standard curve from Epigentek's methylated DNA standard. Values are then represented as methylation percentage relative to vehicle control.

ChIP promoter analysis. Chromatin from NAc tissue punches was immunoprecipitated with an antibody to me3K4H3 (Abcam ab1012) as described previously without modifications[2,6,25]. For the 24-h experiment, samples were amplified using GenomePlex whole genome amplification kit (Sigma), labeled with Cy3 (input) or Cy5 (me3K4H3 enriched), and hybridized to NimbleGen MM8 mouse promoter arrays with three biological replicates used per condition. Each replicate consisted of bilateral NAc punches pooled from ten mice. ChIP-chip analysis was performed as described previously without modification[6,25]. On the basis of these analyses, the *Dnmt3a* promoter was found to have substantially reduced binding at approximately −500 bp upstream of the transcriptional start

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site. ChIPs were then performed on additional sets of 4-h and 24-h cocaine-injected mice and PCRs were performed using primers designed around this region as well as −2,000 bp upstream.

Dendritic spine analysis (GFP immunostaining and single-cell dye filling). For viral-based (GFP immunostaining) spine analysis (**Fig. 4a**), mice were perfused with 4% paraformaldehyde (wt/vol) 4 h following the final cocaine injection and brains sectioned at 100 μm using a Leica vibratome. Slices were immunostained with antibody to GFP (Millipore). Sections were mounted and coded and confocal imaging (Zeiss LSM 710) was performed with the rater blind to experimental conditions. We imaged secondary and tertiary dendrites of NAc medium spiny neurons under a 100× oil-immersion objective at a resolution of 0.027 μm × 0.027 μm × 0.3 μm. Approximately seven to ten neurons were imaged per animal (average total dendritic length = 400 μm per animal, n = 5–6). Dendritic length was measured using ImageJ (US National Institutes of Health) and spine numbers were counted manually by a trained observer who was also blind to experimental conditions. To account for any possible surgical or sampling bias, we annotated anterior/posterior or NAc core/shell location of all images and found no substantial differences in location or region.

Because HSV-GFP (similar to other HSV encoded transgenes) was no longer expressed after 7 d, we performed single-cell dye filling experiments (**Fig. 4b**,**c**). As previously described[50], random cells in NAc shell were impaled with a micropipette containing 5% Lucifer yellow (wt/vol, Molecular Probes) and injected with 1–10 nA of current. Slices were washed in phosphate-buffered saline, mounted and imaged on the confocal microscope using the same imaging parameters as the above GFP experiment. On average, 12 dendrites from six neurons were imaged for each animal (average total dendritic length = 500 μm per animal, n = 4–8). Spine density and spine type analysis were performed using the semi-automated software NeuronStudio (http://research.mssm.edu/cnic/tools-ns.html)[24]. NeuronStudio analysis is not amenable to other dendritic images, such as GFP, Golgi or diolistics. NeuronStudio analyzes single-cell filled deconvolved images in three dimensions and, as an extra advantage, uses an algorithm of voxel clustering and Rayburst sampling to ultimately classify spines into the three major morphologic types: thin, mushroom and stubby. After NeuronStudio processing, a human operator verified that all spines have been appropriately identified and manually corrected any errors in spine characterization. For the operator, the manual aspect of analysis took about 10 min per dendritic image. Because of the labor-intensive nature of three-dimensional analysis, we had a separate NeuronStudio operator for each of the three cell-filling experiments. For each experiment, the NeuronStudio operator randomly analyzed coded images. On finishing analysis, the spine density (as well as spine type) was calculated by dividing the total number of spines present by the dendritic length of the segment. Human-operated NeuronStudio was previously shown to have a range of 68–90% inter-operator variability in spine density counts[24]. Similarly, we found a high degree of inter-operator variability in our three datasets. To control for such variability and to allow for comparison across experiments, we normalized the spine density and spine type data from each experiment with respect to its own control. Data are expressed as percentage change in spine density relative to saline controls.

Statistical analysis. Statistical significance was measured using an unpaired two-tailed Student t test when comparing two groups. One-tailed Student t tests were used for pharmacological verification of changes in global methylation and 7-d, 4-h spine density analysis, as values are expected to fall in an expected sampling distribution. For mRNA analysis, HSV-GFP dendritic spine analysis, submaximal defeat susceptibility and antidepressant tests in defeated mice, two-way ANOVAs were performed, as experiments contained multiple groups. Repeated-measures ANOVAs were used for locomotor sensitization analysis. For ANOVAs, *post hoc* comparisons were performed when appropriate. All error bars represent s.e.m.

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