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A Role for Repressive Histone Methylation in Cocaine-Induced Vulnerability to Stress

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SUMMARY

Substance abuse increases an individual's vulnerability to stress-related illnesses, which is presumably mediated by drug-induced neural adaptations that alter subsequent responses to stress. Here, we identify repressive histone methylation in nucleus accumbens (NAc), an important brain reward region, as a key mechanism linking cocaine exposure to increased stress vulnerability. Repeated cocaine administration prior to subchronic social defeat stress potentiated depressive-like behaviors in mice through decreased levels of histone H3 lysine 9 dimethylation in NAc. Cre-mediated reduction of the histone methyltransferase, G9a, in NAc promoted increased susceptibility to social stress, similar to that observed with repeated cocaine. Conversely, G9a overexpression in NAc after repeated cocaine protected mice from the consequences of subsequent stress. This resilience was mediated, in part, through repression of BDNF-TrkB-CREB signaling, which was induced after repeated cocaine or stress. Identifying such common regulatory mechanisms may aid in the development of new therapies for addiction and depression.

INTRODUCTION

Neuroadaptations to chronic cocaine, in brain areas critical for reward, persist long after the cessation of drug intake and are associated with drug relapse and with emotional signs of withdrawal, including depression-like symptoms (Der-Avakian and Markou, 2010; Nestler, 2005; Shaham and Hope, 2005). The

convergence of aversive and rewarding symptoms suggests shared neural mechanisms, a hypothesis supported by high rates of comorbid mood and substance abuse disorders in humans (Ford et al., 2009). Indeed, it is well known that a mood disorder increases an individual's risk for substance abuse, and this has been widely corroborated in animal models, which illustrate that stress, like cocaine itself, can directly promote subsequent drug taking (Covington et al., 2005; Prasad et al., 1998; Schindler et al., 2010; Shaham et al., 2000; Sorg and Kalivas, 1991; Tidey and Miczek, 1997). Conversely, clinical evidence shows that substance abuse can increase an individual's risk for a mood disorder; however, the neurobiological mechanisms underlying this phenomenon remain largely unexplored.

Chromatin regulation, whereby postmitotic neurons incorporate changes in transcriptional activity without altering DNA sequence, has received increasing attention for its role in mediating the lasting effects of drugs of abuse and stress on brain function (Borrelli et al., 2008; Grayson et al., 2010; Tsankova et al., 2007). One prominent type of chromatin modification, histone H3 lysine 9 dimethylation (H3K9me2), is controlled by a complex interaction between two histone methyltransferases, G9a and G9a-like protein (GLP) (Rice and Allis, 2001; Tachibana et al., 2001). Recent evidence has demonstrated that decreased expression of G9a in nucleus accumbens (NAc), a key brain reward region, along with corresponding reductions in H3K9me2, are important in mediating heightened levels of transcriptional and behavioral responses to repeated cocaine (Maze et al., 2010). Data obtained from postmortem human brain tissue, as well as from animal models, have further indicated a role for histone methylation in the promulgation of depressive-like behaviors and other psychiatric syndromes (Akbarian et al., 2005; Gupta et al., 2010; Schaefer et al., 2009; Tsankova et al., 2006). However, the specific genes at which cocaine- or stress-induced alterations in H3K9me2 influence addiction or depression symptoms have not yet been identified.

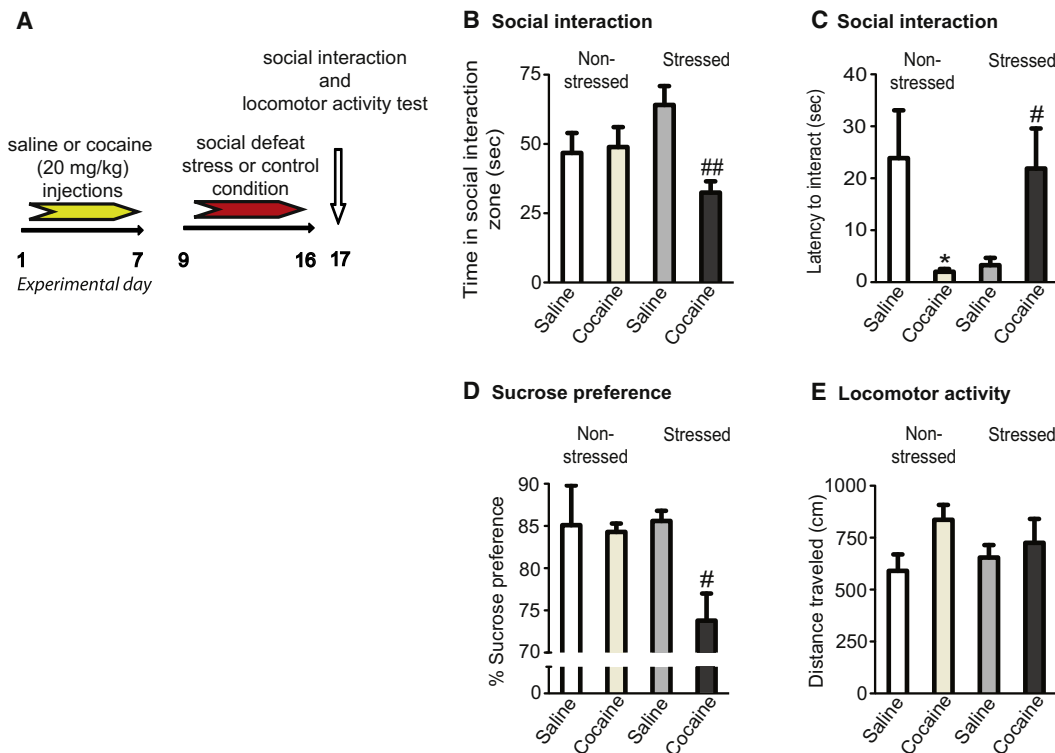


Figure 1. Cocaine Enhances Vulnerability to Social Stress

(A) Time course of saline or cocaine (20 mg/kg/injection) treatment prior to control handling or submaximal (8 days) social defeat stress.

(B) Repeated cocaine prior to submaximal social stress increases social avoidance during a social interaction test.

(C) Repeated cocaine treatment decreases an animal's latency to interact with a target under nonstressed conditions, whereas prior drug administration increases an animal's latency to interact after social stress.

(D) Repeated cocaine prior to submaximal social stress decreases sucrose preference.

(E) Neither repeated cocaine nor social defeat affects locomotor activity in an open field. [#] $p < 0.05$ and ^{##} $p < 0.01$, significant differences from saline injections prior to defeat stress; ^{*} $p < 0.05$, significant differences from nonstressed controls injected with saline. Data are presented as average \pm SEM ($n = 8$ –10/group).

Here, we examine a possible role for H3K9me2 in mediating the effects of repeated cocaine on vulnerability to stress-induced depressive-like behaviors. We first show that, as seen in humans, cocaine increases the susceptibility of mice to chronic social defeat stress, an ethologically valid model of depression (Berton et al., 2006; Kudryavtseva et al., 1991; Rygula et al., 2005). We go on to show that cocaine-induced downregulation of G9a and H3K9me2 in NAc is a key mechanism by which the drug renders the animals more vulnerable to social stress. Subsequent investigation of G9a-dependent molecular mechanisms common to both cocaine- and stress-induced behavioral phenotypes uncovered an essential role for BDNF-TrkB-CREB signaling, which has been implicated in NAc in mediating both addiction- and depression-related phenomena (see Discussion). We show that chronic cocaine induces the small G protein, Ras, which in turn promotes BDNF-TrkB signaling and its subsequent activation of CREB, in NAc and thereby increases vulnerability to social defeat stress. Together, these results provide fundamental insight into how prior exposure to a drug of abuse enhances vulnerability to depression and other stress-related disorders.

RESULTS

Cocaine Promotes Enhanced Susceptibility to Social Stress

To investigate the impact of repeated cocaine on stress vulnerability, we utilized a submaximal version of social defeat. Previous work has shown that 10 days of defeat stress induces several cardinal depressive-like behaviors, such as social avoidance and reduced sucrose preference (Berton et al., 2006; Krishnan et al., 2007). Here, only 8 days of defeat stress were used, which in initial studies did not induce these symptoms. Next, either saline or a sensitizing regimen of cocaine was administered prior to initiating 8 days of defeat stress (Figure 1A). Seven days of repeated cocaine (20 mg/kg/day), immediately followed by 8 days of defeat stress, revealed social avoidance (Figure 1B) and diminished sucrose preference (Figure 1D). This is in contrast to control animals receiving saline prior to chronic stress, which showed no such deleterious behavioral responses. To further verify the potential long-lasting effects of cocaine on behavioral deficits observed after 8 days of defeat stress, animals were re-exposed to a low dose of cocaine (5 mg/kg) 24 hr after the social interaction test (see Figure S1A

available online). Both stressed and nonstressed cocaine-treated animals displayed sensitized locomotor responses to cocaine. The social stress did not, however, potentiate cocaine-induced locomotor activity in cocaine-naïve mice (Figure S1B). Animals exposed to cocaine, in the absence of later social stress, displayed more rapid social interaction (i.e., decreased latency to interact)—an effect of cocaine that was completely reversed by exposure to 8 days of defeat stress (Figure 1C). The effects of cocaine, stress, or the combination of both stimuli had no impact on general levels of locomotor activity (Figure 1E).

Cocaine, when self-administered during binges, increases thresholds for intracranial self-stimulation, indicating a withdrawal syndrome characterized by anhedonia (Markou and Koob, 1991). However, as shown in Figures 1B and 1D, we did not observe an effect of cocaine alone on social interaction or sucrose preference.

Histone Methylation Is Similarly Regulated in NAc by Cocaine and Social Stress

We have shown recently that, following repeated (not acute) cocaine, the repressive histone modification, H3K9me2, and its associated “writer” enzymes, G9a and GLP, are reduced in NAc, leading to the activation of numerous synaptic plasticity-related transcripts, increased dendritic spine density on medium spiny neurons (MSNs), and enhanced cocaine reward (Maze et al., 2010). To validate these earlier findings, animals were treated with either saline or cocaine (20 mg/kg/day) for 7 days. At 24 hr after the final injection, NAc dissections were collected and analyzed for global alterations in G9a and H3K9me2 expression. Consistent with previous data, levels of G9a mRNA (saline versus repeated cocaine, $t_{10} = 2.559$; $p < 0.05$) and protein (Figure 2A) and of H3K9me2 (Figure 2B) were reduced in NAc of animals treated with repeated, but not acute, cocaine. Such changes were absent in dorsal striatum (Figures S2A and S2B). Next, to examine potential similarities between cocaine- and stress-induced regulation of histone methylation in this brain region, the standard 10 day chronic social defeat stress protocol was used (Berton et al., 2006). As previously reported (Krishnan et al., 2007; Vialou et al., 2010), two distinguishable groups of defeated mice, susceptible and unsusceptible, were observed based on a measure of social avoidance, in which susceptible mice displayed decreased social interaction compared to both control and unsusceptible mice (Figure 2C). Ten days after the final defeat, susceptible and unsusceptible mice, as well as non-defeated controls, were analyzed for G9a protein levels in NAc. G9a expression was significantly decreased in NAc of susceptible mice compared to unsusceptible animals (Figure 2D), which were no different from nonstressed controls. These effects were not observed in dorsal striatum (Figure S2C). Consistent with a reduction in G9a expression in susceptible animals, global levels of H3K9me2 were decreased in NAc of these mice compared to both control and unsusceptible mice (Figure 2E). Additionally, *Glp* mRNA, similar to *G9a* mRNA, was significantly reduced in NAc of susceptible mice, a molecular response that was absent in unsusceptible mice (Figure 2H). Numerous other repressive chromatin modifiers, such as *Suv39h1*, many of which form multimeric-binding complexes with G9a and GLP

(Fritsch et al., 2010), were also significantly induced in NAc of unsusceptible mice (Figure 2H), indicating that increased repressive chromatin regulation may contribute to proadaptive responses to stressful stimuli. In dorsal striatum, in contrast, H3K9me2 was significantly increased after social stress in both susceptible and unsusceptible mice (Figure S2D). Regulation of H3K9me2 in NAc after chronic social stress is specific for this mark, which is euchromatic, as global levels of the associated heterochromatic mark H3K9me3 were unaltered in NAc of both susceptible and unsusceptible mice (Figure S3A).

To extend these findings in mice to clinical depression, we evaluated *G9a* and *Glp* levels, as well as other repressive chromatin modifiers, in NAc of postmortem human depressed patients (all symptomatic at their time of death; see Supplemental Experimental Procedures for detailed methods on tissue collection). Similar to results observed in mice susceptible to social stress, *G9a* (Figure 2F) and *Glp* (Figure 2H) mRNA levels were significantly reduced in these patients. Numerous other enzymes involved in transcriptional repression—shown to be significantly induced in NAc of unsusceptible mice—were also downregulated in NAc of human depressed subjects (Figure 2H). Consistent with decreased *G9a* and *Glp* expression in NAc of depressed humans, global levels of H3K9me2 were also significantly reduced (Figure 2G). Unexpectedly, H3K9me3 protein expression was decreased in NAc of human depressed cases (Figure S3B), a finding inconsistent with data from our social defeat model, where no changes occurred in susceptible or unsusceptible mice.

G9a in NAc Promotes Resilience to Chronic Social Stress

We next investigated the role of stress-induced alterations in G9a/GLP and H3K9me2 in NAc in controlling vulnerability to social stress. Animals were subjected to 10 days of chronic social defeat, and 24 hr after the final stress episode following a social interaction test, randomly selected susceptible mice received intra-NAc injections of herpes simplex virus (HSV) vectors expressing either wild-type G9a-GFP, which has previously been demonstrated to induce H3K9me2 in this brain region (Maze et al., 2010), or GFP as a control (Figure 3A). Mice were tested for social interaction 4 days after viral surgery, a time at which maximal transgene expression is seen (Figure 3B), as verified by western blotting (Figure 3C) and quantitative PCR (qPCR) (Figure S4A). As expected, stressed animals overexpressing GFP displayed reduced social interaction compared to GFP-expressing nonstressed controls. In contrast, G9a overexpression, which mimics induction of endogenous G9a in NAc of unsusceptible animals, blocked the chronic stress-induced deficits in social interaction (Figure 3D). GFP and G9a overexpression in both stressed and nonstressed animals had no effect on general locomotor activity (Figure 3E). These data support a role for G9a and H3K9me2 in mediating resilience to chronic social stress.

G9a Repression in NAc Promotes Susceptibility to Social Stress

To directly test whether G9a and H3K9me2 repression in response to repeated cocaine mediates the increased susceptibility to social defeat stress observed under these conditions, we

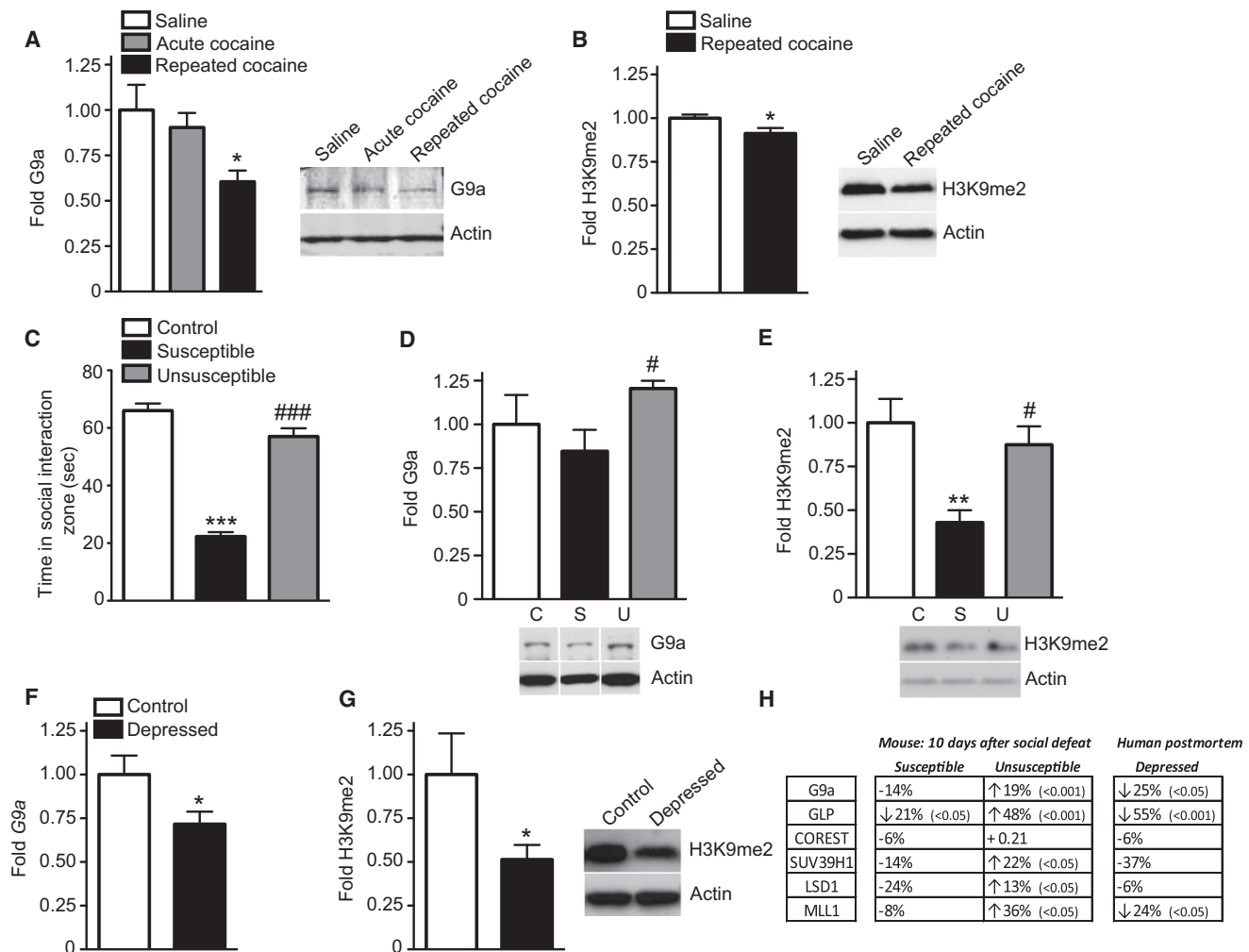


Figure 2. Cocaine, Social Stress, and Clinical Depression Similarly Regulate Repressive Histone Methylation in NAc

Repeated cocaine decreases (A) G9a and (B) H3K9me2 protein levels in NAc.

(C) Mice subjected to chronic (10 days) social stress can be grouped into susceptible and unsusceptible subpopulations.

(D) G9a shows a trend for reduced levels in susceptible mice compared to controls but is significantly reduced in susceptible animals compared to unsusceptible mice.

(E) H3K9me2 expression is reduced in susceptible animals, an effect not seen in unsusceptible animals. For all experiments, $n = 6-7/\text{group}$.

(F) G9a and (G) H3K9me2 protein are reduced in NAc of human postmortem depressed subjects ($n = 8-10/\text{group}$). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, significant differences from control condition; # $p < 0.05$ and ### $p < 0.001$, significant differences from susceptible mice. Data are presented as average \pm SEM.

(H) mRNA expression levels for chromatin-modifying enzymes were quantified in the NAc of control, susceptible, and unsusceptible mice after 10 days of social stress, and compared between clinically depressed and control individuals using postmortem tissue. Significant changes are noted by an arrow and a p value. Increased expression of several genes was observed in unsusceptible mice, as compared to susceptible animals. Likewise, many of these genes were significantly decreased in clinical depression. For mouse work, $n = 8/\text{group}$, and for human analysis, $n = 8-10/\text{group}$.

examined the influence of knocking down G9a in NAc (Maze et al., 2010) on the development of stress-induced depressive-like behaviors. $G9a^{fl/fl}$ mice were injected intra-NAc with HSV vectors expressing GFP or Cre-GFP before being subjected to submaximal (8 days) defeat stress (Figure 4A). G9a knockdown in NAc, which was confirmed immunohistochemically (Figure 4B) and quantitatively via western blotting (Figure 4C) and qPCR (Figure S4B), promoted increased susceptibility to social stress, similar to the effect of repeated cocaine (Figure 4D). This G9a knockdown also reduced sucrose preference after social defeat

(Figure 4E) but had no effect on baseline locomotor activity (Figure 4F).

G9a Overexpression in NAc Prevents Cocaine-Induced Vulnerability to Social Stress

The findings that G9a repression in NAc, which occurs after repeated cocaine, increases an animal's vulnerability to subsequent stress, and that G9a overexpression, which occurs in unsusceptible mice, reverses the behavioral deficits observed in susceptible animals, support the interpretation that

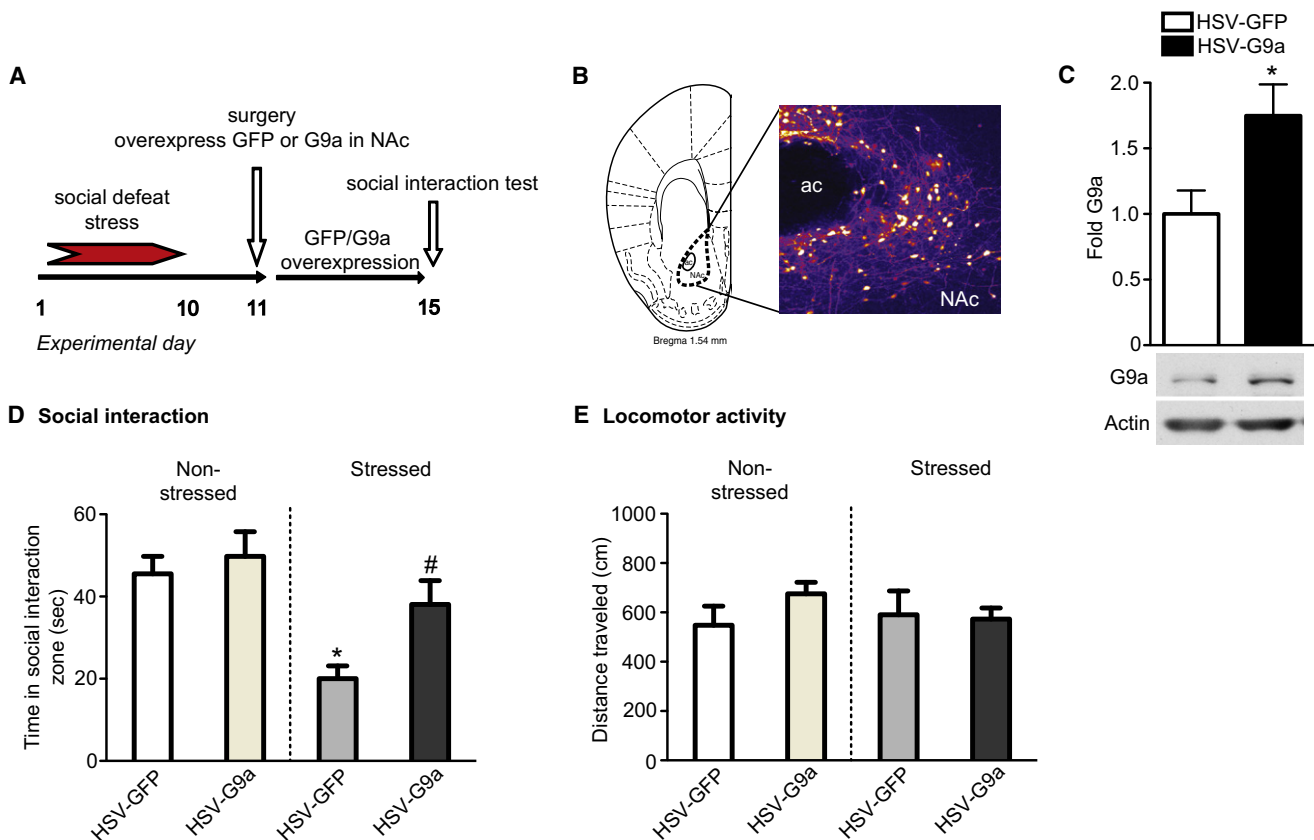


Figure 3. G9a Overexpression in NAc Reverses Social Defeat-Induced Social Deficits

(A) Time course of chronic (10 days) social defeat stress and HSV-GFP/HSV-G9a overexpression in NAc.

(B) Immunohistochemical analysis of HSV-G9a overexpression in NAc.

(C) Western blot analysis of G9a protein in NAc 4 days after HSV infection.

(D) Chronic social defeat stress reduces time spent in the social interaction zone during a social interaction test, an effect that can be reversed by viral overexpression of G9a in NAc.

(E) Neither social stress nor G9a overexpression affects locomotor activity in an open field. * $p < 0.05$, a significant difference from control condition (nondefeated mice infected with GFP); # $p < 0.05$, a significant difference from defeated mice infected with GFP. Data are presented as average \pm SEM ($n = 6$ group).

cocaine-induced repression of G9a and H3K9me2 in this brain region renders animals more vulnerable to future stress experiences. To directly examine this possibility, we increased G9a expression in NAc after repeated cocaine to test whether such gene “replacement” was sufficient to prevent cocaine-induced vulnerability to subsequent stress episodes. A compressed submaximal social defeat protocol was employed to accommodate the time course of HSV expression: animals were injected daily with saline or cocaine (20 mg/kg/day) for 7 days, followed by 8 defeats over a course of 4 days of twice per day (Figure 5A). Using this compressed protocol, animals receiving prior cocaine still exhibited increased susceptibility to repeated social stress, as evidenced by increased levels of social avoidance (Figure 5B), without deficits in general locomotor activity (Figure 5C), similar to observations using the 8 day submaximal protocol.

To test the role of G9a in cocaine-induced vulnerability to social stress, animals were injected once a day for 7 days with cocaine (20 mg/kg/day), before being injected intra-NAc with HSV-GFP or HSV-G9a-GFP, followed by 8 social defeats over 4 days (Figure 5D). As expected, cocaine-treated HSV-GFP

animals displayed increased vulnerability to social stress, as measured by social avoidance behavior. In contrast, cocaine-treated HSV-G9a-GFP animals did not display such deficits (Figure 5E), indicating that increasing G9a expression in NAc after repeated cocaine—and thereby opposing the cocaine-induced repression of endogenous G9a/GLP—is sufficient to prevent drug-induced vulnerability to subsequent stressful experiences without affecting baseline locomotor activity (Figure 5F).

G9a Repression by Cocaine Promotes Increased Vulnerability to Stress through Enhanced BDNF-TrkB Signaling in NAc

To gain insight into the molecular mechanisms by which alterations in G9a/GLP and H3K9me2 in NAc mediate cocaine-induced vulnerability to stress, we focused on BDNF-TrkB signaling, given the considerable overlap between the regulation of this pathway (Figure 6A) in the development of addictive- and depressive-like behaviors (see Discussion). Animals were treated with repeated cocaine (20 mg/kg/day) once daily for 7 days, before being injected intra-NAc with HSV-GFP or

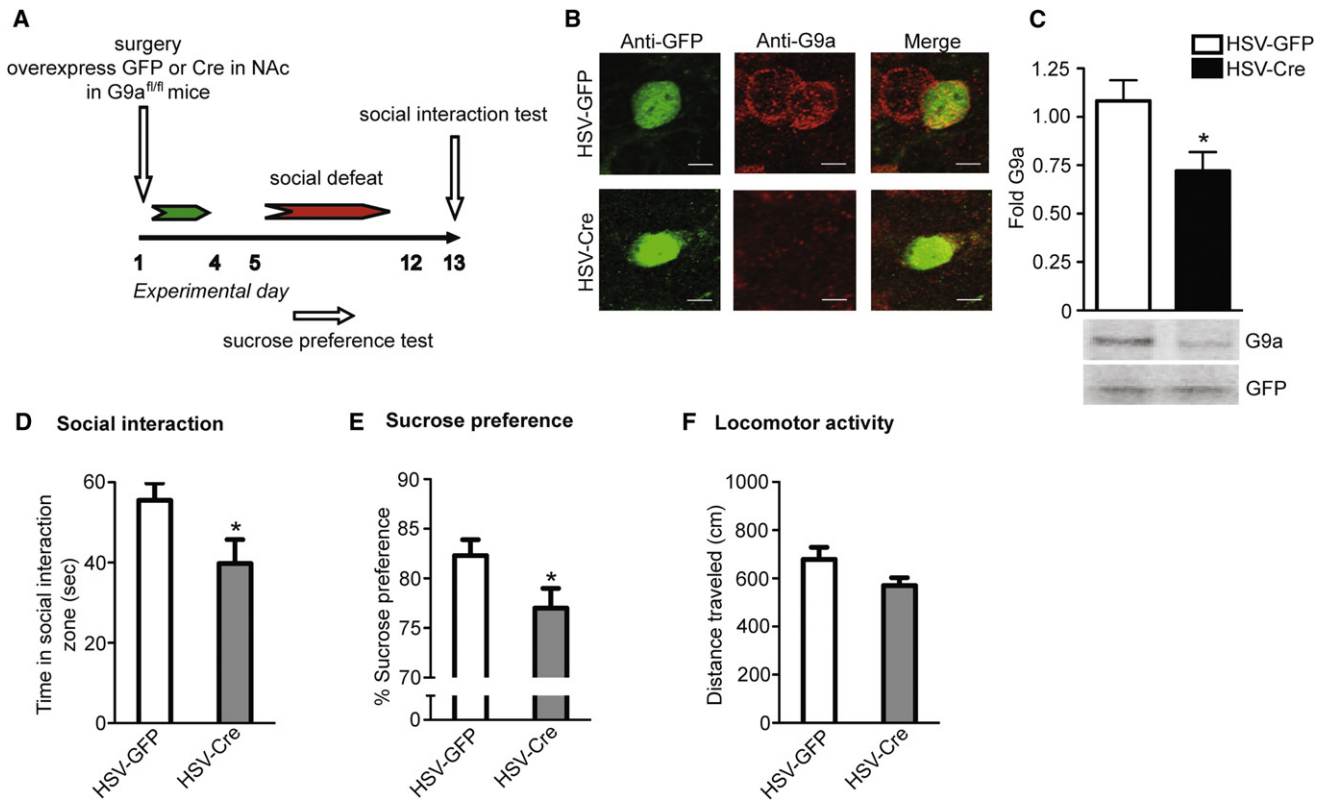


Figure 4. G9a Repression in NAc Promotes Susceptibility to Stress

(A) Time course of Cre-mediated G9a knockdown in NAc and subsequent submaximal (8 days) social defeat stress. (B) Immunohistochemical and (C) western blot validation of G9a knockdown in NAc using HSV-Cre-GFP viruses in *G9a^{fl/fl}* mice. Scale bar in (B), 10 μ M (high-power image). (D) G9a repression in NAc enhances social avoidance in a social interaction test. (E) G9a depletion in NAc promotes anhedonia as measured by sucrose preference. * $p < 0.05$, a significant difference from control condition (defeated mice infected with GFP). Data are presented as average \pm SEM ($n = 6-7$ /group). (F) G9a knockdown does not affect locomotor activity in an open field.

HSV-G9a-GFP, followed by 8 social defeats over 4 days (twice per day) (Figure 6B). As was shown in Figure 5, such G9a “replacement” in NAc after repeated cocaine reversed cocaine’s enhancement of stress vulnerability. At 48 hr after the final defeat experience, virally infected NAc tissue was analyzed for alterations in BDNF-TrkB signaling. Consistent with increased BDNF-TrkB signaling observed in NAc after chronic social defeat stress (see Krishnan et al., 2007), numerous other components of this signaling cascade, not previously examined, were upregulated by social stress in cocaine-experienced animals: including increased levels of phospho-Raf, phospho-MEK1/2, and phospho-CREB (Figure 6C, Figures S5C, S5E, and S5G). Although ERK1/2 has previously been demonstrated to display robust phosphorylation/activation after chronic social stress (Krishnan et al., 2007), such phosphorylation was not observed using our modified defeat protocol, possibly the result of an increased baseline level of phospho-ERK1/2 in NAc due to prior cocaine in these animals, as reported for cocaine previously (Valjent et al., 2000). Alternatively, such differences in ERK1/2 phosphorylation may result from the submaximal defeat protocol used. We also observed induction of total levels of Ras in NAc of mice treated chronically with cocaine followed by repeated social

defeat (Figure 6C). In contrast, total levels of other proteins in this cascade, including BDNF, TrkB, Raf, MEK1/2, ERK1/2, and CREB, were not altered. Also unaltered was Ras-GRF1, a guanine nucleotide exchange factor that activates Ras (Figure 6C, inset). These results suggest that induction of Ras expression per se may be responsible for activating downstream components of this signaling pathway necessary for the cocaine enhancement of depressive-like behaviors (Figure 6C).

Overexpression of G9a, which blocks cocaine-induced vulnerability to stress, significantly reduced cocaine- and stress-mediated increases in Ras expression in NAc, without affecting total protein levels of any of the other members of this pathway examined (Figure 6C and Figures S5A–S5H). NAc-specific overexpression of G9a did, however, significantly reduce the phosphorylation state of all downstream-signaling molecules, including phospho-Raf, phospho-MEK1/2, phospho-ERK1/2, and phospho-CREB, indicating that transcriptional repression of Ras activity by G9a suppresses the activity of this entire signaling cascade. It is important to note that G9a overexpression had no effect on total levels or phosphorylation state of any of the proteins examined in this pathway under control conditions (i.e., HSV-G9a in cocaine- and stress-naive mice),

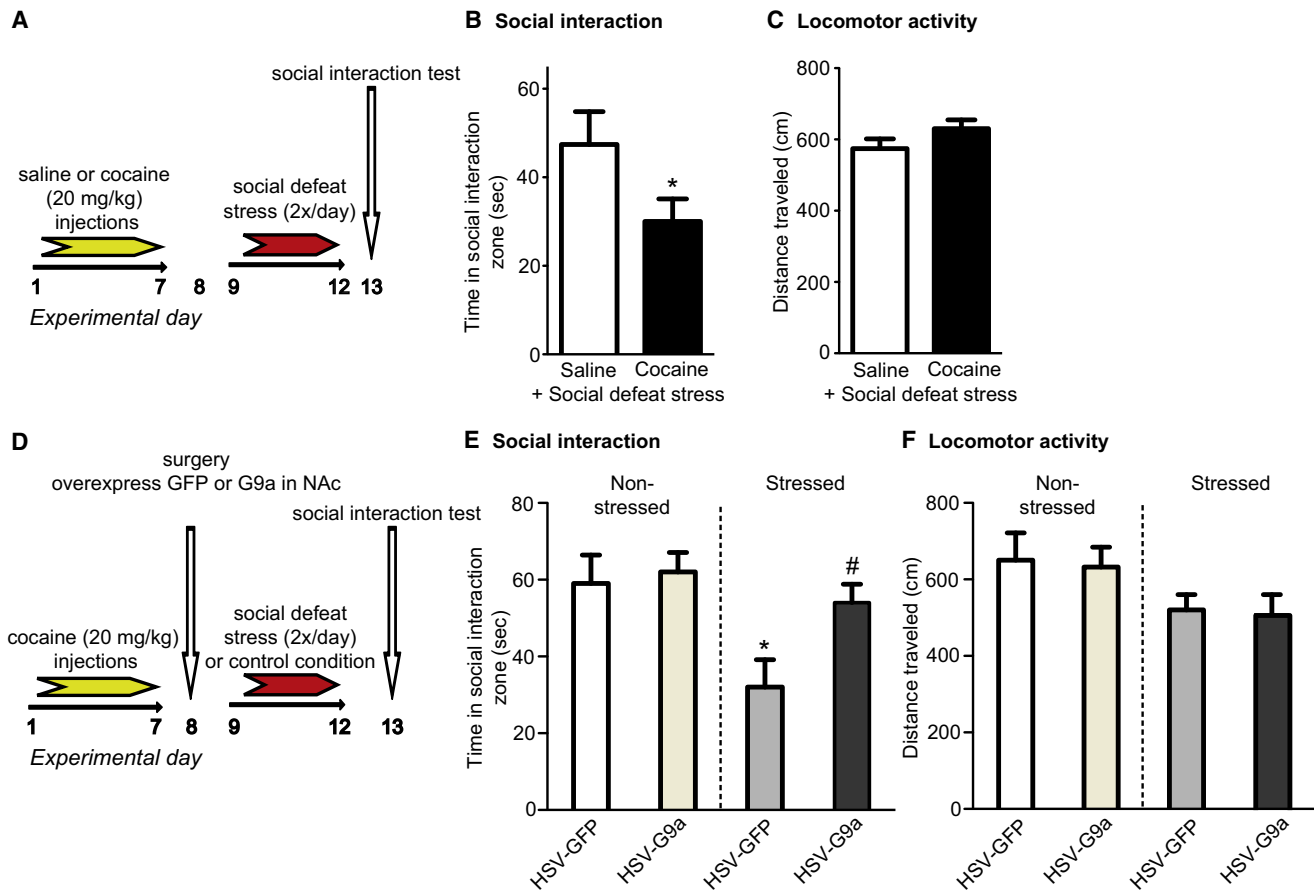


Figure 5. G9a Overexpression in NAc Prevents Cocaine-Induced Vulnerability to Stress

(A) Time course of saline or cocaine (20 mg/kg/injection) prior to control handling or compressed submaximal (8 defeats over 4 days) social defeat stress. (B) Repeated cocaine prior to compressed social stress increases social avoidance during a social interaction test. (C) Repeated cocaine does not affect locomotor activity in an open field following compressed social stress. * $p < 0.05$, significant differences from mice injected with saline ($n = 5\text{--}6/\text{group}$). (D) Time course of saline or cocaine (20 mg/kg/injection) prior to G9a overexpression, followed by compressed social defeat stress. (E) Overexpression of G9a in NAc following repeated cocaine significantly reduces cocaine-induced social avoidance during a social interaction test. (F) Prior cocaine, social stress, or G9a overexpression does not affect locomotor activity in an open field. * $p < 0.05$, a significant difference from control condition (nondefeated mice infected with GFP); # $p < 0.05$, a significant difference from defeated mice infected with GFP ($n = 10$ mice/group). Data are presented as average \pm SEM.

suggesting that G9a acts in a stimulus-dependent manner to block BDNF-TrkB signaling.

To further investigate the possibility that cocaine's induction of Ras contributes to increased stress vulnerability, we examined expression of *H-Ras1*, the *Ras* transcript most predominately expressed in adult brain, in NAc of animals receiving either saline, acute, or repeated cocaine, either immediately after cocaine exposure (1 hr) or 24 hr after the final drug dose. Although *H-Ras1* mRNA expression was unaffected by a single dose of cocaine, it was significantly induced by repeated cocaine at 1 hr, an effect that persisted for 24 hr (Figure 6D). A substantial literature indicates functional differences between NAc and dorsal striatum in the development of stress- and drug-induced behaviors (Saka et al., 2004; Di Ciano et al., 2008; Dias-Ferreira et al., 2009). We thus investigated Ras, ERK, and CREB in dorsal striatum in response to repeated cocaine or stress (Figures

S6A–S6G). Phospho-CREB was decreased in dorsal striatum by acute cocaine, and increased by repeated cocaine, similar to our observations in NAc (Figure S6D). Cocaine had no effect on Ras or ERK in dorsal striatum. Ras protein levels were increased in dorsal striatum after chronic social stress, but only in unsusceptible mice (Figure S6E). In contrast, Ras induction in NAc was observed exclusively in susceptible animals. These data highlight the importance and potential divergence of Ras-ERK-CREB signaling in NAc versus dorsal striatum in chronic drug and stress models (Berton et al., 2006; Krishnan et al., 2007).

In NAc the expression of two related GTPases, *Cdc42* and *Rac1*, which can also be activated in response to TrkB signaling, was unaltered after acute or repeated cocaine (Figures S7A and S7B). Since G9a overexpression in NAc selectively repressed Ras induction after social stress in cocaine-experienced

animals, we investigated whether *H-Ras1* represents a direct target of G9a, and whether *H-Ras1* expression correlates with changes in H3K9me2 promoter binding after either repeated cocaine or social defeat stress. Chromatin immunoprecipitation (ChIP) was performed using anti-G9a, anti-H3K9me2, or anti-acH3K9 antibodies to examine their binding to the *H-Ras1* gene promoter 24 hr after repeated cocaine or social stress. Consistent with changes in Ras expression, H3K9me2 displayed reduced (complemented by increased acH3K9) binding to the *H-Ras1* promoter following repeated (Figure 6E), but not acute (data not shown; $p > 0.05$), cocaine; such reduced binding of H3K9me2 was associated with a similar reduction in G9a binding to the *H-Ras1* promoter after repeated cocaine ($t_6 = 1.960$; $p < 0.05$).

To verify that Ras regulation in NAc influences the development of both addictive- and depressive-like behaviors, mice were socially defeated for 10 days, and their NAc were analyzed for *H-Ras1* expression. *H-Ras1* mRNA was significantly induced in NAc of susceptible, but not unsusceptible, mice 10 days after the last defeat episode (Figure 6F). Like repeated cocaine exposure, social stress reduced H3K9me2 binding to the *H-Ras1* promoter in susceptible mice only, whereas unsusceptible mice displayed increased H3K9me2 binding with no changes observed in acH3K9 promoter association (Figure 6G).

CREB Activity in NAc Promotes Prodepressive Behavioral Phenotypes

To verify that G9a-dependent alterations in Ras-CREB signaling after repeated cocaine or chronic social defeat directly affect behavioral responses to stress, we examined the effects of manipulating CREB on the development of depressive-like behaviors. Although CREB activity in NAc has been implicated in depressive-like behavior in routine assays such as the forced swim and sucrose preference tests (Carlezon et al., 2005), it has not to date been examined in the social defeat paradigm. Moreover, this previous work relied solely on the use of overexpression systems, which are prone to artifact. We thus generated a conditional *Creb^{f/f}* mouse line (see Figure S8 and Supplemental Experimental Procedures for detailed methods) to directly study the role of endogenous CREB in depression-like behavior. Following generation and validation of the line, adult *Creb^{f/f}* mice were injected intra-NAc with adeno-associated virus (AAV) vectors expressing GFP or Cre-GFP. At 18 days after viral injections (a time at which transgene expression is maximal), separate cohorts of animals were subjected to a battery of tests of depressive-like behaviors (Figure 7A). Knockdown of CREB was verified immunohistochemically and by counting CREB+ cells in NAc of AAV-Cre-GFP injected *Creb^{f/f}* mice (Figures 7B and 7C). Consistent with our hypothesis that G9a induction mediates behavioral resilience to chronic social stress in part through downregulation of CREB activity in NAc, AAV-Cre-GFP expressing *Creb^{f/f}* mice displayed consistent antidepressant-like behavioral responses in the social defeat paradigm (i.e., decreased social avoidance), forced swim test (i.e., decreased immobility), and sucrose preference test (i.e., increased sucrose preference), compared to AAV-GFP expressing animals (Figures 7D–7F).

DISCUSSION

Here, we demonstrate that repeated cocaine increases the severity of depressive-like responses to social stress in mice—a phenomenon that parallels high comorbid rates of substance abuse and mood disorders in humans. Furthermore, our data reveal a critical role for repressive histone methylation in NAc in mediating this cocaine-induced vulnerability to social stress. We show that repeated cocaine reduces global levels of H3K9me2 in this brain region, and its associated writer enzymes, G9a and GLP, which enhance susceptibility to subsequent social defeat stress. We demonstrate similar reductions in H3K9me2 and G9a/GLP levels in NAc of depressed humans and of mice subjected to chronic social defeat stress, but only in those animals susceptible to the negative consequences of repeated stress. We then establish that such downregulation of G9a and H3K9me2 in NAc mediates cocaine enhancement of stress vulnerability by demonstrating that local knockout of G9a in this brain region is sufficient to enhance an animal's vulnerability to social stress, while overexpression of G9a in NAc blocks the ability of chronic cocaine to increase stress susceptibility.

An important role for repressive H3K9me2 modifications in the regulation of both cocaine and stress responses comes from recent ChIP-chip studies that characterized altered H3K9me2 binding in NAc, genome wide, in response to repeated cocaine or chronic social defeat stress (Renthal et al., 2009; Wilkinson et al., 2009). Interestingly, we found that a majority of changes in H3K9me2 binding observed in NAc of susceptible mice were reversed by chronic treatment with standard antidepressant treatments and were not observed in unsusceptible animals (Wilkinson et al., 2009). Although chronic cocaine and chronic social defeat stress similarly regulate repressive histone methylation in NAc, development of therapeutics to target enzymes regulating these processes would be difficult, given the ubiquitous nature of histone methyltransferases and demethylases. Therefore, it will be important to identify downstream proteins affected by such alterations in histone methylation, with the hopes that such targets may be more suitable for future drug interventions.

Previous studies from our laboratory have shown that histone acetylation is persistently increased in NAc after chronic social stress (Covington et al., 2009). This effect was interpreted to be adaptive, since mimicking the effect by locally infusing histone deacetylase (HDAC) inhibitors into NAc exerts antidepressant-like actions in several behavioral assays (Covington et al., 2009). Repeated cocaine has also been demonstrated to increase histone acetylation in this brain region, a phenomenon shown to increase the rewarding, reinforcing, and locomotor-activating properties of the drug (Kumar et al., 2005; Renthal et al., 2007; Sanchis-Segura et al., 2009; Schroeder et al., 2008; Sun et al., 2008; Wang et al., 2010). These findings indicate that, in contrast to cocaine repression of G9a and H3K9me2 in NAc, cocaine induction of histone acetylation in this brain region exerts the opposite effect and protects animals from the deleterious consequences of chronic stress.

Numerous biochemical pathways have been implicated in stress- and cocaine-induced behaviors, whereby these stimuli produce similar alterations in the expression or function of many types of signaling proteins. A striking example is the

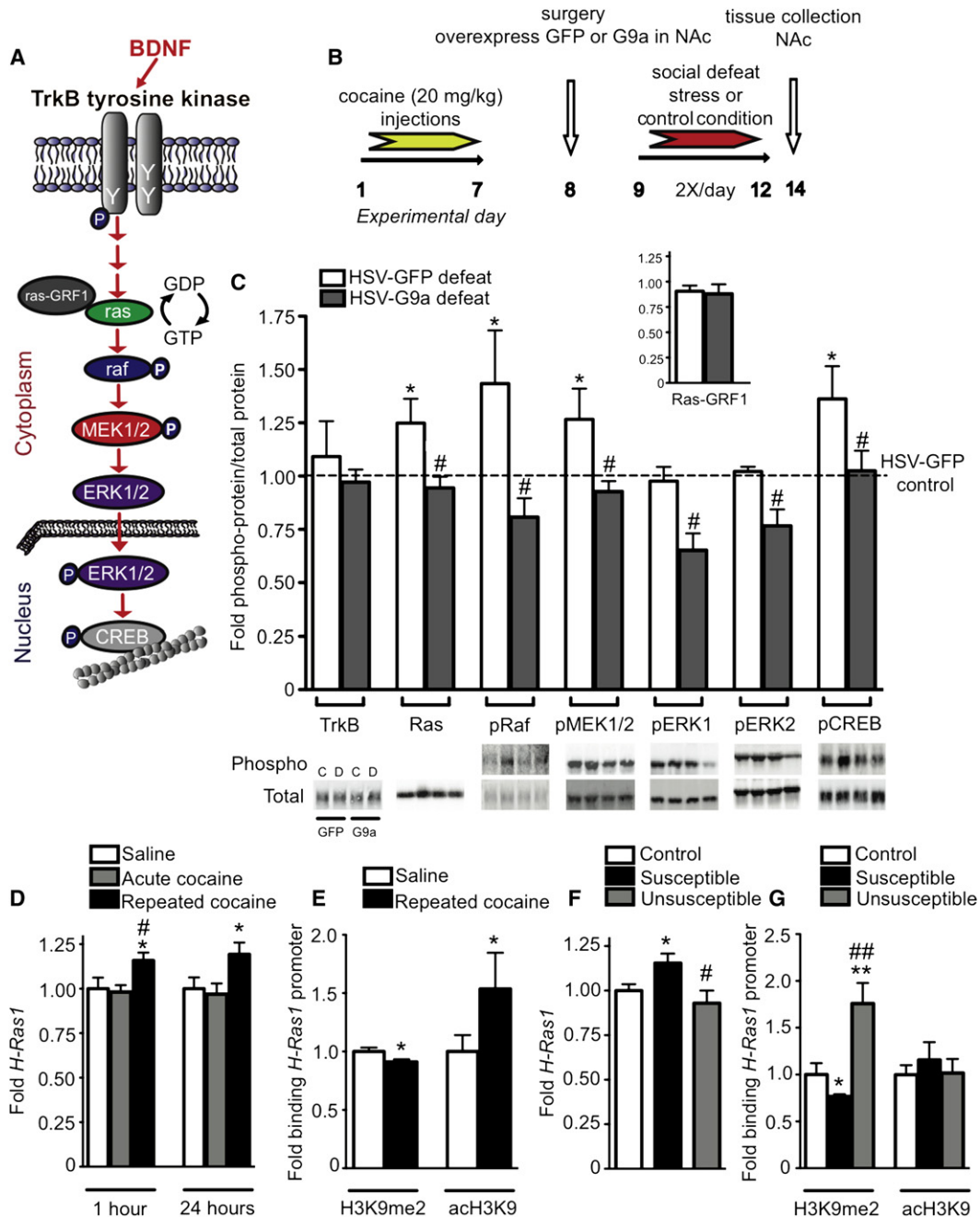


Figure 6. Repeated Cocaine and Chronic Social Stress Induce Ras and Downstream BDNF-TrkB Signaling in NAc

(A) Cartoon depicting stimulus-dependent regulation of the BDNF-TrkB signaling cascade.

(B) Time course of saline or cocaine (20 mg/kg/injection) prior to G9a overexpression, followed by control handling or compressed submaximal (8 defeats over 4 days) social defeat stress and NAc tissue collection ($n = 10$ mice/group).

(C) Compressed social stress following repeated cocaine promotes increased expression of Ras, a member of the BDNF-TrkB signaling cascade, thereby increasing the phosphorylation state of downstream members of the pathway. G9a overexpression, following prior cocaine exposure, prevents stress-induced increases in Ras signaling, and decreases the activity (phospho levels) of downstream signaling molecules, including Raf, MEK1/2, ERK1/2, and CREB. The Ras activator, Ras-GRF1, is unaffected by social stress and G9a overexpression (inset). * $p < 0.05$, a significant difference from control condition (nondefeated mice infected with GFP); # $p < 0.05$, a significant difference from defeated mice infected with GFP ($n = 8-9$ /group).

(D) Repeated, but not acute, cocaine treatment increases *H-Ras1* mRNA expression in NAc both at 1 and 24 hr. # $p < 0.05$, a significant difference from an acute cocaine.

(E) Repeated cocaine reduces H3K9me2 association and increases acH3K9 binding to the *H-Ras1* promoter ($n = 4$ /group; 5 mice pooled/ n).

(F) Like repeated cocaine, chronic (10 days) social stress increases *H-Ras1* mRNA expression in NAc of susceptible mice only ($n = 7$ /group). # $p < 0.05$, a significant difference from susceptible mice.

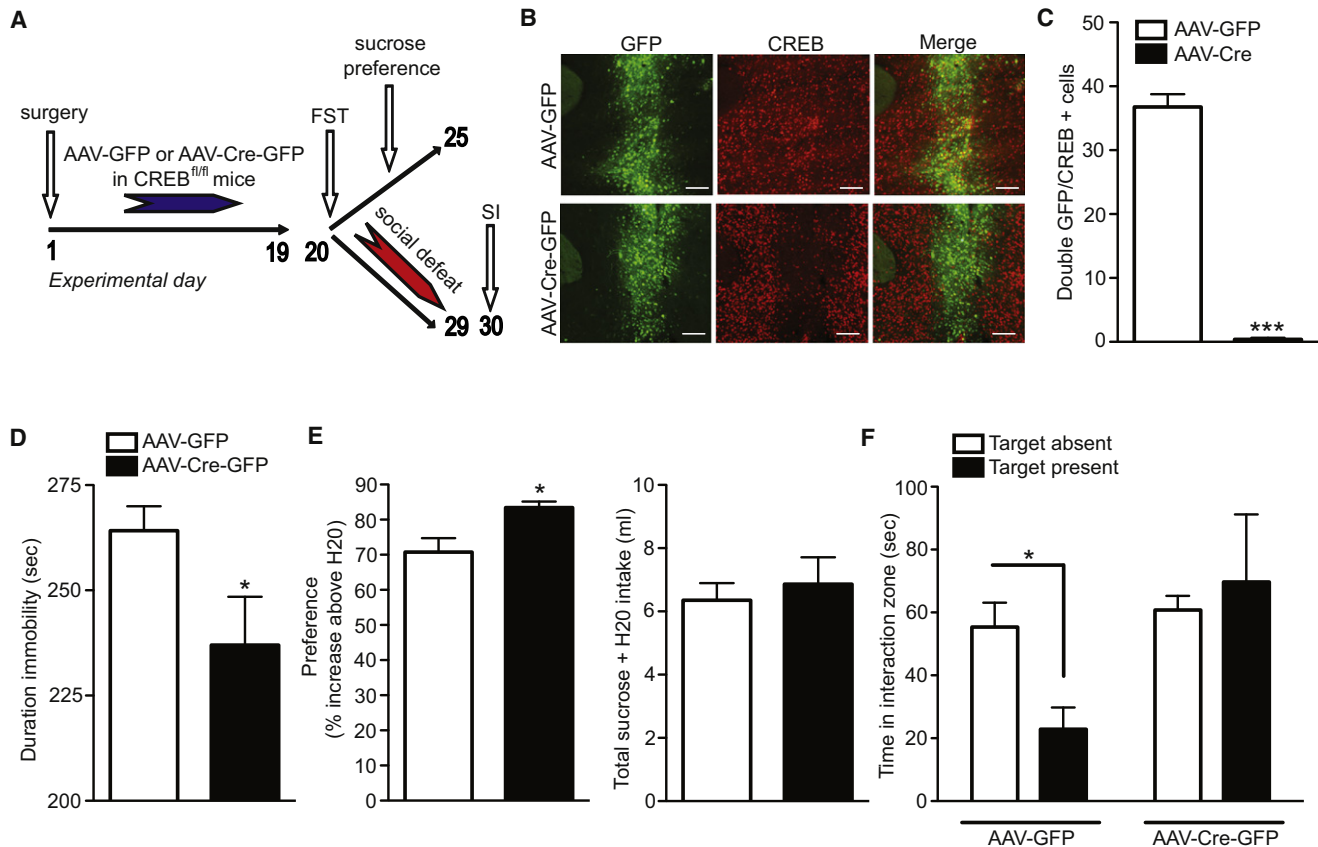


Figure 7. Local Knockdown of Endogenous CREB in NAc Promotes Antidepressant-like Behavioral Phenotypes

(A) Time course of CREB knockdown in *Creb^{fl/fl}* mice and subsequent analyses of depression-associated behaviors.

(B) Immunohistochemical validation of CREB knockdown in *Creb^{fl/fl}* mice using AAV-Cre-GFP viruses. Scale bar, 100 μ M (low-power image).

(C) Quantification of double-labeled GFP and CREB+ cells in NAc following knockdown in *Creb^{fl/fl}* mice using AAV-Cre-GFP viruses. *** $p < 0.001$, a significant difference from AAV-GFP ($n = 5$ /group).

(D) Viral-mediated knockdown of CREB in NAc reduces social avoidance behavior following chronic (10 days) social stress.

(E) Knockdown of CREB in NAc decreases the duration of immobility in the forced swim test.

(F) Knockdown of CREB in NAc increases sucrose preference without affecting total levels of fluid (sucrose plus water) intake. * $p < 0.05$, a significant difference from control condition (defeated mice infected with GFP) ($n = 6$ –8/group). Data are presented as average \pm SEM.

BDNF-TrkB cascade, which is upregulated in NAc by both cocaine and stress exposures and promotes addictive- and depressive-like behaviors (Bahí et al., 2008; Berglind et al., 2009; Berton et al., 2006; Cleck et al., 2008; Eisch et al., 2003; Graham et al., 2007; Green et al., 2010; Grimm et al., 2003; Horger et al., 1999). It should be noted, however, that, although G9a has previously been demonstrated to regulate *Bdnf* mRNA expression in NAc after repeated cocaine (Maze et al., 2010), local BDNF transcription in NAc does not affect behavioral responses to chronic stress (Krishnan et al., 2007). Therefore, it is unlikely that G9a's regulation of local BDNF expression in NAc after repeated cocaine treatment per se can fully account for the increased stress vulnerability observed in cocaine-exposed animals. Rather, our data implicate G9a regulation of

Ras expression in NAc as an important mediator of this phenomenon. We show that Ras is similarly upregulated in NAc by both chronic cocaine and stress, and represents one mechanism through which these two stimuli act to alter cell signaling through manipulations of a common pathway (Figure 8). This is consistent with prior reports of Ras's effect on behavioral responses to cocaine (Fasano et al., 2009; Ferguson et al., 2006; Zhang et al., 2007). Importantly, *H-Ras1* was one of the genes in our previous study that exhibited reduced H3K9me2 binding in NAc of susceptible mice only, with antidepressant treatment fully reversing this effect (Wilkinson et al., 2009). While we ascribe cocaine and stress regulation of Ras and CREB to the BDNF-TrkB signaling cascade, there are many other upstream pathways that could potentially be involved, including other

(G) Susceptibility to social stress reduces H3K9me2 binding to the *H-Ras1* promoter, whereas this repressive mark is increased at the *H-Ras1* promoter in unsusceptible mice (no changes were observed in acH3K9 binding after social defeat stress) ($n = 4$ /group; 5 mice pooled/ n). * $p < 0.05$, a significant difference from saline-injected or control mice. Data are presented as average \pm SEM.

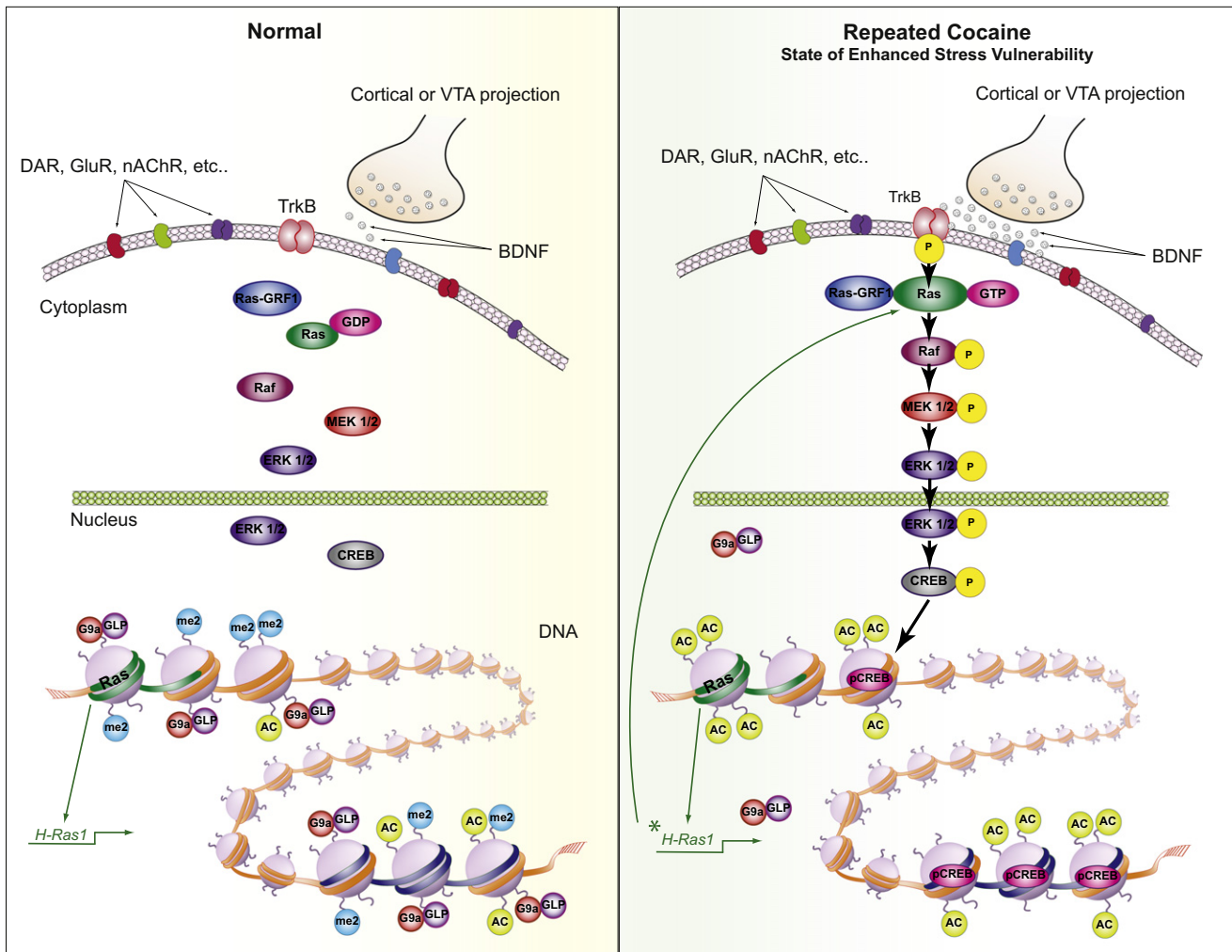


Figure 8. Enhanced Vulnerability to Stress via Cocaine-Induced Priming of BDNF Signaling in NAc

Repeated cocaine increases vulnerability to the depressive-like effects of social defeat stress by priming BDNF signaling through Ras induction in NAc. Under control conditions (left view), BDNF activation of TrkB signaling is limited. However, after repeated cocaine (right view), BDNF-TrkB signaling is elevated in NAc, causing enhanced phosphorylation and activity of downstream-signaling mediators including CREB. This cocaine-initiated adaptive response occurs not only through increased BDNF signaling in NAc but also through increased Ras expression as a result of decreased G9a binding at the *H-Ras1* gene promoter. Chronic stress is associated with similar adaptations in this brain region. Ras also appears to be a target for CREB, creating a positive feed-forward loop, promoting CREB activation and Ras expression as well as depressive-like behavior.

neurotrophic factors, G protein-coupled receptors, and many Ras modulatory proteins, to name a few (Zhang et al., 2007; Flavell and Greenberg, 2008; Meitzen et al., 2011).

Since induction of Ras in NAc by chronic cocaine and chronic stress would be expected to activate CREB, and CREB in this region has previously been shown to oppose cocaine reward and promote depression-like behavior (Barrot et al., 2002; Blendy, 2006; Carlezon et al., 2005; Pliakas et al., 2001), we focused on this protein further. We show that G9a overexpression in NAc, which represses Ras expression, also reduces levels of phospho-CREB in this brain region. Moreover, we show that local knockdown of endogenous CREB in NAc exerts antidepressant actions in the social defeat and other behavioral assays, consistent with several prior studies of CREB action in addiction and depression models. We have also shown that

genome-wide patterns of phospho-CREB binding to gene promoters in NAc of susceptible mice after chronic social defeat stress are reversed by chronic antidepressant treatment and not seen in unsusceptible animals (Wilkinson et al., 2009). Furthermore, microarray analyses of NAc obtained from CREB-overexpressing mice revealed that CREB activity in NAc was sufficient to induce *H-Ras1* expression in this brain region (McClung and Nestler, 2003), similar to that observed here with both chronic cocaine and chronic stress. Likewise, overexpression of mCREB, a dominant-negative form of CREB, reduced *H-Ras1* expression in NAc (McClung and Nestler, 2003) and induced antidepressant-like effects in simple behavioral tests (Barrot et al., 2002; Carlezon et al., 2005; Pliakas et al., 2001). These data indicate a role for CREB activity in the potentiation of Ras expression, in which Ras may act, through a positive

feedback loop, to increase its own expression by enhancing downstream CREB phosphorylation and activity (Figure 8). Taken together, BDNF-TrkB-Ras-CREB signaling in NAc may be one pathway through which both drugs of abuse and stress trigger shared molecular, cellular, and behavioral adaptations (Nestler et al., 2002; Pierce and Bari, 2001; Thomas et al., 2008). The contribution of the core and shell subdivisions of NAc to the phenomena examined here remains unknown. While the core and shell subserve distinct functions in drug and stress models (e.g., Di Ciano et al., 2008), the viral manipulations used in the current study cannot reliably distinguish these subregions, leaving the examination of this important question to future investigations.

Depressive illnesses are among the most prevalent psychiatric disorders in the United States, afflicting ~18% of the total population (Kessler et al., 2003). Only ~40% of all individuals treated with available antidepressants experience a full remission of symptoms, underscoring the high demand for better treatments (Berton and Nestler, 2006; Covington et al., 2010). Developing newer treatments has been limited by a scarcity of knowledge concerning the molecular biology of depression (Krishnan and Nestler, 2008). Experimental probes for mechanisms of cellular function have revealed increasingly complex interactions between enzymes and structural proteins that mediate processes ranging from genomic regulation to cellular morphology to neuronal excitability and synaptic plasticity. Here, we demonstrate that cocaine generates molecular events that become further elevated in response to chronic stress. Such findings may help to explain the large incidence of comorbidity observed for substance abuse and mood disorders, and provide insight into the molecular underpinnings of these illnesses. These studies pave the way for the elucidation of target molecules involved in these processes and the development of improved treatment agents.

EXPERIMENTAL PROCEDURES

Animals

Prior to experimentation, 9- to 11-week-old C57Bl/6 male mice (The Jackson Laboratory, Bar Harbor, ME, USA) were group housed at 5 per cage in a colony room set at constant temperature (23°C) on a 12 hr light/dark cycle (lights on from 0700 to 1900 hr) with ad libitum access to food and water. All protocols involving mice were approved by the Institutional Animal Care and Use Committee (IACUC) at Mount Sinai School of Medicine.

To knock out G9a specifically in NAc neurons, we used mice homozygous for a mutant floxed G9a allele (*G9a^{fl/fl}*) that were fully backcrossed to C57Bl/6J, as described elsewhere (Maze et al., 2010; Sampath et al., 2007; Schaefer et al., 2009). Mice were injected stereotactically in NAc with HSV vectors bicistronically expressing GFP or Cre + GFP under distinct promoters, as described previously (Maze et al., 2010). Immunohistochemistry was used to verify Cre-mediated knockdown of G9a (Figure 4B). HSV vectors were allowed to express in NAc for a minimum of 4 days postsurgery because recombination in *G9a^{fl/fl}* mice was observed to be maximal and stable at this time, consistent with published reports (Maze et al., 2010). G9a overexpression experiments were conducted similarly using HSV vectors expressing either GFP or wild-type G9a plus GFP. HSV vectors have been extensively demonstrated, in numerous previous studies, to only infect neuronal cell bodies within the injected brain area, without affecting glial cells or efferent or afferent neurons.

To induce local deletion of the *Creb* transcript restricted to NAc neurons, mice were stereotactically injected intra-NAc with AAV vectors (serotype 2) expressing GFP or Cre-GFP between the age of 7 and 9 weeks. Immunohisto-

chemical analysis was used to verify the efficiency of Cre-mediated recombination (Figure 7B). AAV-injected animals 18 days postsurgery were used since recombination in *Creb^{fl/fl}* mice was stable and maximal at this time point.

Behavior

Social Defeat Stress

Mice were subjected to 10 days, submaximal (8 days), or compressed submaximal (8 defeats over 4 days) social defeat based on published reports (Berton et al., 2006; Krishnan et al., 2007). In brief, experimental mice were exposed to a novel CD1 aggressor for 5 min daily, and then separated from the aggressor behind a protective barrier, which was perforated to allow for sensory contact for the remainder of the day. During the 5 min physical defeat, visible signs of subordination were observed. Nondefeated control mice were housed two per cage under the same conditions as experimental mice, but without the presence of a CD1 mouse. After the last social defeat episode, experimental and control mice were housed individually.

Social Interaction

Tests for social interaction were performed as previously described (Berton et al., 2006). Briefly, mice were placed within a novel arena that included a small animal cage at one end. Movement (distance traveled, in centimeters) was initially monitored for 250 s for each stressed or control mouse in the absence of a CD1 mouse, immediately followed by an additional 250 s in the presence of a CD1 mouse, which was positioned within the small animal cage. Locomotor activity (distance traveled) and information pertaining to the duration spent in the interaction zone were obtained using EthoVision 3.0 software (Noldus, Attleboro, MA, USA).

Open-Field Test

Open-field assessments were conducted in arenas similar to those used for the social interaction tests (without small cage enclosures). EthoVision video tracking-based methods (Noldus) were used to record the distance traveled and the time spent in the open arena and a delineated "center zone" (34 × 34 cm).

Sucrose Preference Test

Stoppers fitted to 50 ml tubes with ballpoint sipper tubes to prevent leakage (Ancare, Bellmore, NY, USA) were filled with solutions of either 1% sucrose (in drinking water) or drinking water. All animals were acclimatized for 3 days before the two-bottle choice conditions prior to 4 additional days of choice testing (noon to noon) while mice underwent social defeat. Immediately prior to each daily social defeat, fluid levels were noted, and the positions of the tubes were interchanged. Sucrose preferences were calculated as the average percentage of sucrose/water consumed for each of the 4 days.

Forced Swim Test

As previously described (Krishnan et al., 2007), each forced swim test was carried out in a 4 liter beaker containing approximately 3 liters of tap water, at a temperature of 25°C ± 1°C. The duration of time spent immobile in the arena over a 6 min trial was determined using EthoVision video tracking-based methods (Noldus).

Locomotor Activity

Locomotor activity was assessed in a novel cage fitted within a photocell grid device (Med Associates Inc., St. Albans, VT, USA) that counted the number of ambulatory photo beam breaks within 5 min blocks during a 1 hr long period.

Viral-Mediated Gene Transfer

Expression plasmids for Cre recombinase and wild-type G9a were subcloned into HSV or AAV vectors and packaged into high-titer viral particles as previously described (Berton et al., 2006; Maze et al., 2010). Mice were positioned in small animal stereotaxic instruments, under ketamine (100 mg/kg)/xylazine (10 mg/kg) anesthesia, and their cranial surfaces were exposed. Thirty-three gauge syringe needles were bilaterally lowered into the NAc to infuse 0.5 μl of virus at a 10° angle (anterior/posterior + 1.6; medial/lateral + 1.5; dorsal/ventral - 4.4). Infusions occurred at a rate of 0.1 μl/min. Animals receiving HSV injections were allowed to recover for at least 24 hr following surgery.

RNA Isolation, Reverse Transcription, and qPCR

NAc punches were dissected after treatment with cocaine, social defeat, or a combination thereof post-viral surgery and frozen on dry ice. Samples were then homogenized in TRIzol and processed as previously described

(Maze et al., 2010). See Supplemental Experimental Procedures for more details.

Western Blotting

Frozen NAc or caudate putamen (CPu) tissue was homogenized in 30 μ l of homogenization buffer containing 320 mM sucrose, 5 nM HEPES buffer, 1% SDS, phosphatase inhibitor cocktails I and II (Sigma, St. Louis), and protease inhibitors (Roche, Basel, Switzerland) using an ultrasonic processor (Cole Parmer, Vernon Hills, IL, USA). Protein concentrations were determined using a DC protein assay (Bio-Rad, Hercules, CA, USA), and 10–30 μ g of protein was loaded onto 18% or 4%–15% gradient Tris-HCl polyacrylamide gels for electrophoresis fractionation (Bio-Rad). See Supplemental Experimental Procedures for additional methods and listing of antibodies.

Immunohistochemistry

Mice were anesthetized with a lethal dose of chloral hydrate and perfused intracardially with 4% paraformaldehyde before being examined using single or double immunohistochemistry as previously described (Maze et al., 2010). See Supplemental Experimental Procedures for additional methods and listing of antibodies.

ChIP

Freshly dissected NAc punches (14G) were crosslinked with formaldehyde and prepared for ChIP as described previously (Maze et al., 2010). See Supplemental Experimental Procedures for detailed methods.

Drugs

Cocaine-HCl was purchased from Sigma-Aldrich (St. Louis) and used at a concentration of 20 mg/kg/i.p., and mice were immediately returned to their home cage after each injection, unless otherwise noted.

Statistics

See Supplemental Experimental Procedures for detailed statistical methods and statistical results.

SUPPLEMENTAL INFORMATION

Supplemental Information includes eight figures, Supplemental Experimental Procedures, and detailed results and can be found with this article online at doi:10.1016/j.neuron.2011.06.007.

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