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# Rac1 is essential in cocaine-induced structural plasticity of nucleus accumbens neurons

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**Repeated cocaine administration increases the dendritic arborization of nucleus accumbens neurons, but the underlying signaling events remain unknown. Here we show that repeated exposure to cocaine negatively regulates the active form of Rac1, a small GTPase that controls actin remodeling in other systems. Further, we show, using viral-mediated gene transfer, that overexpression of a dominant negative mutant of *Rac1* or local knockout of *Rac1* is sufficient to increase the density of immature dendritic spines on nucleus accumbens neurons, whereas overexpression of a constitutively active *Rac1* or light activation of a photoactivatable form of Rac1 blocks the ability of repeated cocaine exposure to produce this effect. Downregulation of *Rac1* activity likewise promotes behavioral responses to cocaine exposure, with activation of Rac1 producing the opposite effect. These findings establish that Rac1 signaling mediates structural and behavioral plasticity in response to cocaine exposure.**

Experience-dependent structural plasticity in the adult brain has been extensively implicated in long-term adaptations that underlie several psychiatric syndromes, including drug addiction[1–3]. Addiction is marked by long-lasting changes in behavior that persist despite prolonged abstinence[4]. Increasing evidence suggests that morphological changes in neurons that comprise the brain's reward circuitry contribute to these lasting behavioral abnormalities. For example, repeated administration of cocaine or other psychostimulants induces a persistent increase in dendritic spine density and complexity of dendritic branching in medium spiny neurons of the nucleus accumbens (NAc), a key brain reward region[2,5,6]. Dendritic spines are protrusions of the dendritic membrane upon which >90% of excitatory synapses are formed[7]. However, the molecular mechanisms mediating these changes are poorly understood. Whereas there have been several reports directly linking transcriptional mechanisms to cocaine-induced NAc dendritic plasticity[8–13], the events more proximal to spine growth and actin remodeling remain largely unknown.

Dendritic spines are highly plastic and dynamic[7,14], with spine growth and retraction implicated in experience-dependent plasticity in many neural systems[15]. The formation of new spines and reshaping of pre-existing spines is dependent upon remodeling of the actin cytoskeleton, and chronic cocaine administration has been shown to regulate actin turnover in the NAc, as inferred from complex, time-dependent changes in amounts of F-actin and in the phosphorylated states of several actin-binding proteins and the actin-severing protein cofilin[16–18] (see Discussion). In other systems, regulation of actin turnover is governed in large part by small GTPases[19], particularly the Rho family, which includes Rac1, RhoA and CDC42 (ref. 20). Rac1 is involved in dendritic remodeling in cortical and hippocampal neurons both *in vitro*[21–23], as well as *in vivo*[24,25], and Rac1 activation regulates cytoskeleton reorganization, at least in part, through the modulation of cofilin[26,27]. However, despite these insights, causal data directly linking Rac1 signaling to cocaine-induced spine plasticity in NAc is completely lacking. We show here that repeated cocaine exposure primes NAc neurons for transient drug-induced downregulation of Rac1 activity and that such repression of Rac1, through signaling to cofilin, is responsible for the expansion of dendritic spines on NAc neurons and for enhanced cocaine reward.

## RESULTS

### Regulation of Rac1 signaling by cocaine

Because spine plasticity is regulated by the dynamic switching of Rac1 from its inactive GDP-bound form to the active GTP-bound form, we first investigated whether cocaine regulates the activity of Rac1 in the mouse NAc *in vivo*. Immunoprecipitation using an antibody specific for the active form of Rac1 showed that repeated, but not acute, cocaine administration decreased the abundance of active Rac1 15 min after a cocaine challenge, without a change in total Rac1 (**Fig. 1a**). This decrease was highly transient, as amounts of active Rac1 returned to normal within 45 min (**Fig. 1b**) and remained unchanged 24 h after cocaine administration (109.5 ± 10.1 for cocaine-treated mice versus 100.3 ± 9.2 for saline controls; $n = 8$ mice per group; $F_{1,15} = 0.5$; $P > 0.05$; analyzed by ANOVA), consistent with the known stimulus-dependent and transient nature of GTPase

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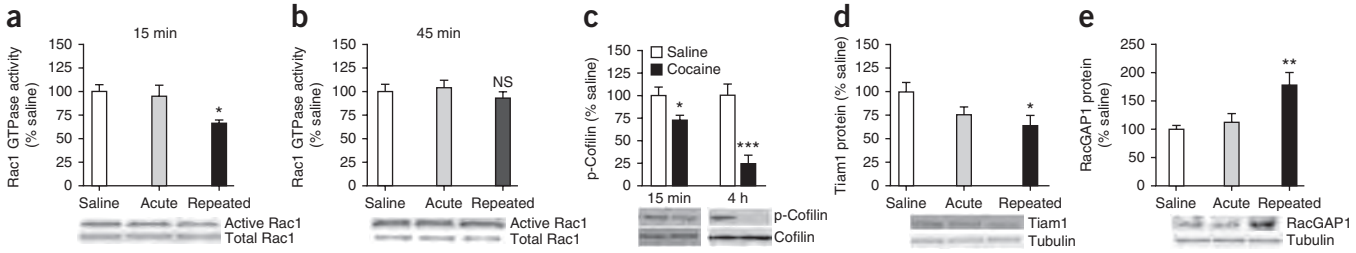


**Figure 1** Cocaine regulation of Rac1 signaling in NAc. (**a**) Repeated, but not acute, cocaine administration transiently decreased Rac1 GTPase activity in NAc 15 min after the final drug injection compared with acute and saline treatments ($n = 7$ or 8 mice per group; $F_{2,21} = 4.50$). (**b**) This decrease in Rac1 GTPase activity was no longer present at 45 min ($n = 7$ or 8 mice per group). At both the 15 and 45 min time points, there were no differences in total amounts of Rac1 ($P > 0.05$, data not shown). (**c**) The inactive (phosphorylated) form of cofilin (p-cofilin) was significantly decreased 15 min after repeated cocaine exposure ($n = 9$ mice per group; $F_{1,17} = 6.29$) and remained decreased at 4 h ($n = 5$ or 6 mice per group; $F_{1,10} = 22.5$). Total amounts of cofilin were unaltered by cocaine at both time points ($P > 0.05$; data not shown). (**d**) At 24 h after the final cocaine treatment, amounts of Tiam1 protein, normalized to tubulin (which was not altered), were significantly lowered by repeated cocaine exposure ($n = 7$ or 8 mice per group; $F_{2,22} = 3.54$). (**e**) RacGAP1 protein, normalized to tubulin, was significantly increased at this same 24-h time point ($n = 8$ or 9 mice per group; $F_{2,14} = 6.73$). Full-length western blots are presented in **Supplementary Figure 2**. Data analyzed by ANOVA; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; NS, not significant ($P > 0.05$); error bars, s.e.m.

signaling in other systems[28,29]. This decrease in GTPase activity was specific to Rac1, as there was no change in activity of the cell division control protein CDC42 (105.9 ± 11.65 for cocaine treated mice versus 100.5 ± 8.43 for saline controls; $n = 6$ mice per group; $F_{1,11} = 0.02$, $P > 0.05$; analyzed by ANOVA). In line with the decreased Rac1 activity observed in response to repeated cocaine exposure, amounts of phosphorylated cofilin, but not total cofilin, also decreased at the 15-min and 4-h time points examined (**Fig. 1c**). Because the phosphorylation of cofilin inhibits its actin-severing properties, these findings suggested that a cocaine challenge after repeated cocaine exposure transiently increases the amount of active cofilin and thereby actin turnover in the NAc.

To identify a mechanism by which cocaine downregulates Rac1 activity, we examined the guanine nucleotide exchange factor (GEF) Tiam1 and the GTPase activating protein (GAP) RacGAP1 (also known as MgcRacGAP). Repeated, but not acute, cocaine administration significantly downregulated Tiam1 expression (**Fig. 1d**), but increased RacGAP1 expression (**Fig. 1e**), in the NAc. These altered expression patterns were more lasting than the changes in Rac1 activity, being observed 24 h after the last cocaine injection, which suggested that they prime the ability of a cocaine challenge to trigger transient suppression of Rac1 activity.

### Rac1 activity in the NAc regulates reward behavior

To assess the behaviorally relevant consequences of decreased Rac1 activity we used the conditioned place preference model (**Fig. 2**), which provides an indirect measure of cocaine reward (see Online Methods). We bilaterally injected herpes simplex virus (HSV) vectors expressing either a dominant-negative (T17N) mutant of Rac1 (HSV–Rac1-dn) or a constitutively active mutant (Q61L) of Rac1 (HSV–Rac1-ca) (ref. 30) into the shell of the mouse NAc. HSV expressing dsRed (a red fluorescent protein) was used as a control. Intra-NAc injection of HSV–Rac1-ca blocked cocaine reward as measured by place conditioning, as well as the acute locomotor-activating effect of the drug, suggesting that cocaine's suppression of Rac1 activity is required for normal rewarding and locomotor responses to the drug (**Fig. 2a**,**c**). Conversely, intra-NAc injection of HSV–Rac1-dn promoted conditioned place preference to cocaine at a drug dose (4 mg per kilogram body weight) that did not induce a preference in HSV-dsRed–injected control mice (**Fig. 2b**), without altering acute locomotor responses to the drug at 7.5 mg kg$^{-1}$ (**Fig. 2c**). Because a decrease in Rac1 activity increases cofilin activity, we overexpressed a constitutively active mutant of cofilin (S3A), which cannot be phosphorylated and therefore increases actin remodeling[31]. Consistent with our HSV–Rac1-dn data, HSV–cofilin-ca injections into the NAc likewise increased the rewarding effects of cocaine without altering locomotor responses to the drug (**Fig. 2b**,**c**). Locomotor responses to saline were not changed by Rac1-ca, Rac1-dn, or cofilin-ca (**Fig. 2d**), and our HSV vectors were able to selectively target the NAc (**Fig. 2e**). NAc medium spiny neuron were infected with HSV–GFP and labeled (**Fig. 2f**).

To further study the role of Rac1 in cocaine-induced behavioral plasticity and to ensure specificity of our Rac1-dn results (since Rac1-dn could conceivably interfere with the functioning of related small GTPases), adult homozygous *lox*P-flanked (floxed) Rac1 mice[32,33] received bilateral intra-NAc injections of HSV vectors expressing Cre recombinase or GFP as a control. Overexpression of Cre significantly decreased *Rac1* mRNA in the NAc as determined by quantitative PCR (qPCR) (21 ± 2.7% decrease compared to HSV–GFP controls; $n = 3$ or 4 mice per group; $t = 2.60$, d.f. = 6, $P < 0.05$; analyzed by two-tailed *t*-test). The relatively small degree of knockdown probably reflects uninfected cells in the grossly dissected tissue, as our HSV vectors target only neurons and Rac1 is highly expressed in non-neuronal cells as well. Indeed, immunohistochemistry confirmed the complete loss of Rac1 from Cre$^+$ medium spiny neurons of the NAc (**Fig. 2g**), whereas expression of GFP alone did not affect Rac1 staining (data not shown). Using a low 4 mg kg$^{-1}$ dose of cocaine, we found that floxed Rac1 mice injected with HSV–Cre showed a significant increase in cocaine preference, +238 ± 80.5 s compared with HSV–GFP-injected floxed Rac1 control mice, which showed no preference for this cocaine dose ($n = 6$ mice per group; $t = 2.35$, d.f. = 10, $P < 0.05$; analyzed by two-tailed *t*-test). Consistent with previous reports of Rac1 cellular localization[34], the Rac1 immunostaining in NAc neurons concentrated at the subplasma membrane of the soma. These data further support the ability of decreased Rac1 activity in NAc to increase sensitivity to cocaine reward.

Because repeated cocaine exposure induced a highly transient decrease in Rac1 activity in NAc that returns to normal within 45 min of a cocaine challenge, we sought to determine whether a more temporally precise blockade of this decrease would also block cocaine reward. To this end, we used a photoactivatable form of Rac1, where Rac1-ca is fused to the photoreactive LOV (light oxygen voltage) domain from phototropin; this prevents the interaction of Rac1 with effector proteins until stimulated with a 458–473 nm light[34].

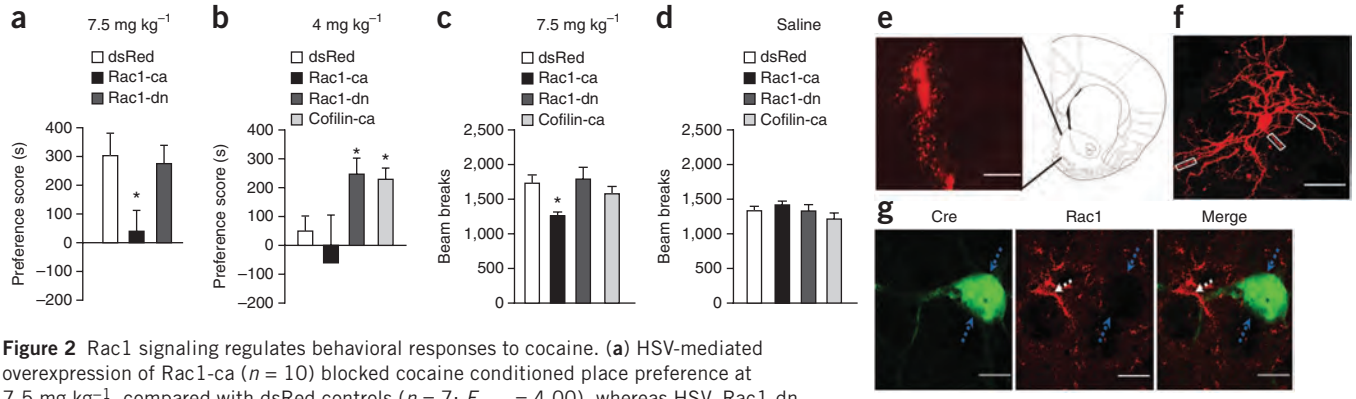


**Figure 2** Rac1 signaling regulates behavioral responses to cocaine. (**a**) HSV-mediated overexpression of Rac1-ca ($n = 10$) blocked cocaine conditioned place preference at 7.5 mg kg$^{-1}$, compared with dsRed controls ($n = 7$; $F_{2,23} = 4.00$), whereas HSV–Rac1-dn ($n = 9$) did not further enhance cocaine's rewarding effects at this higher dose. (**b**) Using a lower dose of cocaine (4 mg kg$^{-1}$), which did not induce a conditioned place preference in control mice ($n = 13$), HSV–Rac1-dn ($n = 8$) induced a robust preference to the drug, compared with the control ($F_{3,37} = 3.33$). Overexpression of a constitutively active form of cofilin (HSV–cofilin-ca; $n = 10$ mice) also increased the reward sensitivity to cocaine, compared with the controls. (**c**,**d**) Rac1-ca ($n = 10$) attenuated locomotor activation following an acute injection of 7.5 mg kg$^{-1}$ cocaine ($F_{3,41} = 3.06$), compared with control (**c**) but had no effect on locomotor responses to a saline challenge ($P > 0.05$) (**d**). (**e**) Anatomical placement of viral infection in NAc after HSV injection. Cartoon shows the location of the injection site, 1.77 mm from bregma. Scale bar, 100 μm. (**f**) A representative HSV-infected NAc medium spiny neuron imaged at original magnification ×40. Rectangular boxes highlight areas used for dendritic spine analysis in **Figure 4**. Scale bar, 50 μm. (**g**) Immunohistochemical images of Cre (green) and Rac1 (red) after HSV–Cre injection into the NAc of a floxed Rac1 mouse. Blue arrows highlight a Cre$^+$ neuron in which Rac1 staining is completely absent; white arrows indicate an adjacent non–Cre-expressing neuron where Rac1 protein is strongly expressed. Representative of sections from four mice. Scale bars, 10 μm. $^*P < 0.05$. ANOVA; error bars, s.e.m.

This tool enabled us to test the behavioral consequences of discrete activation of Rac1 in NAc *in vivo* on a time scale that would functionally reverse the transient decrease in Rac1 caused by cocaine. Mice received intra-NAc injections of HSV–Rac1-photoactivatible (Rac1-pa), HSV–Rac1-pa(C450A) (a mutant that contains the identical LOV domain but is light-insensitive) or control HSV–GFP and were fitted with cannulas to allow the passage of a fiber optic cable[35]. Light activation of Rac1-pa increased the phosphorylated form of cofilin when compared to both HSV–GFP and the light-insensitive mutant Rac1-pa(C450A), without a change in total amounts of cofilin (**Fig. 3a**), demonstrating the efficacy of this construct in the NAc *in vivo*. Moreover, such discrete light activation of Rac1-pa prevented the formation of a conditioned place preference to cocaine (**Fig. 3b**) and suppressed locomotor activity to an acute injection of cocaine (**Fig. 3c**), without altering the locomotor response to saline (**Fig. 3d**), thus paralleling the effects of Rac1-ca. Overexpression of Rac1-pa without light stimulation had no effect on behavioral responses to cocaine. Similarly, overexpression and light stimulation of the control vectors (C450A or GFP) had no effect on place preference or locomotion. Overexpression of these Rac1-pa constructs had no effect on the structure of NAc medium spiny neurons (**Fig. 3e–g**). These data show that a highly temporally discrete and transient activation of Rac1 activity and subsequent downstream signaling cascades in NAc is sufficient to attenuate the rewarding effects of cocaine, providing

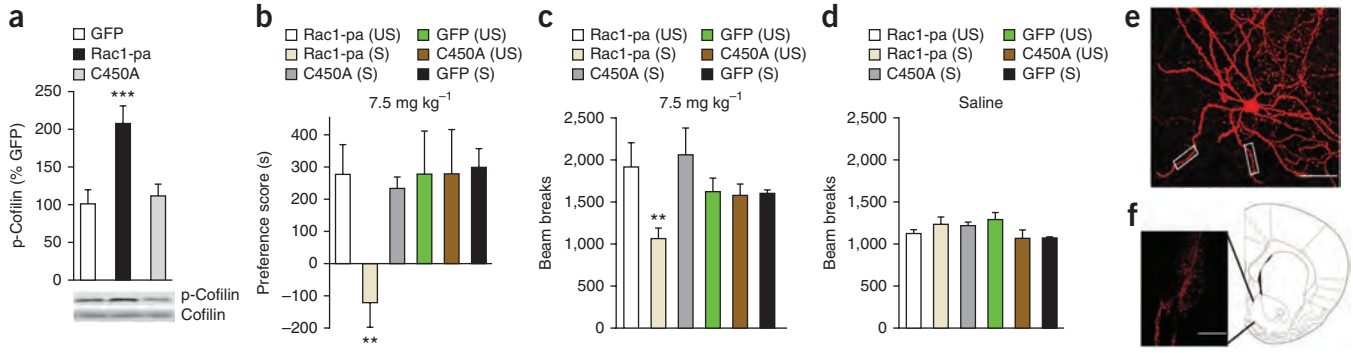


**Figure 3** Temporal regulation of Rac1 signaling regulates behavioral responses to cocaine. (**a**) Optical stimulation of Rac1 activity (by use of Rac1-pa) increased the phosphorylation state of cofilin (p-cofilin) ($F_{2,13} = 8.72$, $^{***}P < 0.005$; $n = 4$ mice), whereas light stimulation of the light-insensitive mutant (C450A) did not alter amounts of p-cofilin ($P > 0.05$), compared with HSV–GFP controls ($n = 5$ mice per group). Full-length western blots are shown in **Supplementary Figure 3**. (**b**) Optical stimulation of Rac1-pa (S) during cocaine pairing (see Online Methods) also prevented the formation of a conditioned place preference ($n = 8$ mice; $F_{5,43} = 2.66$; $^{**}P < 0.01$). Stimulation of mice with the light-insensitive C450A mutant ($n = 7$ mice) or HSV–GFP ($n = 7$ mice) led to cocaine preferences comparable to those in unstimulated (US) control mice ($n = 8$ mice). (**c**,**d**) Light activation of the Rac1-pa construct blocked cocaine-induced increases in locomotion ($n = 7$ or 8 mice per group; $F_{4,45} = 2.43$; $^{**}P < 0.01$) (**c**), but it had no effect on locomotor responses to saline ($n = 7$ or 8 mice per group) (**d**). (**e**) A representative infected NAc medium spiny neuron, imaged at original magnification ×40, infected with HSV–Rac1-pa. Scale bar, 50 μm. (**f**) Anatomical placement of viral infection in NAc after HSV injection. Cartoon shows the location of the injection site, 1.77 mm from bregma. Scale bar, 100 μm. (**g**) A representative HSV–Rac1-pa-C450A–infected NAc medium spiny neuron, imaged at original magnification ×40. Scale bar, 50 μm. Rectangular boxes in **e** and **g** highlight areas used for dendritic spine analysis. Data analyzed by ANOVA; error bars, s.e.m.

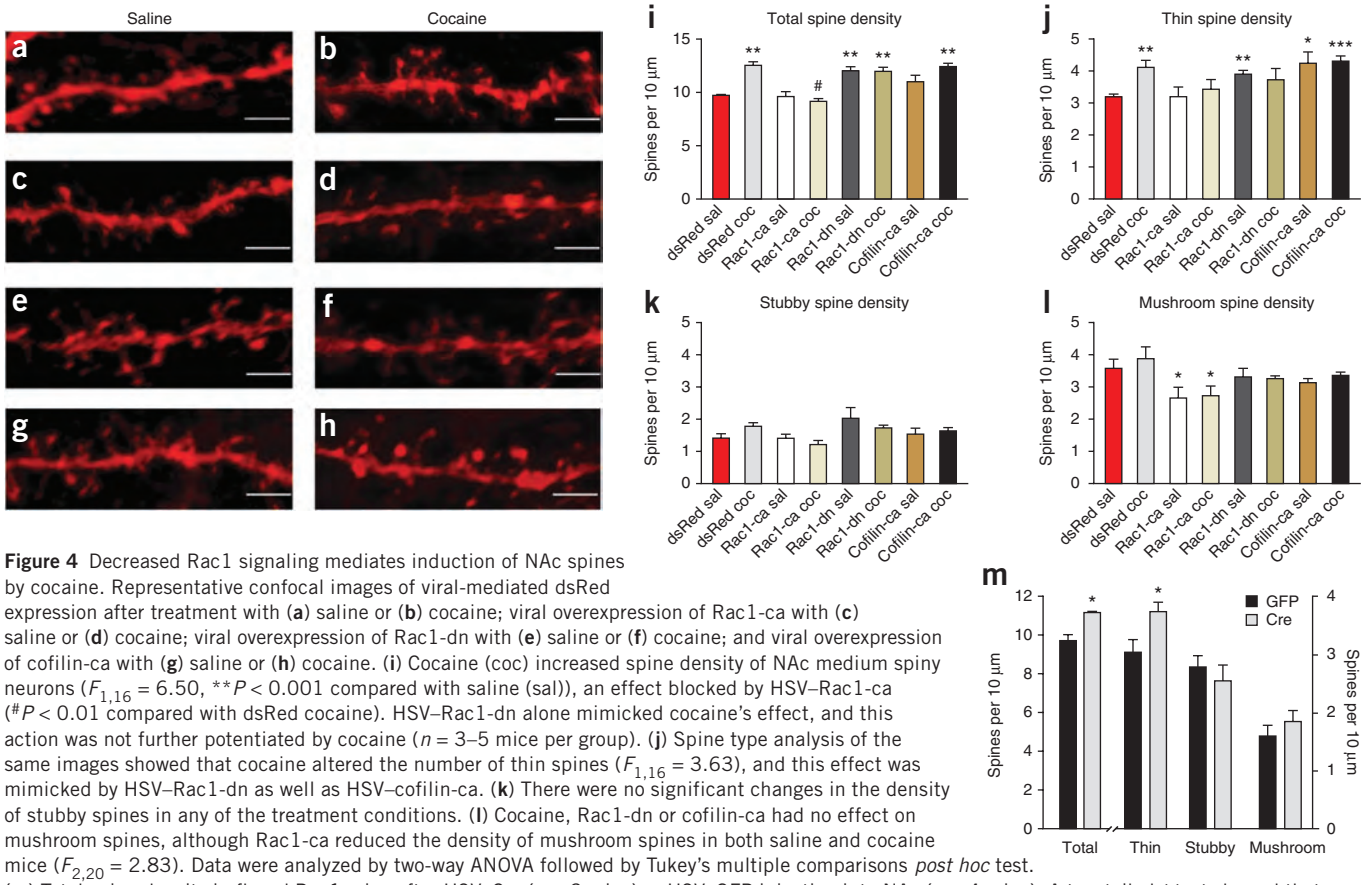


**Figure 4** Decreased Rac1 signaling mediates induction of NAc spines by cocaine. Representative confocal images of viral-mediated dsRed expression after treatment with (**a**) saline or (**b**) cocaine; viral overexpression of Rac1-ca with (**c**) saline or (**d**) cocaine; viral overexpression of Rac1-dn with (**e**) saline or (**f**) cocaine; and viral overexpression of cofilin-ca with (**g**) saline or (**h**) cocaine. (**i**) Cocaine (coc) increased spine density of NAc medium spiny neurons ($F_{1,16} = 6.50$, $^{**}P < 0.001$ compared with saline (sal)), an effect blocked by HSV–Rac1-ca ($^{\#}P < 0.01$ compared with dsRed cocaine). HSV–Rac1-dn alone mimicked cocaine's effect, and this action was not further potentiated by cocaine ($n = 3$–5 mice per group). (**j**) Spine type analysis of the same images showed that cocaine altered the number of thin spines ($F_{1,16} = 3.63$), and this effect was mimicked by HSV–Rac1-dn as well as HSV–cofilin-ca. (**k**) There were no significant changes in the density of stubby spines in any of the treatment conditions. (**l**) Cocaine, Rac1-dn or cofilin-ca had no effect on mushroom spines, although Rac1-ca reduced the density of mushroom spines in both saline and cocaine mice ($F_{2,20} = 2.83$). Data were analyzed by two-way ANOVA followed by Tukey's multiple comparisons *post hoc* test. (**m**) Total spine density in floxed Rac1 mice after HSV–Cre ($n = 3$ mice) or HSV–GFP injection into NAc ($n = 4$ mice). A two-tailed *t*-test showed that such local knockdown of Rac1 increased spine density ($t = 3.71$; d.f. = 5), and this increase was selective for thin spines ($t = 4.69$; d.f. = 5). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; error bars, s.e.m. Scale bars, 10 µm.





further evidence that the cocaine-induced transient downregulation of Rac1 activity in this brain region is required for normal rewarding effects of cocaine.

### Rac1 signaling regulates spine density of NAc neurons

To directly examine the functional consequences of decreased Rac1 activity on spine morphology of NAc medium spiny neurons, we bilaterally injected HSV–Rac1-ca, HSV–Rac1-dn or HSV–cofilin-ca and compared results to injection of the HSV–dsRed control vector. Mice receiving these virus injections were then treated with five doses of cocaine or saline, and spines were analyzed 4 h after the last cocaine dose, to correspond with the transient time scale of HSV expression. We found that with this model, repeated cocaine exposure increased the number of dendritic spines on NAc medium spiny neurons, compared with saline-treated mice, and that this increase was blocked completely by Rac1-ca, which had no effect on spine density under basal conditions. In contrast, overexpression of Rac1-dn induced an increase in spine density in saline-treated mice comparable to that observed in the HSV–dsRed cocaine group (**Fig. 4a–i**). The cocaine-induced increase in spine density in the HSV–dsRed cocaine group, compared to the HSV–dsRed saline group, was largely driven by an increase in thin spines (**Fig. 4j**), and this increase was mimicked by Rac1-dn overexpression, and blocked by Rac1-ca overexpression. In agreement with our Rac1-dn data, overexpression of HSV–cofilin-ca tended to increase total spine density under saline and cocaine conditions (**Fig. 4i**), which was significant when we looked specifically at the number of thin spines (**Fig. 4j**). In contrast, there was no significant effect of repeated cocaine exposure on the density of more consolidated spines (stubby and mushroom) at the time point examined; however, we observed that Rac1-ca by itself, but not Rac1-dn or cofilin-ca, reduced the number of such spines (**Fig. 4k,l**).

To further confirm the role of Rac1 in cocaine-induced spine plasticity in NAc, we repeated our morphological experiments using the floxed Rac1 mice. Consistent with our Rac1-dn results, HSV–Cre knockdown of Rac1 selectively in the NAc increased total spine density, and this increase was largely due to an increase in thin spines, with no changes observed in the density of stubby or mushroom spines (**Fig. 4m**). We next used our photoactivatable Rac1 mutants to further explore the role of Rac1 in regulation of NAc neuron spine density in a more temporally precise manner. Brief light stimulation of mice injected intra-NAc with HSV–Rac1-pa following each injection of cocaine blocked the cocaine induction of spines (9.36 ± 0.306 spines per 10 µm ($n = 5$ mice) versus 9.78 ± 0.106 for GFP saline controls ($n = 4$ mice)) compared with the light-insensitive mutant HSV–Rac1-pa(C450A) (12.0 ± 0.970 spines per 10 µm versus 12.5 ± 0.343 for GFP cocaine; $n = 4$ mice per group; $F_{3,16} = 9.26$, $P < 0.001$; analyzed by ANOVA). These findings demonstrate that preventing the transient decrease in Rac1 activity in the NAc is sufficient to block cocaine-induced structural plasticity of medium spiny neurons.

## DISCUSSION

The findings of this study provide a fundamentally new insight into the molecular basis by which cocaine induces dendritic spine plasticity in the NAc. The data establish that downregulation of Rac1 activity

induced by repeated cocaine exposure is both necessary and sufficient for the cocaine-mediated increase in thin dendritic spines on NAc medium spiny neurons. The transient reduction in Rac1 signaling enhances actin turnover, as evidenced by the decrease in the inactive (phosphorylated) form of cofilin, which leads to more cofilin activity and more thin spines. Our results also directly relate such downregulation of Rac1 signaling and enhanced cofilin activity in the NAc to greater sensitivity to the rewarding effects of cocaine. Indeed, the use of Rac1-pa, which allowed the manipulation of an intracellular signaling protein in real time *in vivo*, made it possible to selectively examine the structural and behavioral consequences of the highly transient cocaine-induced decrease in Rac1 activity in the adult NAc.

While our findings are in agreement with previous results demonstrating the importance of the cofilin pathway and actin dynamics in cocaine-induced structural plasticity, the precise details of cocaine regulation of cofilin phosphorylation are more complicated[16–18]. For example, a previous study[17] reported no change in p-cofilin but an increase in total cofilin in response to a cocaine challenge in rats withdrawing from prior chronic cocaine exposure. This contrasts with our data showing a transient decrease in p-cofilin and no change in total cofilin in response to a cocaine challenge. These apparent discrepancies may be due to several factors that are known to be crucial for drug-induced plasticity, such as the use of rats versus mice, the withdrawal times after the course of chronic cocaine exposure (24 h in our experiments and 3 weeks in ref. 17) or methodological differences in measuring cofilin (total NAc extracts in our study and crude postsynaptic density fractions in ref. 17). Notably, however, the net effect—an increase in cofilin and actin dynamics—is the same in both studies. Moreover, the finding that inhibiting actin turnover by local injection of latrunculin (an actin-binding molecule) into NAc blocks cocaine-induced increases in spine density and locomotor sensitization[18], along with the report of RhoA GTPase downregulation by chronic cocaine[36], further support the role of enhanced actin dynamics in mediating structural and behavioral plasticity in chronic cocaine exposure.

Previous reports have suggested that Rac1 activity promotes spine development, whereas inhibition of Rac1 reduces spine number in other neural systems[37]. However, the role of Rac1 in the regulation of actin dynamics and spine morphology is far more complicated and depends on many factors, such as age and neuronal type, and may even vary between *in vivo* and *in vitro* systems[22,23,25]. Here, we demonstrate that decreased Rac1 signaling in the NAc *in vivo* increases spine formation, particularly of more immature, thin spines through a cofilin-mediated mechanism. Cofilin activity has been shown previously to increase actin depolymerization, nucleation and branching, ultimately leading to thinner spines and new cellular protrusions[38]. However, it should be noted that the changes in Rac1 and cofilin activity observed here may not occur exclusively at the spine, but could instead occur throughout the entire neuron, including the soma, where Rac1 has been shown to regulate gene transcription[39].

Cocaine-induced behavioral and synaptic plasticity has been strongly associated with adaptations in excitatory glutamatergic transmission in the NAc[6,40–43]. For example, at early withdrawal time points after the last cocaine exposure, including those examined here, there is an increase in thin (more highly plastic) spines and synaptic depression[17,44], perhaps representing an increased pool of silent synapses[45]. The role of Rac1 signaling in mediating silent synapse formation, which has not yet been investigated directly, now warrants examination. It will also be important in future studies to determine whether the influence of Rac1 on cocaine regulation of spine plasticity of NAc medium spiny neurons is selective for various subtypes of these neurons, which play distinct roles in the addiction process[35].

Recently, cocaine has been reported to induce kalirin-7, another Rho GEF, and loss of kalirin-7 in knockout mice blocks cocaine's induction of NAc spines and cocaine reward[46]. However, how these findings on kalirin-7 relate to Rac1 is unknown because kalirin-7 induction would be expected to increase Rac1 activity and we show here that downregulation of Rac1, not activation, induces spines and cocaine reward. It is possible that the paradoxical effects seen in kalirin-7 knockout mice are mediated through loss of kalirin-7 in other brain regions or earlier in development or perhaps mediated through actions of kalirin-7 on Rho GTPases other than Rac1.

Whereas repeated administration of opiate drugs of abuse, like repeated administration of psychostimulants, causes sensitized behavioral responses, opiates decrease dendritic spine density on NAc medium spiny neurons, in contrast to the induction seen with psychostimulants[5]. Virtually nothing is known about the effect of opiate drugs on activity of the Rac1-cofilin pathway in the NAc, and the role of actin dynamics in mediating opiate-induced addictive behaviors remains unexplored. Moreover, whereas several previous studies that blocked cocaine-induced increases in spine density, through a variety of pharmacological and molecular manipulations, concomitantly observed blunted behavioral effects of cocaine[10,13,18], other studies have seen the opposite[9,11,12,16]. These findings highlight the need for further research to carefully delineate the likely complex and time-dependent role of spine plasticity of NAc medium spiny neurons in mediating distinct aspects of behavioral adaptations to cocaine. One key consideration is that most studies that have examined drug-induced morphological plasticity in the NAc have relied, as we do in the present investigation, on the use of investigator-administered drug. An important subject for future studies is to determine whether the molecular changes seen using such noncontingent drug administration are the same as those that occur with drug self-administration models, highlighting the importance of investigating Rac1 signaling in these additional models of drug addiction[47–49].

Together, our data support a scheme whereby repeated administration of cocaine leads to downregulation of the Rac1 GEF Tiam1 and upregulation of the Rac GAP (RacGAP), thereby priming NAc medium spiny neurons for transient reductions in Rac1 activity in response to a subsequent cocaine challenge. This cascade (**Supplementary Fig. 1**) may be a mechanism by which chronic cocaine exposure induces long-term changes in plasticity in NAc, and this provides new directions for the development of therapies for cocaine addiction.

## METHODS

Methods and any associated references are available in the online version of the paper.

*Note: Supplementary information is available in the online version of the paper.*

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**AUTHOR CONTRIBUTIONS**

D.M.D., S.J.R. and E.J.N. were responsible for overall study design. D.M.D., H.S., K.C.D., C.D. and I.M. designed and conducted GTPase activity assays and analyzed the data. D.M.D., M.E.C., J.W.K. and D.F. carried out the stereotaxic surgeries and behavioral experiments. D.M.D., M.S.M.-R., D.D.-W., V.G. and H.S. carried out and analyzed the western blots. D.M.D., D.C. and V.G. scanned, counted and analyzed the spine data. D.M.D., M.K.L., H.S., K.N.S., G.E.H., S.J.R., Y.O. and K.M.H. designed and did the necessary cloning and conducted the optical Rac1-pa experiments. Y.Z. provided the floxed Rac1 mice and expertise in Rac1 signaling; R.L.N. constructed and provided the viral vectors for gene transfer. D.M.D. 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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1. Garey, L.J. *et al.* Reduced dendritic spine density on cerebral cortical pyramidal neurons in schizophrenia. *J. Neurol. Neurosurg. Psychiatry* **65**, 446–453 (1998).
2. Russo, S.J. *et al.* The addicted synapse: mechanisms of synaptic and structural plasticity in nucleus accumbens. *Trends Neurosci.* **33**, 267–276 (2010).
3. Soetanto, A. *et al.* Association of anxiety and depression with microtubule-associated protein 2- and synaptopodin-immunolabeled dendrite and spine densities in hippocampal CA3 of older humans. *Arch. Gen. Psychiatry* **67**, 448–457 (2010).
4. Hyman, S.E., Malenka, R.C. & Nestler, E.J. Neural mechanisms of addiction: the role of reward-related learning and memory. *Annu. Rev. Neurosci.* **29**, 565–598 (2006).
5. Robinson, T.E. & Kolb, B. Structural plasticity associated with exposure to drugs of abuse. *Neuropharmacology* **47**, 33–46 (2004).
6. Kalivas, P.W. The glutamate homeostasis hypothesis of addiction. *Nat. Rev. Neurosci.* **10**, 561–572 (2009).
7. Nimchinsky, E.A., Sabatini, B.L. & Svoboda, K. Structure and function of dendritic spines. *Annu. Rev. Physiol.* **64**, 313–353 (2002).
8. Deng, J.V. *et al.* MeCP2 in the nucleus accumbens contributes to neural and behavioral responses to psychostimulants. *Nat. Neurosci.* **13**, 1128–1136 (2010).
9. LaPlant, Q. *et al.* Dnmt3a regulates emotional behavior and spine plasticity in the nucleus accumbens. *Nat. Neurosci.* **13**, 1137–1143 (2010).
10. Maze, I. *et al.* Essential role of the histone methyltransferase G9a in cocaine-induced plasticity. *Science* **327**, 213–216 (2010).
11. Norrholm, S.D. *et al.* Cocaine-induced proliferation of dendritic spines in nucleus accumbens is dependent on the activity of cyclin-dependent kinase-5. *Neuroscience* **116**, 19–22 (2003).
12. Pulipparacharuvil, S. *et al.* Cocaine regulates MEF2 to control synaptic and behavioral plasticity. *Neuron* **59**, 621–633 (2008).
13. Russo, S.J. *et al.* Nuclear factor kappa B signaling regulates neuronal morphology and cocaine reward. *J. Neurosci.* **29**, 3529–3537 (2009).
14. Trachtenberg, J.T. *et al.* Long-term *in vivo* imaging of experience-dependent synaptic plasticity in adult cortex. *Nature* **420**, 788–794 (2002).
15. Holtmaat, A. & Svoboda, K. Experience-dependent structural synaptic plasticity in the mammalian brain. *Nat. Rev. Neurosci.* **10**, 647–658 (2009).
16. Toda, S., Shen, H.W., Peters, J., Cagle, S. & Kalivas, P.W. Cocaine increases actin cycling: effects in the reinstatement model of drug seeking. *J. Neurosci.* **26**, 1579–1587 (2006).
17. Shen, H.W. *et al.* Altered dendritic spine plasticity in cocaine-withdrawn rats. *J. Neurosci.* **29**, 2876–2884 (2009).
18. Toda, S., Shen, H. & Kalivas, P.W. Inhibition of actin polymerization prevents cocaine-induced changes in spine morphology in the nucleus accumbens. *Neurotox. Res.* **18**, 410–415 (2010).
19. Halpain, S. Actin and the agile spine: how and why do dendritic spines dance? *Trends Neurosci.* **23**, 141–146 (2000).
20. Penzes, P. & Jones, K.A. Dendritic spine dynamics—a key role for kalirin-7. *Trends Neurosci.* **31**, 419–427 (2008).
21. Hayashi-Takagi, A. *et al.* Disrupted-in-Schizophrenia 1 (DISC1) regulates spines of the glutamate synapse via Rac1. *Nat. Neurosci.* **13**, 327–332 (2010).
22. Tashiro, A., Minden, A. & Yuste, R. Regulation of dendritic spine morphology by the rho family of small GTPases: antagonistic roles of Rac and Rho. *Cereb. Cortex* **10**, 927–938 (2000).
23. Tashiro, A. & Yuste, R. Regulation of dendritic spine motility and stability by Rac1 and Rho kinase: evidence for two forms of spine motility. *Mol. Cell Neurosci.* **26**, 429–440 (2004).
24. Oh, D. *et al.* Regulation of synaptic Rac1 activity, long-term potentiation maintenance, and learning and memory by BCR and ABR Rac GTPase-activating proteins. *J. Neurosci.* **30**, 14134–14144 (2010).
25. Luo, L. *et al.* Differential effects of the Rac GTPase on Purkinje cell axons and dendritic trunks and spines. *Nature* **379**, 837–840 (1996).
26. Yang, N. *et al.* Cofilin phosphorylation by LIM-kinase 1 and its role in Rac-mediated actin reorganization. *Nature* **393**, 809–812 (1998).
27. Edwards, D.C., Sanders, L.C., Bokoch, G.M. & Gill, G.N. Activation of LIM-kinase by Pak1 couples Rac/Cdc42 GTPase signalling to actin cytoskeletal dynamics. *Nat. Cell Biol.* **1**, 253–259 (1999).
28. Shirazi Fard, S., Kele, J., Vilar, M., Paratcha, G. & Ledda, F. Tiam1 as a signaling mediator of nerve growth factor-dependent neurite outgrowth. *PLoS ONE* **5**, e9647 (2010).
29. Miyamoto, Y., Yamauchi, J., Tanoue, A., Wu, C. & Mobley, W.C. TrkB binds and tyrosine-phosphorylates Tiam1, leading to activation of Rac1 and induction of changes in cellular morphology. *Proc. Natl. Acad. Sci. USA* **103**, 10444–10449 (2006).
30. Nobes, C.D. & Hall, A. Rho GTPases control polarity, protrusion, and adhesion during cell movement. *J. Cell Biol.* **144**, 1235–1244 (1999).
31. Marinissen, M.J. *et al.* The small GTP-binding protein RhoA regulates c-Jun by a ROCK-JNK signaling axis. *Mol. Cell* **14**, 29–41 (2004).
32. Chen, L., Melendez, J., Campbell, K., Kuan, C.Y. & Zheng, Y. Rac1 deficiency in the forebrain results in neural progenitor reduction and microcephaly. *Dev. Biol.* **325**, 162–170 (2009).
33. Gu, Y. *et al.* Hematopoietic cell regulation by Rac1 and Rac2 guanosine triphosphatases. *Science* **302**, 445–449 (2003).
34. Wu, Y.I. *et al.* A genetically encoded photoactivatable Rac controls the motility of living cells. *Nature* **461**, 104–108 (2009).
35. Lobo, M.K. *et al.* Cell type-specific loss of BDNF signaling mimics optogenetic control of cocaine reward. *Science* **330**, 385–390 (2010).
36. Kim, W.Y., Shin, S.R., Kim, S., Jeon, S. & Kim, J.H. Cocaine regulates ezrin-radixin-moesin proteins and RhoA signaling in the nucleus accumbens. *Neuroscience* **163**, 501–505 (2009).
37. Hering, H. & Sheng, M. Dendritic spines: structure, dynamics and regulation. *Nat. Rev. Neurosci.* **2**, 880–888 (2001).
38. Ghosh, M. *et al.* Cofilin promotes actin polymerization and defines the direction of cell motility. *Science* **304**, 743–746 (2004).
39. Bosco, E.E., Mulloy, J.C. & Zheng, Y. Rac1 GTPase: a "Rac" of all trades. *Cell Mol. Life Sci.* **66**, 370–374 (2009).
40. Kalivas, P.W., Volkow, N. & Seamans, J. Unmanageable motivation in addiction: a pathology in prefrontal-accumbens glutamate transmission. *Neuron* **45**, 647–650 (2005).
41. Schmidt, H.D. & Pierce, R.C. Cocaine-induced neuroadaptations in glutamate transmission: potential therapeutic targets for craving and addiction. *Ann. NY Acad. Sci.* **1187**, 35–75 (2010).
42. Thomas, M.J., Kalivas, P.W. & Shaham, Y. Neuroplasticity in the mesolimbic dopamine system and cocaine addiction. *Br. J. Pharmacol.* **154**, 327–342 (2008).
43. Wolf, M.E. The Bermuda Triangle of cocaine-induced neuroadaptations. *Trends Neurosci.* **33**, 391–398 (2010).
44. Thomas, M.J., Beurrier, C., Bonci, A. & Malenka, R.C. Long-term depression in the nucleus accumbens: a neural correlate of behavioral sensitization to cocaine. *Nat. Neurosci.* **4**, 1217–1223 (2001).
45. Huang, Y.H. *et al. In vivo* cocaine experience generates silent synapses. *Neuron* **63**, 40–47 (2009).
46. Kiraly, D.D. *et al.* Behavioral and morphological responses to cocaine require Kalirin7. *Biol. Psychiatry* **68**, 249–255 (2010).
47. Chen, B.T. *et al.* Cocaine but not natural reward self-administration nor passive cocaine infusion produces persistent LTP in the VTA. *Neuron* **59**, 288–297 (2008).
48. McCutcheon, J.E., Wang, X., Tseng, K.Y., Wolf, M.E. & Marinelli, M. Calcium-permeable AMPA receptors are present in nucleus accumbens synapses after prolonged withdrawal from cocaine self-administration but not experimenter-administered cocaine. *J. Neurosci.* **31**, 5737–5743 (2011).
49. McFarland, K., Lapish, C.C. & Kalivas, P.W. Prefrontal glutamate release into the core of the nucleus accumbens mediates cocaine-induced reinstatement of drug-seeking behavior. *J. Neurosci.* **23**, 3531–3537 (2003).

## ONLINE METHODS

**Mouse colonies.** All mice were housed four to five per cage in a colony with a 12-h light-dark cycle (lights on from 7:00 a.m. to 7:00 p.m.) at a constant temperature (23 °C) with *ad libitum* access to water and food. All mouse protocols were approved by the Mount Sinai School of Medicine IACUC.

**Cocaine treatments.** Male 8- to 10-week-old C57BL/6J mice received seven daily intraperitoneal injections of saline, acute cocaine treatments (six saline injections plus one 20 mg/kg cocaine-HCl injection) or repeated cocaine treatments (seven 20 mg/kg cocaine-HCl injections). Mice were used 15 min, 45 min, 4 h, or 24 h after the last injection.

**Vector injections.** We used the bicistronic p1005+ HSV vector, expressing dsRed alone or with Rac1-ca or Rac1-dn. Cofilin-ca (S3A), Rac1-pa, and Rac1-pa (C450A) were expressed with the same HSV vector where GFP replaced dsRed. Vectors were prepared according to published methods[9,10,13,50]. Extensive prior work has shown that HSV vectors infect only neurons, and in the NAc, most of the neurons infected are medium spiny neurons, which comprise ~95% of all neurons in this region[50]. Mice were anesthetized with ketamine (100 mg/kg)-xylazine (10 mg/kg) and placed in a small-animal stereotaxic instrument, and their skull surface was exposed. For Rac1 and cofilin vectors, thirty-three gauge syringe needles were used to unilaterally infuse 0.5 µl of virus into the NAc of both hemispheres at a 10° angle (AP + 1.6; ML + 1.5; DV − 4.4) at a rate of 0.1 µl/min. For Rac1-pa experiments, mice were cannulated as previously published[35], and 0.5 µl of either HSV–Rac1-pa, HSV–Rac1-pa(C450A) or HSV–GFP was injected into the NAc of the right hemisphere at a 0° angle (AP + 1.4; ML + 1.3; DV − 4.3) at a rate of 0.1 µl/min. Next, a 20-gauge cannula, 4 mm in length from the cannula base, was implanted into the right hemisphere (AP + 1.4; ML + 1.3; DV − 3.9). Instant adhesive was placed between the base of the cannula and the skull, and skull fixture adhesive was used to cement the cannula in place.

To induce local deletion of the Rac1 transcript restricted to NAc neurons, we used mutant mice homozygous for a floxed Rac1 allele. The floxed allele contains *lox*P sites flanking exon 1 including the translation start site[33]. Mice were stereotaxically injected into the NAc with HSV–GFP or HSV–Cre–GFP between the age of 10 and 13 weeks. All injection/cannula placements (including drug and saline injections) were verified, and the <5% of all mice with misplaced sites were excluded from subsequent analyses.

**GTPase activity assay.** Active Rac1 or CDC42 was immunoprecipitated using a monoclonal antibody that specifically recognizes the GTP-bound but not GDP-bound form of these proteins (NewEast Biosciences; 1:1000). The immunoprecipitate was then western blotted for total Rac1 (NewEast Biosystems; 1:500) or total CDC42 (NewEast Biosystems; 1:500).

**Dendritic spine analysis.** To study the role of Rac1 in the regulation of NAc medium spiny neuron morphology *in vivo*, we used methods previously described with the following modifications[9,10,13]. Briefly, two days after viral vector injections, when HSV expression is maximal, mice were intraperitoneally injected five times over 3 days with either cocaine (20 mg/kg) or saline. For the Rac1-pa spine studies, 10 min following each cocaine injection, mice were stimulated with 473 nm blue laser diode for 20 min. Mice were killed by transcardial perfusion 4 h after the last treatment, and brains were later sectioned at 100 µm on a vibratome. Sections were then immunostained using an antibody against GFP (Invitrogen; 1:500) or DsRed (Clontech; 1:500). All HSV images were captured with a laser confocal microscope with a 100× oil-immersion objective. Images were acquired with the pinhole set at 1 arbitrary unit and a 1024 × 1024 frame size. Dendritic length was measured using Image J software (NIH), and spine numbers were counted blind by the primary experimenter, as slides were coded before experimental scanning. The average number of spines per 10 µm of dendrite was calculated. We measured the number of spines on 1 or 2 neurites per neuron equaling at least 300 µm of secondary dendrites from dsRed-expressing NAc medium spiny neurons. We examined six to eight neurons per mouse with three or four mice per group (seven groups), after which an average value was obtained for each mouse for statistical analysis. Spine type analysis was carried out with the semi-automated software NeuronStudio (http://research.mssm.edu/cnic/tools-ns.html)[9].

**Western blotting.** NAc punch dissections were homogenized in 30 µl of homogenization buffer containing 320 mM sucrose, 5 nM HEPES buffer, 1% (wt/vol) SDS, phosphatase inhibitor cocktails I and II (Sigma), and ultra cocktail mini protease inhibitors tablets (Roche), using an ultrasonic processor. Protein concentrations were determined using a DC protein assay and 30 µg of protein were loaded onto Tris-HCL polyacrylamide gels for electrophoresis. The samples were then transferred to a nitrocellulose membrane and blocked for one hour in Odyssey blocking buffer. Blocked membranes were incubated overnight at 4 °C in Odyssey blocking buffer with the following primary antibodies: 1:500 phospho-cofilin and 1:1000 cofilin (Cell Signaling); 1:500 RacGAP1 (Tocris), and 1:300 Tiam1 (Sigma); 1:500 total Rac1 and total CDC42 (NewEast Biosciences). After thorough washing, the membranes were incubated with respective infrared dye (IRDye)-conjugated secondary antibodies (LI-COR Biosciences; 1:5000 to 1:10,000) dissolved in Odyssey blocking buffer for 1 h at room temperature. All total proteins were normalized to tubulin (Cell Signaling; 1:50,000). The blots were imaged with the Odyssey Infrared Imaging system and quantified by ImageJ.

**Quantitative PCR.** NAc punch dissections were homogenized in Trizol (Invitrogen) and processed according to the manufacturer's instructions. RNA was purified with RNeasy microcolumns (Invitrogen). The RNA was reverse transcribed with a Bio-Rad iScript kit. cDNA was quantified by qPCR using SYBR Green. Each reaction was run in triplicate and analyzed following the ΔΔCt method as previously described[10]. The following primer pairs were used to confirm Rac1 knockdown:

| | |
|---|---|
| *Rac1* F: | CAGTGAATCTGGGCCTATGG |
| *Rac1* R: | ACAGTGGTGTCGCACTTCAG |
| *Gapdh* F: | AGGTCGGTGTGAACGGATTTG |
| *Gapdh* R: | TGTAGACCATGTAGTTGAGGTCA. |

**Behavioral assays.** To examine the effect of Rac1 and cofilin on cocaine reward, we used an unbiased conditioned place preference model similar to one used in a previous study[13]. Mice were conditioned for 2 d to intraperitoneal saline injections in one chamber for 30 min during a morning session and to 7.5 or 4 mg/kg intraperitoneal cocaine injections in the opposite chamber for 30 min during an afternoon session. The day after the second conditioning, mice were tested for place preferences during a 20-min session where they were allowed to freely explore all three chambers. Locomotor activity was recorded automatically by infrared photobeams 30 min after an acute intraperitoneal injection of saline or cocaine (7.5 mg/kg).

**Light activation of Rac1-pa.** For *in vivo* validation of the Rac1-pa constructs, mice were stereotaxically injected with HSV–Rac1-pa or an HSV expressing the light-insensitive mutant, HSV–Rac1-pa(C450A), and cannulated as previously published[35]. Three days later, an optic fiber was placed in the cannula. We used a 200-µm core optic fiber that was modified such that when the optic fiber was secured *in vivo* to the cannula, the fiber was flush with the length of the cannula with ~50 µm of the stripped 200-µm core exposed beyond the cannula[35]. Optic fibers were attached through an FC/PC adaptor to a 473-nm blue laser diode and continuous light was generated through a stimulator. Optic fiber light intensity was measured using a light sensor; light intensity was ~15 mW/mm$^2$ at the tip of the fiber. For conditioned place preference experiments, mice were injected with either HSV–Rac1-pa, HSV–Rac1-pa(C450A), or HSV–GFP. The 473-nm light was turned on for the duration of the cocaine pairing. Conditions were identical during the saline pairing without light stimulation. To ensure specificity of the Rac1-pa construct, a group of mice was injected with the same constructs and underwent identical treatment except that they remained unstimulated (that is, cannulas and laser implantation but no light stimulation) during cocaine training.

**Statistics.** Two way ANOVAs (viral vector × drug injection) were used for dendritic spine analysis followed by Tukey's multiple comparisons *post hoc* analysis when appropriate. One-way ANOVAs were used for behavioral experiments and Rac1 activity assays followed by planned comparisons as *post hoc* analysis. Student's *t*-tests (two-sample, two-tailed) were used for verification of gene knockdown, as well as spine and behavioral analysis of floxed Rac1 mice. These tests were chosen on the basis of the design of each experiment according to established standards in the field.

50. Barrot, M. *et al.* CREB activity in the nucleus accumbens shell controls gating of behavioral responses to emotional stimuli. *Proc. Natl. Acad. Sci. USA* **99**, 11435–11440 (2002).