

# Dynamic BDNF activity in nucleus accumbens with cocaine use increases self-administration and relapse

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**A single exposure to cocaine rapidly induces the brief activation of several immediate early genes, but the role of such short-term regulation in the enduring consequences of cocaine use is poorly understood. We found that 4 h of intravenous cocaine self-administration in rats induced a transient increase in brain-derived neurotrophic factor (BDNF) and activation of TrkB-mediated signaling in the nucleus accumbens (NAc). Augmenting this dynamic regulation with five daily NAc BDNF infusions caused enduring increases in cocaine self-administration, and facilitated relapse to cocaine seeking in withdrawal. In contrast, neutralizing endogenous BDNF regulation with intra-NAc infusions of antibody to BDNF subsequently reduced cocaine self-administration and attenuated relapse. Using localized inducible BDNF knockout in mice, we found that BDNF originating from NAc neurons was necessary for maintaining increased cocaine self-administration. These findings suggest that dynamic induction and release of BDNF from NAc neurons during cocaine use promotes the development and persistence of addictive behavior.**

Cocaine administration activates convergent dopamine and glutamate receptor signaling in NAc neurons leading to the phosphorylation of cyclic AMP response element-binding protein (CREB) and the induction of multiple downstream CREB-regulated genes<sup>1–3</sup>. BDNF, a prominent CREB-regulated gene, mediates many enduring changes in neuroplasticity and synaptogenesis. The two major sources of BDNF protein in the NAc originate from dopaminergic and glutamatergic afferents from the ventral tegmental area and prefrontal cortex, respectively<sup>4–7</sup>. However, recent evidence indicates that BDNF is also produced locally in NAc neurons via CREB-regulated gene expression<sup>8,9</sup>. Under normal conditions, BDNF mRNA is expressed at very low levels in striatal neurons, but its expression is markedly increased by seizure activity<sup>10</sup>, acute cocaine<sup>11,12</sup> or through local D1 receptor activation in striatal neurons<sup>13</sup>.

NAc infusions of antibodies directed against BDNF or TrkB reduce methamphetamine-induced dopamine release and locomotor behaviors, indicating that endogenous BDNF causes a direct presynaptic facilitation of dopaminergic transmission in the NAc<sup>14</sup>. Conversely, local NAc infusions of BDNF enhance the development of locomotor sensitization to cocaine, and lead to a prolonged and indirect potentiation of instrumental responses for conditioned stimuli associated with natural rewards<sup>15</sup>. These studies suggest that regulation of endogenous BDNF during cocaine use could contribute to the development of addictive behavior that persists long after BDNF levels return to normal.

In this study, we found that both BDNF protein and TrkB-mediated signal transduction were transiently elevated in the NAc of rats immediately following intravenous cocaine self-administration, but

returned to control levels after 24 h. We studied the effects of this dynamic BDNF regulation on the development of addiction-like increases in cocaine self-administration, and on the propensity for relapse during withdrawal. We also determined whether BDNF originating from NAc neurons was necessary for promoting addiction-like increases in cocaine self-administration.

## RESULTS

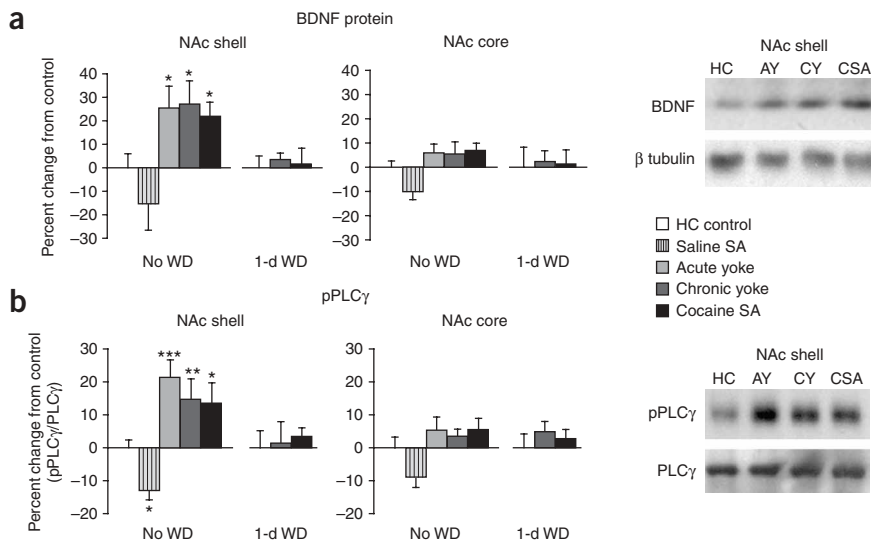
### Cocaine use induces BDNF and TrkB signaling in NAc

We tested whether BDNF protein levels in striatal subregions would change immediately following 4 h of intravenous cocaine self-administration in rats with chronic self-administration experience (4 h per d for 18 d). BDNF protein levels were elevated specifically in the NAc shell, but not in the NAc core or caudate putamen (data not shown), and returned to basal control levels after 1-d withdrawal from self-administration, compared with untreated home-cage controls ( $F_{4,68} = 3.638$ ,  $P = 0.010$ ; **Fig. 1a**). This BDNF regulation was similar (22–27%) whether cocaine injections were delivered in a reinforcing context (contingent upon lever-press behavior), or in chronic yoked animals receiving the same number and temporal pattern of cocaine injections passively throughout the course of training. In addition, the BDNF response to intravenous cocaine failed to differ with either acute or chronic administration, as yoked animals receiving cocaine for the first time (acute yoke) showed a similar 26% increase in BDNF. Thus, dynamic regulation of BDNF in the NAc shell reflects a generalized pharmacological response to cocaine that remains constant from initial to chronic cocaine use.

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**Figure 1** Transient increases in BDNF protein and TrkB signaling in NAC following intravenous cocaine self-administration (CSA). **(a)** BDNF protein levels were increased in the NAC shell, but not in the core, immediately after CSA, but not after 1-d withdrawal (WD). Similar regulation was induced by either acute (AY) or chronic (CY) yoked-administration. **(b)** PLC $\gamma$  phosphorylation also increased in the NAC shell, but not in the core, immediately after cocaine yoked-administration or self-administration (SA). Data from cocaine-exposed groups ( $N = 11$ – $12$  per group) and saline self-administering animals ( $N = 8$ ) are expressed as mean  $\pm$  s.e.m. percentage of untreated home cage (HC) controls ( $N = 26$ ). \*  $P < 0.05$ , \*\*  $P < 0.01$  and \*\*\*  $P < 0.001$ , compared with untreated controls. Representative blots are shown to the right.

We also asked whether natural rewards would regulate BDNF levels in the NAC in animals trained to self-administer sucrose pellets. BDNF levels in the NAC shell and core failed to differ immediately after initial acquisition or after 5 subsequent sucrose self-administration sessions (data not shown), indicating that BDNF increases in the NAC are not related to instrumental learning or generalized exposure to natural rewards.

We then determined whether increased BDNF levels would be accompanied by functional activation of TrkB receptors, potentially reflecting cocaine-induced release of BDNF in the NAC. Activated TrkB receptors directly phosphorylate phospholipase C $\gamma$  (PLC $\gamma$ ) at tyrosine 783 (ref. 16). PLC $\gamma$  phosphorylation was increased by 14–22% specifically in the NAC shell immediately following cocaine administration ( $F_{4,60} = 8.252$ ,  $P < 0.001$ ), and this effect was no longer apparent after 1-d withdrawal (Fig. 1b). Increases in PLC $\gamma$  phosphorylation also failed to differ with acute or chronic cocaine administered by passive yoked injections, paralleling regulation in NAC BDNF levels, but was slightly reduced in animals self-administering saline, reflecting procedural effects unrelated to cocaine.

### Temporal regulation of BDNF and TrkB signaling in NAC

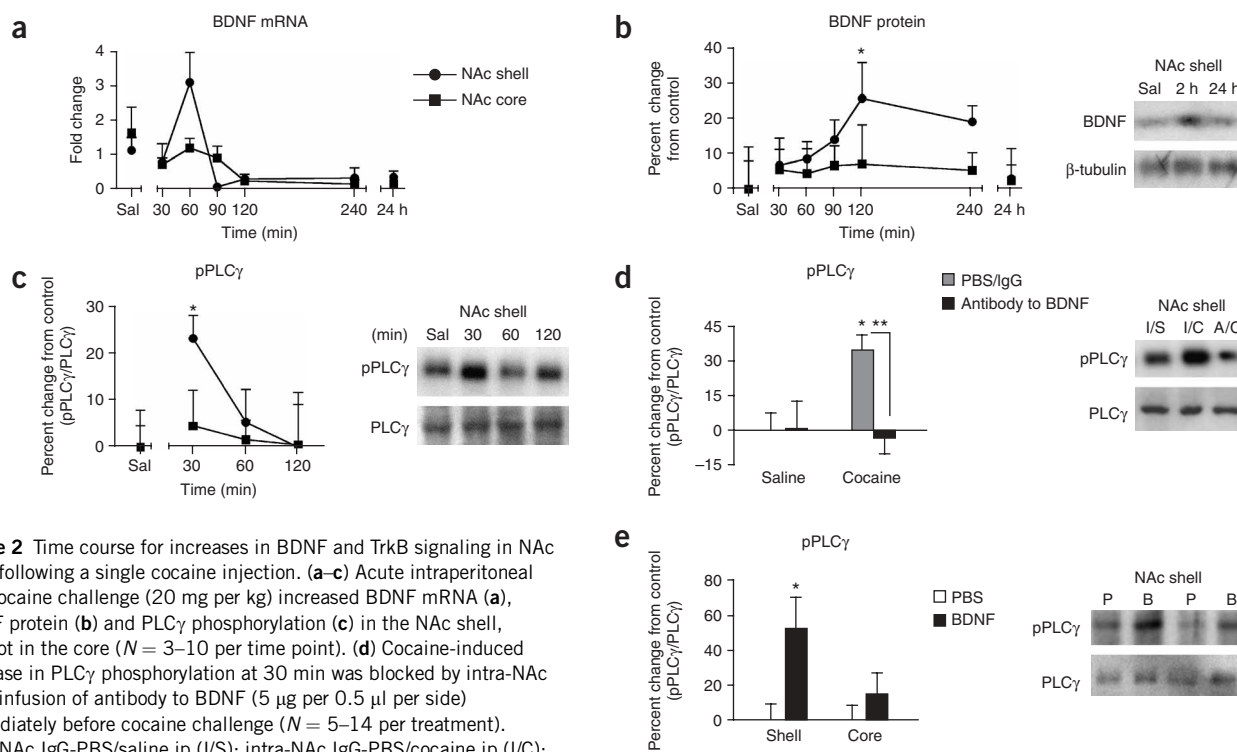
To further characterize cocaine regulation of BDNF, we determined a time course for induction of BDNF mRNA, BDNF protein and PLC $\gamma$  phosphorylation in naive rats that received a single 20 mg per kg of body weight (intraperitoneal) cocaine or saline injection. The cocaine injection induced a twofold peak increase in BDNF mRNA after 60 min in the NAC shell (Fig. 2a) that declined below basal levels (saline injection) at 90 min postinjection. In contrast, there was a 26% peak increase in BDNF protein at 120 min after cocaine injection ( $F_{2,23} = 3.401$ ,  $P < 0.05$ ), and BDNF remained elevated for at least 4 h, but declined to basal levels after 24 h (Fig. 2b). These findings are similar to the BDNF regulation observed in the NAC shell of self-administering

animals, and suggest that cocaine-induced increases in BDNF protein are at least partially derived from BDNF synthesis in NAC neurons. These data also agree with recent reports that intraperitoneal cocaine increases BDNF gene transcription in striatal regions with single or repeated treatments<sup>2,11,12,17</sup>. Such dynamic regulation of BDNF differs from the gradual increases of BDNF protein that accumulate in the NAC after prolonged cocaine withdrawal, as the latter is not accompanied by increases in BDNF mRNA, and thus may not reflect BDNF derived from local protein synthesis in NAC neurons<sup>12,18</sup>.

Notably, a 23% peak increase in PLC $\gamma$  phosphorylation was found only 30 min after the cocaine injection (Fig. 2c), thereby preceding the rise in BDNF protein levels and declining completely after 2 h ( $F_{3,23} = 3.001$ ,  $P < 0.05$ ). To determine whether cocaine-induced PLC $\gamma$  phosphorylation was mediated by local BDNF release, we infused an antibody directed against BDNF (5.0  $\mu$ g per 0.5  $\mu$ l per side) into the NAC shell immediately before cocaine challenge, and found that cocaine-induced increases in PLC $\gamma$  phosphorylation were completely abolished when compared with the contralateral side receiving IgG or phosphate-buffered saline (PBS) infusions (Fig. 2d;  $F_{3,33} = 6.074$ ,  $P = 0.002$ ). Together, these results indicate that presynthesized BDNF is released by cocaine and activates TrkB receptor-mediated signal transduction in the shell, but is subsequently replenished by newly and locally synthesized BDNF protein. Moreover, BDNF release is evidently sustained throughout cocaine self-administration, as PLC $\gamma$  phosphorylation remained elevated after a 4-h self-administration session (Fig. 1b). A single intra-NAC shell infusion of human recombinant BDNF (2.5  $\mu$ g per 0.5  $\mu$ l per side) induced a similar 52% increase in PLC $\gamma$  phosphorylation 30 min later compared with a contralateral infusion of the PBS vehicle ( $F_{1,13} = 7.204$ ,  $P = 0.019$ , Fig. 2e), and this effect did not spread to adjacent core tissue. Therefore, we used intra-NAC shell infusions of BDNF or antibody to BDNF to augment or to block dynamic regulation of endogenous BDNF, respectively, in the cocaine self-administration tests described below.

### Dynamic BDNF increases cocaine self-administration

We tested the effects of five daily bilateral NAC-shell infusions of BDNF or anti-BDNF on cocaine self-administration on a fixed-ratio five reinforcement schedule where five lever presses produced a cocaine injection. Animals were trained to self-administer cocaine (500  $\mu$ g per kg per injection) in daily 4-h sessions for 3–4 weeks until cocaine intake stabilized. During the next week, animals received BDNF (2.5  $\mu$ g per side), antibody to BDNF (5.0  $\mu$ g per side) or PBS vehicle/IgG (5  $\mu$ g per side) infusions in the NAC shell immediately following five consecutive sessions to augment or to attenuate dynamic regulation of BDNF (Fig. 3a, left). Neither infusions of BDNF nor antibody to BDNF in the NAC shell substantially altered baseline cocaine self-administration during the treatment regimen. Similarly, BDNF infusions given immediately before the test sessions had no acute effect on baseline cocaine self-administration (data not shown).



**Figure 2** Time course for increases in BDNF and TrkB signaling in NAc shell following a single cocaine injection. (**a–c**) Acute intraperitoneal (ip) cocaine challenge (20 mg per kg) increased BDNF mRNA (**a**), BDNF protein (**b**) and PLC $\gamma$  phosphorylation (**c**) in the NAc shell, but not in the core ( $N = 3–10$  per time point). (**d**) Cocaine-induced increase in PLC $\gamma$  phosphorylation at 30 min was blocked by intra-NAc shell infusion of antibody to BDNF (5  $\mu\text{g}$  per 0.5  $\mu\text{l}$  per side) immediately before cocaine challenge ( $N = 5–14$  per treatment). Intra-NAc IgG-PBS/saline ip (I/S); intra-NAc IgG-PBS/cocaine ip (I/C); intra-NAc antibody to BDNF/cocaine ip (A/C). (**e**) NAc shell infusion of BDNF (2.5  $\mu\text{g}$  per 0.5  $\mu\text{l}$  per side) increased PLC $\gamma$  phosphorylation in the NAc shell, but not in the core, 30 min postinfusion ( $N = 8–10$  per treatment). PBS (P); BDNF (B). Data are expressed as mean  $\pm$  s.e.m. \*  $P < 0.05$  and \*\*  $P < 0.01$ , compared with intraperitoneal saline (at peak time point) or with contralateral IgG-PBS infusions.

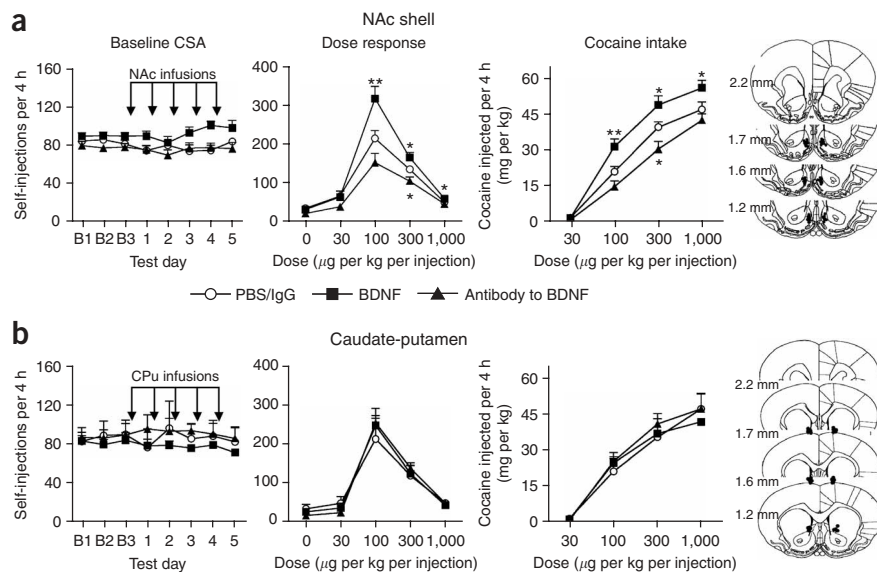
Three days following the final NAc infusion, animals were tested in a between-session dose-response procedure with each injection dose of cocaine being available for 4 h in 1 of 5 consecutive daily tests. Cocaine is self-administered with an inverted, U-shaped dose-response curve on fixed-ratio reinforcement schedules, and a descending limb whereby higher injection doses prolonged the duration of cocaine reward, resulting in fewer self-injections over time than with lower injection doses. Prior NAc-shell infusions of BDNF led to a vertical shift in the inverted, U-shaped dose-response function (treatment by dose interaction,  $F_{8,216} = 7.128$ ,  $P < 0.001$ ), including higher peak self-administration rates and increased cocaine intake on the descending limb of the curve compared with PBS/IgG-infused controls (**Fig. 3a**). The increase in cocaine intake is more apparent when these dose-response data were transformed into cocaine intake (number of injections  $\times$  dose per injection) over the entire test session (**Fig. 3a**, third panel). A vertical shift in the self-administration dose-response curve is thought to reflect a transition to more addicted biological states in animal models of cocaine addiction<sup>19–21</sup>. Thus, augmenting transient elevations in BDNF-TrkB receptor activity produced by daily cocaine self-administration subsequently facilitates this transition.

Conversely, blocking dynamic increases in endogenous BDNF with five daily postsession infusions of antibody to BDNF subsequently lead to a downward shift in the cocaine self-administration dose-response curve, including significantly reduced cocaine intake at the 300  $\mu\text{g}$  per kg dose ( $F_{2,54} = 8.777$ ,  $P = 0.001$ ) and a reduction in peak self-administration rates at the 100  $\mu\text{g}$  per kg dose that approached significance ( $P = 0.07$ ). Because a downward shift in the dose-response curve is opposite to changes associated with the development of cocaine addiction, and can be produced by lesions of NAc projection regions (for example<sup>22</sup>), these results suggest that transient increases in

endogenous BDNF in the NAc shell during ongoing cocaine use at least partially contribute to maintaining higher cocaine intake. Neither of these changes were found when BDNF or antibody to BDNF was infused 2.0 mm dorsal in the caudate putamen (**Fig. 3b**), indicating a specific interaction in the mesolimbic dopamine system.

In separate experimental groups, we studied the role of dynamic BDNF regulation in cocaine self-administration using a progressive-ratio schedule of reinforcement. In progressive-ratio testing, each successive cocaine injection requires a progressively greater number of lever-press responses, and the highest ratio of lever presses per cocaine injection achieved before animals quit self-administration altogether reflects the degree of effort exerted by animals to maintain self-administration. NAc infusions of BDNF or antibody to BDNF were administered after five daily cocaine self-administration sessions as described above (**Fig. 4a**). Beginning 3 d after the last infusion, cocaine self-administration on the progressive-ratio schedule was measured at two cocaine injection doses (see Methods). Control animals receiving PBS/IgG infusions into the NAc shell worked harder for the higher injection dose of cocaine (750  $\mu\text{g}$  per kg), performing  $112 \pm 26$  lever presses per injection for the last infusion, compared with only  $63 \pm 16$  lever presses per injection for the lower dose (250  $\mu\text{g}$  per kg). Thus, the higher dose of cocaine is more reinforcing to rats.

Repeated intra-NAc BDNF infusions led to a profound increase in the amount of effort animals would exert to obtain cocaine on the progressive-ratio reinforcement schedule (**Fig. 4b**), achieving higher response/injection ratios at both injection doses when compared with PBS/IgG-infused controls (treatment by dose interaction,  $F_{2,18} = 3.428$ ,  $P = 0.05$ ). BDNF-treated animals exerted markedly greater effort to maintain self-administration at the higher injection dose. Cumulative responses for representative animals show that the BDNF-treated



**Figure 3** Dynamic regulation of NAc BDNF produces enduring increases in cocaine intake. **(a)** Repeated postsession infusions of BDNF (2.5  $\mu\text{g}$  per 0.5  $\mu\text{l}$  per side) or antibody to BDNF (5  $\mu\text{g}$  per 0.5  $\mu\text{l}$  per side) in the NAc shell (left) produced opposite changes in CSA (fixed ratio 5) dose-response and dose-intake curves (middle) 3–7 d after cessation of treatment ( $N = 17$ –20 per treatment). **(b)** Similar treatments infused dorsally in the caudate putamen had no effect on CSA ( $N = 6$ –7 per treatment). Right, infusions sites in medial NAc shell or caudate putamen. Data are expressed as mean  $\pm$  s.e.m. \*  $P < 0.05$  and \*\*  $P < 0.01$ , compared with PBS/IgG-treated rats.

PBS/IgG-treated controls ( $F_{2,53} = 9.570$ ,  $P < 0.001$ ), but declined to similar levels by the fifth extinction test (treatment by test session interaction,  $F_{8,212} = 5.166$ ,  $P < 0.001$ ) (Fig. 5a, left). These data indicate that NAc infusions of BDNF ending 18 d earlier enhanced the ability of the cocaine-associated

environment to elicit cocaine-seeking behavior in withdrawal.

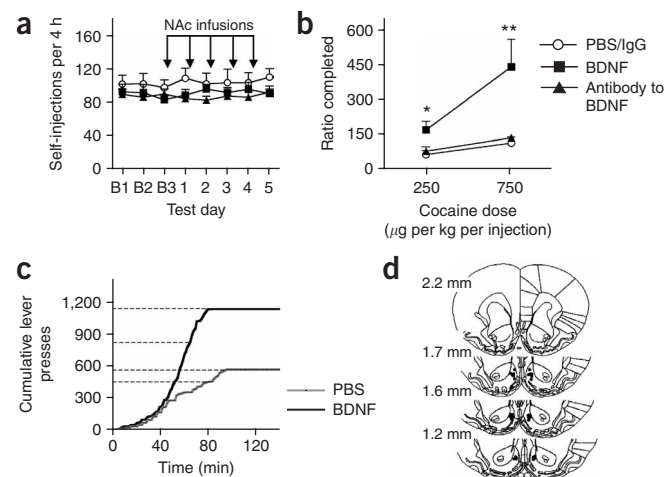
The following week, extinguished animals were subjected to reinstatement testing, where the ability of five different priming stimuli to trigger a return to cocaine-seeking behavior was measured. Priming stimuli consisted of discrete cues specifically associated with previous cocaine injections, intravenous priming injections of cocaine (saline, 500 and 2,000  $\mu\text{g}$  per kg), and brief exposure to intermittent footshock stress (Fig. 5a, right), as similar stimuli trigger craving responses in human cocaine addicts<sup>23,24</sup>. Augmenting dynamic BDNF regulation during prior cocaine self-administration with BDNF infusions led to substantially greater cocaine-seeking behavior being elicited by intravenous priming injections of cocaine compared with PBS/IgG-treated controls, but saline priming was ineffective in both groups, indicating a specific enhancement in reinstatement mechanisms (treatment by dose,  $F_{4,88} = 4.233$ ,  $P = 0.004$ ). Similarly, BDNF-treated animals showed enhanced cocaine seeking triggered by exposure to discrete cues associated with previous cocaine injections ( $F_{2,60} = 4.144$ ,  $P = 0.021$ ), and markedly enhanced cocaine-seeking behavior following a 30-min period of randomly presented intermittent footshock stress ( $F_{2,51} = 6.607$ ,  $P = 0.003$ ). Thus, augmenting transient

rat performed 328 responses for the final cocaine injection at this high dose, but the PBS/IgG-infused control achieved a maximal response ratio of only 146 responses per injection (Fig. 4c). Thus, augmenting transient increases in NAc BDNF subsequently enhanced the motivation for cocaine when reinforcement was withheld, an effect associated with the transition to addiction<sup>21</sup>. In contrast, postsession infusions of antibody to BDNF did not alter responding for cocaine on the progressive-ratio schedule, possibly reflecting insufficient blockade of endogenous BDNF during prior cocaine self-administration, or that augmenting endogenous BDNF levels is needed to facilitate responding on more difficult progressive-ratio schedules.

### Dynamic BDNF enhances the propensity for relapse

To determine whether BDNF regulation during cocaine use would subsequently alter the propensity for relapse in withdrawal, we measured cocaine-seeking behavior after a period of forced abstinence. Animals treated with BDNF, antibody to BDNF or PBS/IgG during cocaine self-administration and tested in fixed-ratio dose-response (Fig. 3) remained in their home cages for the first 10 d of cocaine withdrawal. On days 11–15, animals were placed in the test chambers under extinction conditions for 4 h per d, and the magnitude of cocaine-seeking behavior in the absence of reinforcement was assessed by the amount of responding at the lever that previously delivered cocaine injections. Animals receiving prior intra-NAc BDNF infusions showed a 54% increase in drug-paired lever responding with initial exposure to the cocaine-associated test chambers compared with

**Figure 4** Repeated NAc BDNF infusions increase motivation for cocaine injections. **(a)** Animals received repeated postsession infusions of BDNF (2.5  $\mu\text{g}$  per 0.5  $\mu\text{l}$  per side) or antibody to BDNF (5  $\mu\text{g}$  per 0.5  $\mu\text{l}$  per side) in the NAc shell during fixed-ratio CSA. **(b)** BDNF-treated animals subsequently achieve a higher ratio of lever-press responses per cocaine injection before ceasing SA altogether in progressive-ratio testing at two different cocaine injection doses ( $N = 7$  per treatment). **(c)** Individual cumulative response records for a PBS/IgG control and a BDNF-treated rat self-administering cocaine at 750  $\mu\text{g}$  per kg per injection. Dotted lines denote number of responses completed for the final cocaine injection earned. **(d)** Localization of infusions sites in medial NAc shell. Data are expressed as mean  $\pm$  s.e.m. \*  $P < 0.05$  and \*\*  $P < 0.01$ , compared with PBS/IgG-treated rats.



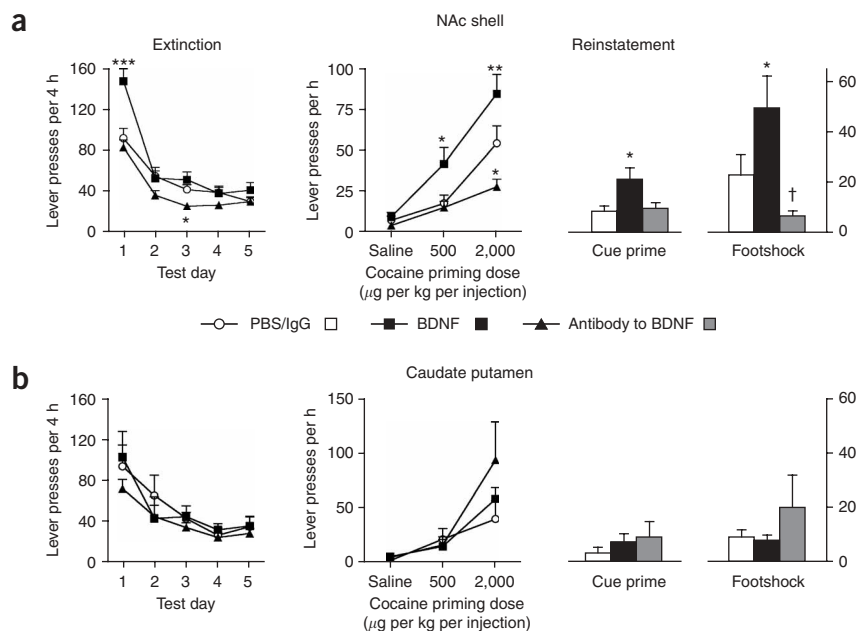
BDNF increases in the NAc shell during cocaine self-administration increased the propensity for relapse triggered by conditioned, pharmacological and stressful stimuli up to 4 weeks after the final BDNF treatment.

In contrast, neutralizing the endogenous rise in BDNF for only five self-administration sessions subsequently attenuated relapse induced by cocaine priming ( $F_{2,44} = 7.780$ ,  $P = 0.001$ ) and footshock stress ( $T_{36} = 2.068$ ,  $P = 0.046$ ), and facilitated extinction from cocaine self-administration by the third day of testing ( $F_{2,53} = 5.748$ ,  $P = 0.005$ ) compared with PBS/IgG-treated controls. Cue-induced reinstatement was unaltered, possibly as a result of a floor effect. These data suggest that endogenous BDNF regulation in the NAc shell during daily cocaine use promotes subsequent relapse after prolonged cocaine withdrawal. Similar infusions of BDNF or antibody to BDNF in the caudate putamen during prior cocaine self-administration failed to affect responding in either extinction or reinstatement testing (Fig. 5b), indicating a specific role for NAc BDNF in relapse behaviors.

We then asked whether regulation of self-administration and relapse behaviors by BDNF and antibody to BDNF would generalize to natural rewards. Rats trained to self-administer sucrose pellets on an identical fixed-ratio schedule received a similar 5-d postsession infusion regimen of BDNF and antibody to BDNF in the NAc shell. There was no effect of BDNF or antibody to BDNF infusions on the rate of sucrose pellet self-administration the following week, and no effect on subsequent extinction responding (Fig. 6), although response rates were twofold higher than in the initial cocaine extinction tests, potentially precluding BDNF effects. Following extinction, however, BDNF manipulations had no effect on reinstatement of sucrose seeking triggered by non-contingent sucrose-pellet priming when response rates were similar to cocaine priming in cocaine-trained animals. These data indicate that altered responding for cocaine with BDNF or antibody to BDNF involved motivation-related rather than performance-related influences, and suggest that BDNF in the NAc does not have a prominent role in maintaining natural reward-seeking behavior.

### BDNF knockout in NAc neurons reduces cocaine reinforcement

In addition to local BDNF synthesis in NAc neurons, other potential sources of BDNF protein in the NAc include dopaminergic and glutamatergic afferents, as discussed above. We studied the role of local BDNF that was derived solely from NAc neurons on cocaine self-administration behavior. We infused an adeno-associated viral vector (AAV) encoding CRE-recombinase tagged with green fluorescent protein (CreGFP) into the NAc of floxed BDNF mice (1 kb of the single coding exon flanked by *loxP* sites) to produce a highly localized knockout of BDNF protein derived exclusively from NAc neurons in adult animals. Neurons infected with AAV-CreGFP were localized in the medial NAc without evidence for gliosis or scarring (Fig. 7a,b). BDNF mRNA was virtually undetectable by real-time polymerase chain

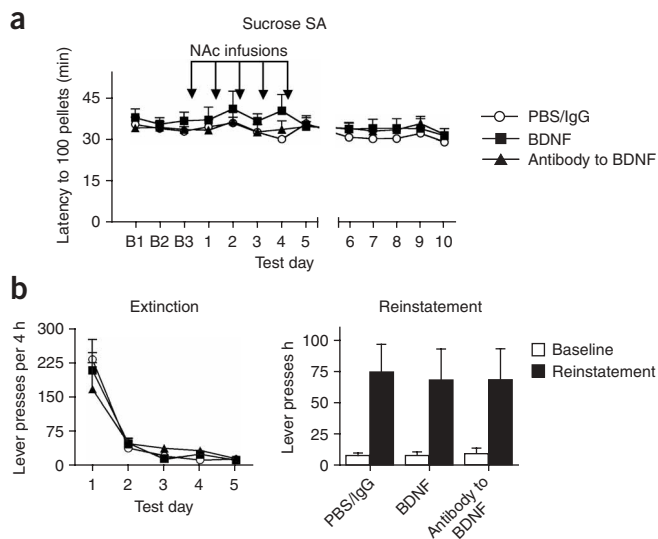


**Figure 5** Dynamic regulation of NAc BDNF during SA produces enduring facilitation of relapse in cocaine withdrawal. (a) Repeated infusions of BDNF (2.5 µg per 0.5 µl per side) or antibody to BDNF (5 µg per 0.5 µl per side) in the NAc shell during prior CSA training (shown in Fig. 3) produced opposite effects on cocaine-seeking behavior in extinction and reinstatement testing conducted 3–4 weeks later ( $N = 14–20$  per treatment). BDNF-treated rats showed greater nonreinforced responding at the drug-paired lever during the initial extinction test (left), following intravenous priming injections of cocaine (second from left), during exposure to cues associated with delivery of prior cocaine injections (third from left), and immediately after 30 min of randomly presented footshock stress (right). Neutralizing endogenous BDNF with prior infusions of antibody to BDNF attenuated reinstatement of drug-paired lever responding induced by cocaine priming and footshock stress. (b) Similar treatments previously infused dorsally in the caudate putamen (Fig. 3) had no effect on responding in extinction and reinstatement testing ( $N = 6–7$  per treatment). Data are expressed as mean  $\pm$  s.e.m. \*  $P < 0.05$ , \*\*  $P < 0.01$  and \*\*\*  $P < 0.001$ , compared with PBS/IgG-treated rats.

reaction (RT-PCR) in laser-captured GFP-expressing neurons infected with AAV-CreGFP compared with neurons infected with the AAV-GFP control vector (Fig. 7c). BDNF protein levels were substantially reduced by 46% in microdissected tissue surrounding the AAV-CreGFP-infected region compared with tissue infected with AAV-GFP (Fig. 7d,  $F_{1,6} = 33.940$ ,  $P = 0.001$ ). Therefore, a substantial proportion of BDNF is derived from local protein synthesis in NAc neurons.

Floxed BDNF mice were given similar bilateral intra-NAc infusions of AAV-CreGFP and AAV-GFP 14 d before the onset of a serial behavioral testing procedure (see Methods). Localized BDNF knockout in NAc neurons had no effect on initial spontaneous lever-press behavior in the absence of reinforcement, and  $\sim 50\%$  of both AAV-CreGFP- and AAV-GFP-infused mice acquired sucrose pellet self-administration (fixed ratio 3) on the first day of acquisition testing (Fig. 8a, left center). Following acquisition, the latency to consume the 30-pellet allotment was similar in both groups (data not shown). These data indicate that mice with localized BDNF knockout in NAc neurons had normal instrumental learning.

After intravenous catheterization, acquisition of cocaine self-administration was tested daily in 8–10 1-h sessions on a fixed-ratio 1 schedule of reinforcement using a relatively high training dose (500 µg per kg per injection). There were no major differences in overall cocaine self-administration during acquisition training, with 9 of 9 BDNF knockout and 7 of 9 GFP-expressing controls ultimately



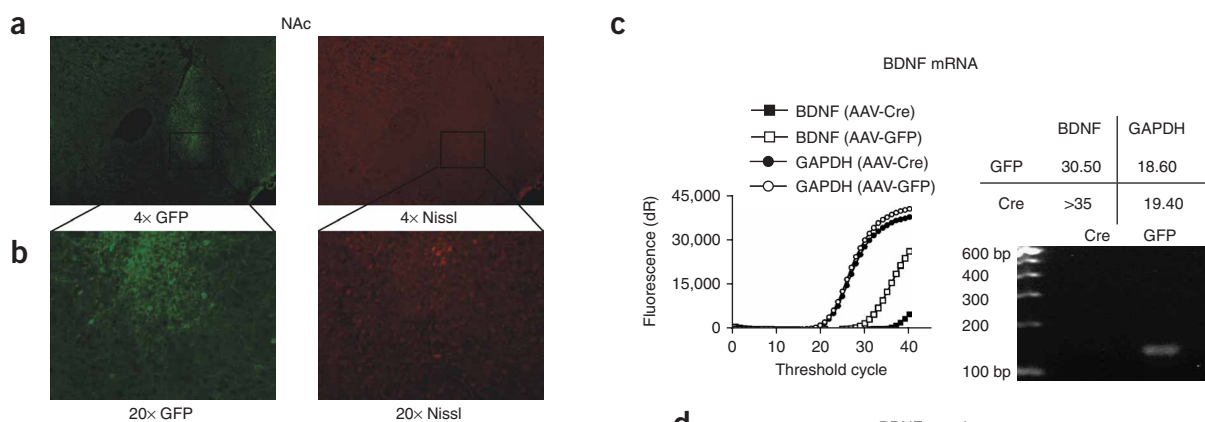
**Figure 6** NAc BDNF and antibody to BDNF infusions fail to alter motivation for natural reward. **(a)** Repeated postsession infusions of BDNF (2.5  $\mu$ g per 0.5  $\mu$ l per side) or antibody to BDNF (5  $\mu$ g per 0.5  $\mu$ l per side) in the NAc shell (left) had no effect on sucrose SA rates (fixed ratio 5) 2–6 d after cessation of treatment ( $N = 6$  per treatment). **(b)** Repeated NAc infusions of BDNF or antibody to BDNF in the NAc shell during prior sucrose SA training (shown in **a**) had no effect on sucrose-seeking behavior in extinction and reinstatement tests conducted 3–4 weeks later ( $N = 6$  per treatment). Reinstatement of nonreinforced responding at the sucrose-paired lever was elicited by noncontingent presentation of sucrose pellets every 2 min for 1 h. Data are expressed as mean  $\pm$  s.e.m.

acquiring cocaine self-administration, and with similar latencies of  $\sim 6$  d to meet acquisition criteria (number of sessions until  $> 15$  cocaine injections for three consecutive days with  $\geq 3:1$  active to inactive lever-response ratio) (Fig. 8a, right). Thus, localized BDNF knockout failed to affect the acquisition of cocaine self-administration under conditions of relatively free access.

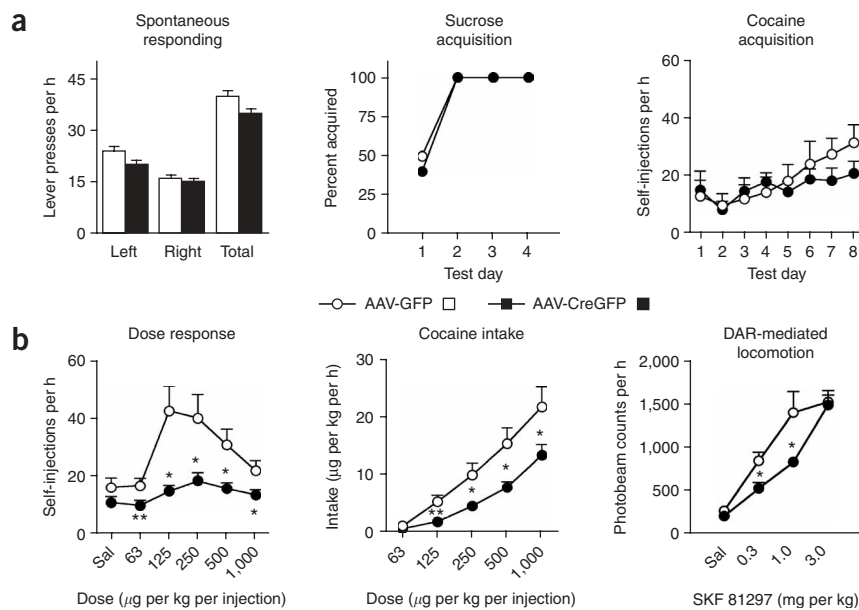
In mice that met acquisition criteria, the response requirement was incrementally increased to five lever presses per injection (fixed ratio 5) until they showed stable self-administration rates. Mice were subsequently tested in a between-session dose-response procedure. AAV-GFP-infused control mice showed the typical inverted, U-shaped self-administration dose-response curve with a dose threshold of 125  $\mu$ g

per kg per injection for maintaining cocaine self-administration (Fig. 8b). In contrast, the dose-response curve was flat and shifted downward in AAV-CreGFP-infused mice compared with GFP-expressing controls (treatment by dose,  $F_{5,65} = 4.237$ ,  $P = 0.002$ ), and overall cocaine intake was reduced across all injection doses (treatment by dose,  $F_{4,52} = 3.245$ ,  $P = 0.019$ ). These data indicate that BDNF derived solely from NAc neurons maintains sensitivity to cocaine reinforcement under the more demanding fixed-ratio 5 reinforcement schedule, and is critical for vertical shifts in the dose-response curve that would reflect a transition to more addicted biological states. However, as reflected in acquisition testing, localized BDNF knockout failed to completely abolish cocaine reinforcement, as these mice self-administered cocaine at rates greater than saline (effect of dose in knockouts,  $F_{5,40} = 3.717$ ,  $P = 0.007$ ).

To determine whether attenuation of cocaine reinforcement was related to a reduction in postsynaptic dopamine-receptor function, we measured locomotor responses to the directly acting dopamine-receptor agonist SKF 81297 in cocaine-naïve mice treated with AAV vectors. Localized knockout of BDNF in NAc neurons significantly reduced dopamine receptor-mediated locomotion in AAV-CreGFP mice compared with AAV-GFP-expressing controls ( $F_{1,8} = 4.205$ ,  $P < 0.05$ ), an



**Figure 7** AAV-Cre-mediated BDNF knockout in NAc neurons. **(a,b)** Localized expression of Cre-GFP fusion protein after intra-NAc infusions of AAV-CreGFP in floxed BDNF mice. **(c)** Quantitative RT-PCR in laser-captured GFP-positive neurons showed elimination of detectable BDNF mRNA in pooled cells that were infected with AAV-CreGFP compared with cells that were infected with AAV-GFP (control). Representative amplification curves for BDNF and GAPDH mRNA from individual pooled samples are shown (mean of three replicates). Mean CT values (table) represent pooled cells from  $N = 3$ –4 animals per treatment. BDNF RT-PCR products (150 bp) separated on agarose gel were readily detectable in cells infected with AAV-GFP, but not in cells infected with AAV-CreGFP. **(d)** Immunoblots from tissue infected with AAV-CreGFP showed a 46% reduction in BDNF protein levels compared with AAV-GFP-infected tissues ( $N = 4$  animals per treatment). Data are expressed as mean  $\pm$  s.e.m.  $** P < 0.01$ , compared with AAV-GFP-treated animals.



**Figure 8** Localized inducible knockout of BDNF in NAc neurons reduces cocaine reinforcement. **(a)** Intra-NAc infusions of AAV-CreGFP in floxed BDNF mice had no effect on spontaneous lever-press behavior, or on acquisition of sucrose (fixed ratio 3) or cocaine (fixed ratio 1; 500 μg per kg per injection) SA compared with AAV-GFP controls. **(b)** Following acquisition, the dose-response and dose-intake curves for CSA (fixed ratio 5) were shifted downward in floxed BDNF mice that received intra-NAc AAV-CreGFP infusions. AAV-CreGFP infusions also attenuated dopamine-receptor (DAR)-mediated locomotion. Data are expressed as mean ± s.e.m. \* $P < 0.05$  and \*\* $P < 0.01$ , compared with AAV-GFP-infused mice ( $N = 6-9$  per treatment).

expression of BDNF mRNA in NAc neurons, which was sufficient for maintaining a substantial percentage of the BDNF protein in NAc tissue. Moreover, cocaine self-administration increased BDNF mRNA expression, indicating that NAc neurons are a likely source of dynamic BDNF protein regulation, and

our results establish a role for BDNF that is derived exclusively from these neurons in regulating sensitivity to cocaine reinforcement. Additional sources of BDNF translocated from dopaminergic and cortico-striatal afferents are known to regulate dopamine D3 receptor expression in NAc neurons that also could contribute to sensitization processes<sup>6,29</sup>.

Behavioral studies have shown that heterozygous BDNF knockout throughout the brain delays the development of cocaine sensitization<sup>15</sup> and attenuates the rewarding effects of cocaine in conditioned place preference<sup>30</sup>. In dopamine cell-body regions such as ventral tegmental area or substantia nigra, continuous local infusion of BDNF increases amphetamine-induced behavioral activation<sup>15,31,32</sup>, and a single infusion of BDNF in the ventral tegmental area administered after withdrawal from cocaine self-administration produces a delayed facilitation in responding for cocaine-associated cues<sup>33</sup>. These behavioral effects could result from local TrkB receptor signaling that potentiates excitatory synaptic input to midbrain dopamine neurons<sup>34</sup>, leading to enhanced dopamine release in the NAc in response to conditioned or pharmacological stimulation<sup>35</sup>.

In the NAc, continuous BDNF infusion also facilitates the development of locomotor sensitization to cocaine, while augmenting responding for conditioned rewards<sup>15</sup>, but these behavioral effects could involve retrograde BDNF transport from dopamine terminals to cell bodies in the ventral midbrain<sup>36,37</sup>. However, intra-NAc infusions of antibody to BDNF or to TrkB attenuate methamphetamine-induced locomotion<sup>14</sup> and we found that BDNF deletion from NAc neurons attenuates dopamine receptor function, suggesting that local TrkB-mediated signaling in NAc neurons contributes to the behavioral consequences of cocaine-induced BDNF regulation. TrkB receptors activate CREB-regulated gene expression that could maintain sensitivity to cocaine reinforcement, as we previously reported that a transient loss of NAc CREB also shifts the dose-response for cocaine self-administration downward<sup>8</sup>. Notably, striatal CREB regulates ΔFosB accumulation that occurs with chronic cocaine<sup>2</sup>, and striatal overexpression of ΔFosB increases the motivation for cocaine in progressive-ratio testing, but does not increase cocaine intake with unrestricted access<sup>38</sup>. Together, these results suggest that BDNF-induced TrkB signaling would represent a critical upstream event

effect that was overcome at the highest dose of SKF 81297 tested (Fig. 8b, right panel). These results indicate that BDNF originating from NAc neurons maintains sensitivity to postsynaptic dopamine-receptor function.

## DISCUSSION

Our results suggest that dynamic regulation of BDNF synthesis and release in the NAc during ongoing cocaine use contributes to the development and maintenance of cocaine addiction. Thus, either self- or experimenter-administered cocaine induced transient increases in BDNF and in TrkB-mediated signaling in NAc neurons, and augmenting this regulation led to a delayed, but prolonged, increase in cocaine intake under conditions of relatively unrestricted access. These changes resulted in a vertical shift in the self-administration dose-response curve reminiscent of addiction-like phenotypic changes in other models of cocaine addiction<sup>19-21</sup>. Augmenting prior BDNF regulation also enhanced the level of effort animals would exert to earn cocaine injections when response demands were increased, and increased cocaine-seeking behavior in the absence of reinforcement in extinction and reinstatement testing conducted during withdrawal. These latter effects suggest that dynamic elevations in NAc BDNF levels during cocaine use would facilitate relapse to cocaine seeking even after periods of prolonged abstinence.

Notably, neutralizing dynamic regulation in endogenous BDNF with antibody to BDNF or with localized BDNF knockout in NAc neurons produced a downward shift in the cocaine self-administration dose-response curve, and attenuated relapse to cocaine seeking elicited by cocaine priming and exposure to stress, along with a reduction in postsynaptic dopamine-receptor responsiveness in NAc neurons. Taken together, these findings suggest that dynamic regulation of BDNF levels in the NAc shell may contribute to increased cocaine consumption and an enduring propensity for relapse, two major criteria for delineating cocaine addiction from pre-morbid drug use in humans<sup>25</sup>.

Previous studies using *in situ* hybridization or northern blot suggest that BDNF mRNA is absent<sup>4,26,27</sup> or expressed at relatively low levels<sup>28</sup> in dopamine terminal regions like the NAc under normal conditions. Using more sensitive RT-PCR procedures, we detected a relatively low

governing multiple pathways that differentially contribute to the manifestation of addictive behavior.

Our results suggest that transient increases in BDNF-induced TrkB receptor signaling with even a single cocaine exposure could lead to indirect and persistent effects that facilitate the development of cocaine addiction. BDNF treatment increases neurite outgrowth in cultured striatal neurons<sup>39</sup>, an effect linked to TrkB-regulated gene expression<sup>40</sup>. Thus, dynamic regulation of BDNF during cocaine use could be involved in dendritic arborization that occurs specifically in the shell, but not core, of the NAc<sup>41,42</sup>, and may involve BDNF regulation of actin polymerization<sup>43</sup>. These morphological changes are consistent with the delayed behavioral response that we found with BDNF treatment, and ultimately could underlie the enduring increase in the motivation for cocaine via disturbances in cortical and dopaminergic input to NAc neurons.

In contrast to neuroadaptations that develop with chronic drug use, our results suggest that even transient cellular responses to acute cocaine exposure can have a prominent and enduring impact on addictive behavior. Moreover, since blocking endogenous BDNF signaling over relatively few self-administration sessions attenuated the development of many long-term aspects of addictive behavior, such dynamic regulation may be essential to the maintenance of cocaine addiction, thereby opening a potential window for treatment in active cocaine abusers. Thus, therapeutic disruption of local BDNF synthesis or TrkB receptor signaling could prevent or reverse a cascade of pathological events resulting from dynamic regulation of BDNF in NAc neurons.

## METHODS

**Animals.** Male Sprague-Dawley rats initially weighing 275–325 g (Charles River) and male floxed BDNF mice (initially 8–10 weeks old), maintained on a mixed background<sup>44</sup> and bred in our transgenic core facilities (University of Texas Southwestern), were individually housed in a climate-controlled environment (21 °C) on a 12-h light-dark cycle (lights on at 7:00 a.m.). Animals were maintained according to the US National Institutes of Health Guide for the Care and Use of Laboratory Animals and protocols approved by the University of Texas Southwestern Institutional Animal Care and Use Committee.

**Self-administration procedures.** Animals were trained to lever press for intravenous cocaine hydrochloride injections (500 µg per kg per 50-µl injection) or the saline vehicle (as a procedural control) in daily test sessions (light cycle) as previously described<sup>8,45</sup> (see **Supplementary Methods** online). Brain tissue from animals self-administering cocaine 4 h per d for 18 d was compared with yoked rats that received the same amount and temporal pattern of passive cocaine injections throughout training (chronic yoke). Another study group received yoked saline injections on all but the final session, when they received cocaine injections for the first time (acute yoke). Tissues from experimental groups were compared with age- and group-matched home cage controls that were handled daily. Animals trained to self-administer cocaine developed stable self-administration by the third week of training, and on the final training day (day 18) cocaine intake ranged from 25.5 mg per kg to 56.5 mg per kg among individual self-administering rats (and yoked partners). Rats were killed by microwave irradiation (5 kW, 1.5 s, Murimachi Kikai) either immediately (no withdrawal) or 1 d after the final test session.

Two additional groups of rats were maintained at 85% original body weight and trained to lever press for 45-mg sucrose pellets for 18 d under similar conditions, and killed either immediately following initial acquisition (100 pellets earned per session) or after five sessions of acquired sucrose self-administration. For temporal analysis of BDNF regulation, naive rats were habituated with five daily saline injections (intraperitoneal) before receiving an acute cocaine challenge (20 mg per kg), and killed 30 min to 24 h later. Tissue for mRNA analysis was collected by rapid decapitation without microwave irradiation.

## Measurement of BDNF and PLC $\gamma$ phosphorylation in NAc homogenates.

NAc shell, core and caudate putamen tissue samples were obtained as described<sup>8,45</sup>. For mRNA determination, tissue samples were immediately homogenized in RNA-STAT60 (IsoTex Diagnostics) and RNA was extracted according to the manufacturer's instructions. DNAase-treated (Ambion) RNA samples were converted to cDNA using Superscript III (Invitrogen). Cycle thresholds (CT) were calculated from triplicate reactions using the second derivative of the reaction. BDNF CT values were normalized by subtracting GAPDH CT values, which show no regulation by cocaine. Fold changes were calculated by subtracting mean normalized control CT values from CT values for individual samples. Primer sequences for BDNF and GAPDH were 5'-TCATA CTTCGGTTGCATGAAGG-3', 5'-AGACCTCTCGAACCTGCC-3', and 5'-AA CGACCCCTTCATTGAC-3', 5'-TCCACGACATACCTCAGCAC-3', respectively.

BDNF protein and PLC $\gamma$  phosphorylation levels were determined in 20-µg protein samples subjected to SDS-PAGE (BDNF, 15.0% acrylamide; phosphoPLC $\gamma$ , 7.0% acrylamide) using a Tris-Tricine-SDS buffer (Bio-Rad), followed by electrophoretic transfer to PVDF membranes. BDNF was immunolabeled with rabbit antibody to BDNF (1:1,000; Cat # sc-546) or rabbit antibody to phosphoPLC $\gamma$  (1:1,000; Cat # sc-12943), from Santa Cruz Biotechnology, overnight at 4 °C in blocking buffer, and washed and labeled with species-specific peroxidase-conjugated secondary antibody (1:10,000; BioRad) for 1 h at 23 °C. Following chemiluminescence detection, BDNF blots were reprobed for  $\beta$ -tubulin (1:100,000, Cat # 05-661, Upstate Biotechnology) and phosphoPLC $\gamma$  blots were reprobed for total PLC $\gamma$  (1:1,000, Cat # 05-163, Upstate Biotechnology) as internal standards. Immunoreactivity was quantified by densitometry under conditions that were linear over at least a threefold concentration range. Each sample was compared with the mean of 5–6 untreated control samples to allow for normalization across blots.

In some experiments, rats under chloral hydrate anesthesia (200 mg per kg) received unilateral infusions of antibody to BDNF (5.0 µg per 0.5 µl per side) in the NAc shell through 30-gauge microsyringes immediately before cocaine challenge, with contralateral infusions of IgG (5.0 µg) or the PBS vehicle. Other animals received unilateral infusions of 2.5 µg per 0.5 µl per side human recombinant BDNF (a generous gift from Regeneron Pharmaceuticals), with contralateral PBS infusions. NAc core and shell tissues were collected 30 min later for analysis of PLC $\gamma$  phosphorylation. PhosphoPLC $\gamma$  levels with either IgG or PBS infusions were similar and pooled into a single control group.

**Localized BDNF knockout in NAc neurons.** We infused an AAV that expressed Cre recombinase into the NAc of male mice homozygous for a floxed allele (exon 5) encoding the BDNF gene<sup>44</sup>. GFP and an N-terminal enhanced GFP-Cre recombinase fusion protein<sup>46</sup> were prepared in AAV vectors using a triple-transfection helper-free method as described previously<sup>47</sup>. Two weeks after NAc AAV infusions (see **Supplementary Methods**), brains were dissected, rapidly frozen, and 9-µm coronal sections were dehydrated in RNAase-free 95% and 100% ethanol and xylene for 20 s. Approximately 60–100 GFP-expressing cells from nine consecutive sections per mouse were collected by laser capture (PixCell Iie, Arcturus), and pooled for subsequent RNA extraction (PicroPure RNA isolation kit, Molecular Devices). Following two rounds of linear RNA amplification (RiboAmp HS RNA Amplification, Molecular Devices), samples were subjected to quantitative RT-PCR as described above. BDNF PCR products (150 bp) were visualized under ultraviolet light after separation on a 1.5% metaphore agarose gel with ethidium bromide. Neither GFP nor CreGFP infection altered GAPDH mRNA levels compared with extracts purified from NAc homogenates (data not shown).

**Data analysis.** BDNF protein and PLC $\gamma$  phosphorylation were analyzed by one-factor ANOVA, followed by Fisher's least significant difference *post hoc* comparisons. Behavioral data were analyzed by two-factor ANOVA with repeated measures on dose or test session. Significant interactions were followed by univariate ANOVA at each dose or test session, which was also used for cue, stress and sucrose reinstatement data, or acquisition latency data, and followed by Fisher's least significant difference tests. Behavioral responses with intra-NAc PBS or IgG were similar, and so these data were pooled as a single control.



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

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#### AUTHOR CONTRIBUTIONS

D.L.G. conducted the majority of the experiments and all of the data analysis, with assistance in tissue generation from R.K.B. and S.E. R.J.D. provided the AAV viral vectors and M.R. provided the floxed BDNF mice. D.W.S. supervised the project and co-wrote the manuscript with D.L.G.

#### COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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