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# Different Roles of BDNF in Nucleus Accumbens Core versus Shell during the Incubation of Cue-Induced Cocaine Craving and Its Long-Term Maintenance

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## Abstract

Brain-derived neurotrophic factor (BDNF) contributes to diverse types of plasticity, including cocaine addiction. We investigated the role of BDNF in the rat nucleus accumbens (NAc) in the incubation of cocaine craving over 3 months of withdrawal from extended access cocaine self-administration. First, we confirmed by immunoblotting that BDNF levels are elevated after this cocaine regimen on withdrawal day 45 (WD45) and showed that BDNF mRNA levels are not altered. Next, we explored the time course of elevated BDNF expression using immunohistochemistry. Elevation of BDNF in the NAc core was detected on WD45 and further increased on WD90, whereas elevation in shell was not detected until WD90. Surface expression of activated tropomyosin receptor kinase B (TrkB) was also enhanced on WD90. Next, we used viral vectors to attenuate BDNF-TrkB signaling. Virus injection into the NAc core enhanced cue-induced cocaine seeking on WD1 compared with controls, whereas no effect was observed on WD30 or WD90. Attenuating BDNF-TrkB signaling in shell did not affect cocaine seeking on WD1 or WD45 but significantly decreased cocaine seeking on WD90. These results suggest that basal levels of BDNF transmission in the NAc core exert a suppressive effect on cocaine seeking in early withdrawal (WD1), whereas the late elevation of BDNF protein in NAc shell contributes to incubation in late withdrawal (WD90). Finally, BDNF protein levels in the NAc were significantly increased after ampakine treatment, supporting the novel hypothesis that the gradual increase of BDNF levels in NAc accompanying incubation could be caused by increased AMPAR transmission during withdrawal.



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## Introduction

Relapse after abstinence from cocaine is often induced by exposure to cocaine-associated cues. In rats, cue-induced cocaine seeking progressively increases over the first months of withdrawal from extended-access cocaine self-administration and remains elevated even 6 months after the last exposure (Neisewander et al., 2000; Grimm et al., 2001; Lu et al., 2004b; c; Sorge and Stewart, 2005; Pickens et al., 2011). This phenomenon, termed incubation, is reminiscent of findings in cocaine addicts (Gawin and Kleber, 1986). In parallel with incubation of cocaine craving, brain-derived neurotrophic factor (BDNF) protein levels show a time-dependent increase in the nucleus accumbens (NAc), ventral tegmental area (VTA), and amygdala (Grimm et al., 2003). This suggests that BDNF may contribute to incubation.

Other results also implicate BDNF in cocaine seeking but indicate regional differences in its function. For example, elevation of BDNF in the medial prefrontal cortex (mPFC) suppresses cocaine seeking (Berglind et al., 2007, 2009; Sadri-Vakili et al., 2010; Whitfield et al., 2011), whereas elevation of BDNF in the VTA (Lu et al., 2004a) and NAc shell (Graham et al., 2007; Graham et al., 2009) facilitates cocaine seeking. Similar studies have not been performed in the NAc core, and BDNF's role in seeking after long withdrawals (>15 d) has not been examined in either NAc subregion. Core-shell differences are possible given different effects of cocaine exposure on BDNF and TrkB expression in these regions (Filip et al., 2006; Graham et al., 2007; Graham et al., 2009).

Another important adaptation accompanying incubation is the accumulation of GluA2-lacking $Ca^{2+}$-permeable AMPA receptors (CP-AMPARs) in NAc synapses (Conrad et al., 2008). In contrast to the $Ca^{2+}$-impermeable GluA1A2-containing AMPARs that dominate in the NAc of drug-naive rats (Reimers et al., 2011), CP-AMPARs exhibit higher conductance and can be identified based on inward rectification resulting from voltage-dependent polyamine block (Isaac et al., 2007; Liu and Zukin, 2007). CP-AMPARs are first detected in the NAc core on approximately withdrawal day 30 (WD 30) (Wolf and Tseng, 2012), and their blockade on WD45 prevents the expression of incubated cue-induced cocaine seeking (Conrad et al., 2008). They also accumulate in shell (Mameli et al., 2009; McCutcheon et al., 2011). Previous *in vitro* studies showed that BDNF promotes synaptic delivery of CP-AMPARs (Caldeira et al., 2007, Li and Keifer, 2008, 2009; Keifer and Zheng, 2010), and our *in vivo* study demonstrated that BDNF infusion into the NAc core transiently increased GluA1 surface expression without affecting GluA2 or GluA3, suggesting an increase in CP-AMPARs (Li and Wolf, 2011a).

In light of these previous findings, we tested the hypothesis that elevated BDNF transmission in the NAc underlies incubation of cocaine craving and accompanying CP-AMPAR accumulation. Contrary to our hypothesis, we found that BDNF in NAc shell contributes to incubation only in late withdrawal, whereas core BDNF initially suppresses incubation. Additionally, time course data raised the possibility that enhanced AMPAR transmission during incubation triggers the elevation of BDNF, rather than *vice versa*. A final experiment explored this possibility using ampakines (Lynch and Gall, 2006) to elevate AMPAR transmission.

## Materials and Methods

### Subjects

Male Sprague-Dawley rats (250–275 g) were purchased from Harlan and housed with food and water available *ad libitum*. Rats were allowed to acclimate 4–7 d before any experimental procedures. For self-administration experiments, rats were housed individually

in a reversed light cycle room (12 h/12 h light/dark), with lights on at 19:00 h. All self-administration sessions and cue-induced cocaine-seeking tests were performed in the dark phase of the cycle with food and water available throughout the sessions. For other experiments, rats were housed in pairs in a normal light cycle room (12 h/12 h light/dark), with lights on at 07:00 h. All procedures were approved by the Institutional Animal Care and Use Committee of Rosalind Franklin University of Medicine and Science.

### Catheter implantation and self-administration training

Rats were anesthetized by isoflurane gas (Henry Schein). Then a Silastic catheter (Plastics One) was inserted into the right internal jugular vein and passed subcutaneously to the mid-scapular region. Rats also received the analgesic banamine (2 mg/kg, s.c.) before surgical procedures, and catheters were flushed daily with sterile 0.9% saline solution. Self-administration training sessions started 5–7 d after surgeries and continued for 10 consecutive days (6 h/d) as previously described (Conrad et al., 2008). Briefly, rats were placed in self-administration chambers (MED Associates) equipped with two nose-poke holes. Responding in one hole (active) resulted in an intravenous infusion of cocaine (0.5 mg/kg in 32 $\mu$l/infusion) or saline (32 $\mu$l/infusion) paired with the illumination of a cue light. Responding in the other hole (inactive) had no consequences. Rats were brought back to their home cages after each 6 h training session. A fixed ratio 1 schedule was used to deliver the cocaine with a 20 s timeout period. Responses in both holes (active and inactive) and total infusions were recorded for each training session.

### Cue-induced cocaine-seeking tests

After the 10 day cocaine self-administration training described above (Catheter implantation and self-administration training), rats were housed in their home cages and handled several times per week during the withdrawal period. On the test day, rats were brought back to the self-administration training boxes, and cue-induced cocaine seeking was tested under extinction conditions, in which responding in the active hole led to no cocaine infusion but only the presentation of the light cue previously paired with drug delivery during the training sessions. Responses in the active hole were recorded and used as a measure of cocaine seeking. All seeking tests lasted 30 min, except the test shown in Figure 6*C* (60 min).

### Viral vectors and intracranial infusion

For behavioral studies, rats in all 6 cohorts (see Figs. 6 to Fig. 9) received bilateral intracranial viral vector infusions into either NAc core or shell immediately after the catheter implantation. The coordinates for NAc core were as follows: anteroposterior (AP) +1.2 mm; lateral (L) +2.6 mm (6° angle); and dorsoventral (DV) −7.0 mm (Paxinos and Watson, 1998). The coordinates for NAc shell were as follows: AP +1.2 mm; L +1.5 mm (6° angle); and DV −7.0 mm. Anesthetized by isoflurane gas, rats were mounted onto a stereotaxic frame and viral vectors were infused directly through Hamilton syringes. Our prior results showed that the virus injection volumes used in our studies produce an area of infection confined to the targeted region; that is, injections aimed at the core did not lead to appreciable virus spread into the shell (Li and Wolf, 2011b). The lentiviruses (LVs) used in this study (kind gifts from Drs. Amine Bahi and Jean-Luc Dreyer) were LV-GFP ($8 \times 10^9$ IU/ml), LV-TrkBsiRNA ($8 \times 10^9$ IU/ml), and LV-TrkBT1 ($8 \times 10^9$ IU/ml). They were infused into the NAc at a rate of 0.2 $\mu$l/min (4 $\mu$l/side) with 5 min allowed for diffusion before withdrawing the syringe. In a previous study, the expression and functional efficacy of these viral vectors were demonstrated ~20 d after their injection into the NAc (Bahi et al., 2008). Even as long as 12 weeks after intra-NAc injection of LV-TrkBsiRNA, there is still a robust decrease (~60%) in TrkB mRNA levels in the NAc [control, 100 ± 22.12 ($n = 7$); LV-TrkBsiRNA, 43.09 ± 6.29 ($n = 10$); $p < 0.05$, *t* test (Bahi and Dreyer, unpublished

observations)]. These studies, as well as our present results, verify that LV expression persists over the timeframe of our experiments. The adeno-associated virus (AAV) vectors used in this study (kind gifts from Drs. Joost Verhaagen and Eric M. Ehlert) were AAV-GFP ($1 \times 10^9$ TU/ml), AAV-TrkB.T1 with FLAG ($3.6 \times 10^9$ TU/ml), and AAV-TrkB.T1 with GFP ($3 \times 10^9$ TU/ml). We pooled the latter two viruses to generate the AAV-TrkB.T1 group. AAV viral vectors were infused at a rate of 0.1 $\mu$l/min (0.5 $\mu$l/side) with 10 min allowed for diffusion. Their expression and functional efficacy have been established in previous studies (Eisch et al., 2003; Wit et al., 2006). For example, cotransfection of AAV-TrkB and AAV-TrkB.T1 *in vitro* significantly reduced BDNF-induced phosphorylation of TrkB (Wit et al., 2006). Furthermore, FLAG immunostaining on WD90 confirmed virus expression throughout the timeframe of our experiments (see Figs. 7 and 9). For biochemical studies (see Fig. 10), we used LV-GFP and LV-BDNF-GFP made by Dr. Amy W. Lasek (Ernest Gallo Clinic and Research Center, Department of Neurology, University of California, San Francisco, Emeryville, California). Expression of these viruses throughout the timeframe of the experiments was confirmed by GFP or BDNF immunohistochemistry (21 d after virus injection; see Fig. 10*A*) or immunoblotting (47 d after virus injection; see Fig. 10*B*). The goal of the LV-BDNF-GFP experiment was to produce a long-term elevation of BDNF levels in the NAc core or shell (47 d), dissect NAc tissue, and use a protein crosslinking assay to determine whether AMPAR surface expression was elevated. To selectively and accurately dissect the region of NAc expressing LV-GFP, we used a GFP flashlight and filter glasses as described previously (Li and Wolf, 2011b). However, the GFP signal produced by LV-BDNF-GFP was not strong enough to visualize. To overcome this, we used a mixture of LV-BDNF-GFP and LV-GFP (8:1, v/v) to produce a stronger GFP signal, enabling us to use the GFP flashlight to identify and dissect the region of viral expression. Therefore, after rats were anesthetized with ketamine-xylazine mixture (80 and 10 mg/kg, respectively), either LV-GFP virus alone or the mix of LV-BDNF-GFP ($1.2 \times 10^7$ pg/ml) and LV-GFP ($7.1 \times 10^7$ pg/ml) was infused directly into NAc core or shell with Hamilton syringes (0.2 $\mu$l/min; 3 $\mu$l/side; 5 min diffusion time) using the same coordinates described above. Forty-seven days after the infusion, GFP-expressing regions from NAc core or shell were collected for the protein crosslinking assay (see Biotinylation and protein crosslinking).

## Histology

At the end of each behavioral experiment, rats were deeply anesthetized and transcardially perfused first with 0.9% saline and then with 4% paraformaldehyde (PFA; 158127, Sigma) in PBS. Brains were postfixed in 4% PFA overnight, and then 40 $\mu$m sections were obtained by cryostat. For experiments using LV-GFP, LV-TrkBsiRNA, and LV-TrkB.T1, sections were mounted on gelatin-coated slides and locations of viral injection sites were determined after Cresyl Violet (C1791, Sigma) staining. Only rats with both injection tips in the target region (NAc core or shell) were included for statistical analysis. For experiments using AAV-GFP or AAV-TrkB.T1, sections were processed for either FLAG or GFP immunohistochemistry, as described previously (Lobbestael et al., 2010), to verify viral expression and injection placement. Briefly, sections were first blocked with 10% donkey serum in PBS-T (PBS with 0.1% Triton X-100) and then incubated with primary antibody (FLAG: 1:5000, F7425, Sigma; GFP: 1:5000, ab290, Abcam) overnight. After three PBS-T washes, sections were incubated with a Cy3-AffiniPure Donkey Anti-Rabbit IgG (1: 500 in PBS, 711–165-152, Jackson ImmunoResearch Laboratories) for 2 h at room temperature and then mounted with PVA-DABCO coverslipping solution. Slides were examined by epifluorescence microscopy (Eclipse C600; Nikon), and only rats with viral expression in the target region were included in the analysis.

### BDNF immunohistochemistry

Forty-five days or 90 d after the last day of saline or cocaine self-administration training, rats were decapitated and brains were fixed in 2.5% PFA in PBS on ice for 2 h. Then brains were transferred to 30% sucrose in 2.5% PFA and fixed for another 24 h at 4°C. Sections (40 $\mu$m) were obtained using a cryostat and stored in cryoprotectant (23.8% glycerol and 28.6% ethylene glycol in 0.1 M PBS) at −20°C. For immunohistochemistry, free-floating sections were first washed in PBS for 15 min and then blocked with 10% donkey serum with 0.1% Triton X-100 for 1 h. Next, sections were incubated with primary antibody (1:1000 in 4% donkey serum; AB1779, Millipore) overnight at 4°C. After a 30 min wash in PBS, sections were incubated with a Cy3-AffiniPure Donkey Anti-Rabbit IgG (1: 500 in PBS, 711–165-152, Jackson ImmunoResearch Laboratories) for 3 h at room temperature and then mounted with PVA-DABCO coverslipping solution. In preliminary control studies (data not shown), we validated the specificity of the BDNF immunostaining by omitting primary antibody. We also verified that BDNF immunoreactivity was high in cortex but low in the NAc of drug-naive rats, which was consistent with previous studies (Conner et al., 1997). Visualization of BDNF immunoreactivity was accomplished by epifluorescence microscopy (Eclipse C600; Nikon). NIS Elements software was used to acquire images and analyze staining intensity. For quantification, slices were altas matched and those at the AP level of ~1.20 mm from bregma were sampled. On each slice (two slices/rat), the mean intensity of two representative regions from core and two representative regions from shell was measured (as shown in Fig. 3*D*). The mean density of the anterior commissure was used for background subtraction. All images were taken with the same exposure time. To determine whether our results might have been affected by a change in the volume of the NAc in cocaine versus saline rats, we also measured the volume of NAc on WD45. After a fixation protocol similar to that described above, brain slices (40 $\mu$m) were serially collected. Then every fourth section was selected, yielding a series of 8 equally spaced sections that spanned the NAc. The volume of the NAc was estimated according to Cavalieri principles (Coggeshall, 1992).

The same BDNF immunofluorescence protocol described above was used to verify expression of LV-BDNF-GFP 3 weeks after virus infusion into the NAc. BDNF immunoreactivity and GFP (expressed by the virus) were visualized by confocal microscopy (Fluoview; Olympus).

### Biotinylation and protein crosslinking

For biotinylation, rats were decapitated at different withdrawal times (WD14, WD25, WD48, and WD90) after saline or cocaine self-administration training. Then NAc tissue from both hemispheres was collected and processed for biotinylation of surface-expressed proteins using sulfo-NHS-S-S-Biotin (Thermo Scientific). For each animal, a portion of biotinylated NAc tissue was set aside without further processing and used to measure total TrkB expression. The remainder of biotinylated tissue was purified using NeutrAvidin agarose beads (catalog no. 29204, Thermo Scientific), generating a bound fraction that was used to analyze TrkB surface expression. Both purified samples (bound fractions) and unprocessed samples were analyzed as described below (Western blotting). Biotinylation and purification methods have been described previously (Ferrario et al., 2011). For protein crosslinking, rats were decapitated 47 d after intracranial infusion of LV-GFP or LV-BDNF-GFP into either NAc core or shell. Then NAc core or shell subregions were dissected and processed for $BS^3$ [bis-(sulfosuccinimidyl)suberate] crosslinking as previously described (Boudreau and Wolf, 2005; Ferrario et al., 2010; Boudreau et al., 2012). The samples were further processed to distinguish surface and intracellular AMPAR subunits as described below (Western blotting).

### RNA extraction and real-time PCR

Bilateral NAc core and shell tissue, as well as mPFC tissue, were collected separately 45 d after discontinuing either saline or cocaine self-administration. RNA was extracted with TRIzol reagent (15596–026, Invitrogen), according to the protocol from the manufacturer. After cleaning with the DNA-free Kit (AM1906, Invitrogen), 1 $\mu$g of RNA from each sample was transcribed into cDNA using High-Capacity cDNA Reverse Transcription Kit (4368814, Applied Bio-systems). To measure total BDNF mRNA levels and BDNF exon IV transcript levels, quantitative RT-PCR was performed using TaqMan Gene Expression Master Mix (4369016, Invitrogen) and normalized for each sample to the GAPDH mRNA levels. The primer and probe sets of TaqMan Gene Expression Assay for total BDNF and GAPDH were purchased from Applied Biosystems (total BDNF ID: Rn02531967_s1; GAPDH ID Rn01775763_g1). The BDNF exon IV-specific primer and probe set was made by TaqMan Custom Assay & Oligo Service based on a previous study (Liu et al., 2006) measuring BDNF exon IV transcript levels. The 96-well plates with 25 $\mu$l of reaction mixture in each well were first incubated at 50°C for 2 min and then at 95°C for another 10 min, followed by 40 cycles (95°C for 10 s and then 60°C for 1 min) of PCR. All samples were run in triplicate, and real-time fluorescence was detected by 7500 Real-time PCR System (Applied Biosystems). Threshold cycles ($C_t$) were analyzed by 7500 System SDS Software (Applied Biosystems), and a relative quantification method ($\Delta\Delta C_t$) was used to calculate target gene expression according to the Guide to Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR (Applied Biosystems).

### Ampakine treatment

The regimen for ampakine treatment was taken from a previous study (Rex et al., 2006) in which rats received 2 intra-peritoneal (i.p.) injections per day (one between 08:00 h and 09:00 h; the other between 14:00 and 15:00 h) for 8 consecutive days. For the first 4 d, all rats received vehicle injections [16.7% 2-hydroxypropyl-$\beta$-cyclodextrin (H107, Sigma) in 0.9% sterile saline]. After this acclimatization period, rats in the vehicle group continued to receive vehicle injections for the next 4 d, whereas rats in the ampakine group received injections of the ampakine CX929 (5 mg/kg dissolved in vehicle solution; provided by Cortex Pharmaceuticals). Twenty-two hours after the last injection, both NAc and hippocampal tissue from a randomly chosen hemisphere were dissected and homogenized in lysis buffer as previously described (Boudreau and Wolf, 2005) and processed as described below (Western blotting). NAc and hippocampal tissue from the other hemisphere were collected and processed for mRNA extraction, followed by RT-PCR to measure BDNF mRNA levels as described above (RNA extraction and real-time PCR). Rats were housed in pairs with one animal in the vehicle group and the other in the ampakine group.

### Western blotting

All samples (10–20 $\mu$g of per lane) were processed for Western blotting as previously described (Boudreau and Wolf, 2005; Ferrario et al., 2011; Boudreau et al., 2012) with the following primary antibodies: BDNF (sc-546, 1:500; Santa Cruz Biotechnology); phosphorylated TrkB (pTrkB, Tyr 706/707; 4621, 1:1000; Cell Signaling), TrkB (07–225, 1:2000; Millipore); GluA1 (PA1–37776, 1: 1000; Thermo Scientific); GluA2 (L21/32, 1:200; University of California Davis/National Institutes of Health NeuroMab Facility, Davis, California). After incubation with either HRP-conjugated anti-rabbit or anti-mouse IgG (1:10000; Invitrogen), proteins were visualized by film using the ECL detection system (GE Healthcare) and quantified by TotalLab (Life Sciences Analysis Essentials). GAPDH (CB1001, 1:10000; Calbiochem) was used as a loading control for BDNF, GluA1, and GluA2. The levels of pTrkB were normalized to TrkB levels, which were determined with the phosphorylation-independent TrkB antibody. The levels of TrkB in biotinylated samples

(bound fraction) were normalized to total protein levels, which were determined with Ponceau-S stain (P7170, Sigma).

### Statistical analysis

Unpaired *t* tests (two-tailed) were conducted to compare mean values between two groups as indicated in each figure, except where otherwise noted. Two-way repeated-measure ANOVA with days × virus pretreatment as factors was used to compare daily total infusions across the 10 d of cocaine self-administration training. Significance was set at $p < 0.05$.

## Results

### BDNF protein levels are increased in the NAc of cocaine rats on WD45

We began by focusing on WD45, a time when incubation of cue-induced cocaine craving and synaptic accumulation of CP-AMPARs have been demonstrated in the rat NAc after extended access cocaine self-administration (6 h/d for 10 d) (Conrad et al., 2008). Immunoblotting was used to compare BDNF protein levels in NAc tissue obtained on WD45 after this same regimen of cocaine or saline self-administration. We found a significant increase in BDNF protein levels in the cocaine group (Fig. 1, $p < 0.05$), consistent with a previous report showing increased BDNF levels on WD30 and an even greater increase on WD90 (Grimm et al., 2003). Self-administration training data from the cocaine and saline rats shown in Figure 1 were published previously, as were biochemical results indicating increased levels of CP-AMPARs in the NAc of the cocaine group (Ferrario et al., 2011).

### Surface expression of phosphorylated TrkB is increased in cocaine rats on WD90

The time-dependent increase in BDNF protein that occurs in the NAc during incubation (Grimm et al., 2003) (Fig. 1) could theoretically lead to time-dependent changes in TrkB expression or activation in the NAc. To test this, we used NAc tissue that was collected and biotinylated at different withdrawal time points (WD25, WD48, and WD90) after saline or cocaine self-administration training using the same regimen described above. Then we measured surface and total levels of TrkB and phosphorylated TrkB (pTrkB; Tyr706/707) by immunoblotting. Phosphorylation at Tyr706/707 is necessary for TrkB activation and subsequent activation of downstream pathways (Segal et al., 1996; Cunningham et al., 1997; Huang and Reichardt, 2003). Although surface TrkB, total TrkB, and total pTrkB protein levels did not differ between cocaine and saline groups at any withdrawal time point (Fig. 2*B–D*), surface pTrkB levels were significantly elevated in the NAc of cocaine rats compared with saline controls by WD90 (Fig. 2*A*, $p < 0.05$, one tail). This could indicate that gradual increases in BDNF signaling (Fig. 1; Grimm et al., 2003; also see Figs. 3 and 4) eventually lead to increased levels of TrkB activation during withdrawal.

### Time-dependent increases in BDNF levels follow different time courses in the NAc core versus shell

Our studies of BDNF and TrkB described above (Figs. 1 and 2, respectively) were performed in whole NAc, rather than core and shell subregions, for two reasons. First, we were extending a prior study performed in whole NAc (Grimm et al., 2003). Second, our goal was to test a hypothesized link between BDNF and CP-AMPAR accumulation during incubation, and we knew that CP-AMPARs accumulated in both core and shell subregions of the NAc during incubation, although the effect is less robust in the shell (Conrad et al., 2008; McCutcheon et al., 2011). However, because previous evidence has suggested distinct BDNF–cocaine interactions in the NAc core versus shell (Graham et al., 2007; Graham et

al., 2009), we next examined whether BDNF levels are differently affected in the NAc core and shell during incubation of cocaine craving.

As in prior studies, rats self-administered saline or cocaine for 6 h/d for 10 d (Fig. 3*A*). They were killed on WD45, and brain sections were processed for BDNF immunohistochemistry. To quantify BDNF immunoreactivity in the NAc core and shell, we sampled two sections per animal. In each section, we measured the mean density of BDNF immunoreactivity in two regions of core and two regions of shell (Fig. 3*B*, squares). We observed a significant increase in BDNF staining in the NAc core of cocaine rats on WD45 (~150% of saline control values; $p < 0.05$), but no group difference in the NAc shell (Fig. 3*C*, *D*). Because core composes the majority of our whole NAc dissection, this explains the increased BDNF levels detected in whole NAc by immunoblotting in Figure 1. To determine whether BDNF levels eventually increase in the NAc shell, we prepared another cohort of rats and measured BDNF immunoreactivity in the NAc core and shell on WD90. At this withdrawal time, cocaine rats showed an even more robust increase in BDNF staining in the core (~270% of control) and now showed a significant increase in the shell (~180% of control) (Fig. 4*B*, *C*; $p < 0.05$). In parallel with these studies, we compared the volume of the NAc in saline and cocaine rats (on WD45) and found no difference (Fig. 3*E*), indicating that our quantification of BDNF staining in the two groups was not influenced by volume changes. The different time courses of BDNF elevation in core and shell indicate that BDNF signaling in these subregions of the NAc may play different roles in the incubation of cocaine craving. Therefore, all subsequent experiments examined each subregion separately.

### BDNF mRNA levels are unchanged in the NAc core and shell on WD45

BDNF mRNA is present in the NAc (Filip et al., 2006; Graham et al., 2007; Prakash et al., 2008), raising the possibility that the increased BDNF protein levels observed in the NAc during incubation could be the result of increased BDNF mRNA levels. To test this hypothesis, we used real-time PCR to measure BDNF mRNA levels in the NAc core and shell of cocaine and saline groups killed on WD45 after self-administration training. The training data are shown in Fig. 5*A*. Based on results in Figure 3, if increased BDNF protein is the result of increased BDNF mRNA levels, an increase in BDNF mRNA levels would be expected in core but not shell on WD45.

Multiple promoters of the BDNF gene as well as different transcriptional control mechanisms led to multiple BDNF transcripts. We first used a primer set that detects exon VIII, common to all BDNF transcripts (Liu et al., 2006), to measure total BDNF mRNA levels. We found no changes in either NAc subregion of cocaine rats compared with saline rats (Fig. 5*B*). Previous studies have shown that exon IV was selectively elevated both in the ventral striatum after acute cocaine exposure and in the mPFC 7 d after cocaine self-administration (Liu et al., 2006; Sadri-Vakili et al., 2010). In the mPFC, cocaine self-administration also increased the association of BDNF exon IV with acetylated histone H3 (Sadri-Vakili et al., 2010), which facilitates an open chromatin configuration and enhances gene transcription (Robison and Nestler, 2011). Moreover, methyl-CpG-binding protein 2, a transcription factor that is a key regulator of BDNF gene expression (Chen et al., 2003; Martinowich et al., 2003; Im et al., 2010), showed decreased association with mPFC BDNF exon IV on WD7 after cocaine self-administration training (Sadri-Vakili et al., 2010). Based on these previous findings, we also measured BDNF exon IV expression in the NAc core and shell on WD45. However, similar to BDNF total mRNA, the level of BDNF exon IV did not differ between saline and cocaine groups (Fig. 5*C*). These data suggest that the cocaine-induced increase in BDNF protein is not the result of an increase in BDNF mRNA levels in the NAc. Although it remains possible that cocaine increased the translation of existing BDNF mRNA in NAc neurons, it is more likely that other mechanisms explain the increase in BDNF protein levels in the NAc after incubation (see Discussion).

### Attenuating BDNF-TrkB signaling in the NAc core before cocaine self-administration training enhances cocaine seeking on WD1

To study whether BDNF-TrkB signaling in the NAc core plays a role in the incubation of cocaine craving, rats were infused with LV-TrkBsiRNA or LV-GFP (control) into the NAc core before cocaine self-administration training (Fig. 6*A*). Efficacy of this LV-TrKBsiRNA was previously demonstrated (Bahi et al., 2008; see also Materials and Methods). Two cohorts of rats were generated: one destined for a test of cue-induced cocaine seeking on WD1 (Cohort 1) and the other destined for testing on WD30 (Cohort 2). As shown in Figure 6*B*, cocaine infusions over 10 d of self-administration training did not differ between rats that received prior infusion of LV-GFP versus LV-TrkBsiRNA. However, cue-induced cocaine seeking on WD1 showed a 70% increase in LV-TrkBsiRNA rats compared with LV-GFP rats (Fig. 6*C*, $p < 0.05$). On WD30, LV-GFP rats showed incubation compared with WD1 (Fig. 6*C*, $p < 0.05$), whereas LV-TrkB-siRNA rats showed only a trend ($p = 0.11$) toward a further increase compared with their elevated WD1 values. Thus, attenuating TrkB signaling in the NAc core before cocaine self-administration enhanced cue-induced craving on WD1, suggesting that BDNF-TrkB signaling exerts a negative influence on cue-induced cocaine craving during early withdrawal.

Although LV-GFP rats and LV-TrkBsiRNA rats showed no significant difference in cue-induced cocaine seeking behavior on WD30 (Fig. 6*C*), BDNF protein levels in the NAc increase markedly between WD30 and WD90 (Grimm et al., 2003; Figs. 3 and 4) and incubation is maintained over this period (Grimm et al., 2001; Lu et al., 2004b; c). This suggests the possibility that elevation of BDNF levels may be important for a late phase of incubation, rather than its development during the first month after discontinuing cocaine self-administration. Indirect support for a relationship between BDNF and the persistence of incubation is provided by comparing the duration of incubation of cue-induced cocaine and sucrose seeking. Whereas incubation of cocaine seeking is at peak levels between WD30 and WD90 and is accompanied by increased BDNF levels, incubation of sucrose seeking in rats is much less persistent, returning to basal levels by WD90, and is not associated with increased BDNF levels in the NAc (Grimm et al., 2003; Lu et al., 2004c).

To test the hypothesis that elevation of BDNF levels may be important for the incubation at long withdrawal times, such as WD90, we used an AAV expressing a truncated form of TrkB (TrkB.T1) that acts as a dominant negative to attenuate BDNF-TrkB signaling. This virus, referred to hereafter as AAV-TrkB.T1, has been shown to attenuate BDNF-TrkB signaling both *in vivo* and *in vitro* (Eisch et al., 2003; Wit et al., 2006; see Materials and Methods for more details). We used AAV-TrkB.T1 for two reasons. First, we had exhausted our supply of LV-TrkBsiRNA performing WD1/WD30 experiments in core (Fig. 6) and shell (see below). Furthermore, the FLAG or GFP tags on the AAVs provide a simple means of verifying that their expression lasts through WD90.

AAV-TrkB.T1 or AAV-GFP (control) was injected into the NAc core before cocaine self-administration training (Fig. 7*A*). In this experiment, the same rats (Cohort 3) were used to test cocaine seeking on WD1 and WD90. For our prior experiment (Fig. 6), we had used different cohorts on each test day, but we subsequently verified that 2 or 3 tests can be performed in the same animals without affecting incubation (data not shown), as also found previously (Lu et al., 2004a; Li et al., 2008; Lu et al., 2009). No difference in cocaine infusions was observed between AAV-TrkB.T1 and AAV-GFP groups during self-administration training (Fig. 7*B*). Consistent with results obtained using LV-TrkBsiRNA to decrease BDNF-TrkB signaling in the NAc core (Fig. 6*C*), we observed a 70% increase on WD1 in rats previously infused with AAV-TrkB.T1 compared with AAV-GFP rats (Fig. 7*C*, $p < 0.05$). However, both AAV-GFP and AAV-TrkB.T1 rats showed incubation (significantly greater seeking on WD90 compared with WD1 within each group) and

cocaine seeking on WD90 did not differ between the two groups (Fig. 7*C*), suggesting that knocking down BDNF-TrkB signaling in the NAc core did not affect this late phase of incubation of cocaine craving. The fact that TrKB.T1 rats exhibited incubation on WD90 relative to WD1 in this experiment, but not on WD30 relative to WD1 in the experiment shown in Figure 6, most likely reflects the longer duration of the former experiment, permitting a difference to emerge relative to WD1. After the WD90 test, we assessed expression of AAV-TrkB.T1 by FLAG immunohistochemistry. As shown in Figure 7*D*, strong FLAG signals were observed in the NAc core, indicating that the virus was still expressing on WD90. Together, our behavioral results suggest that basal levels of BDNF signaling in the NAc core (i.e., levels present before the withdrawal-dependent increase in BDNF protein) exert a suppressive effect on cue-induced cocaine seeking on WD1 because attenuating BDNF-TrkB signaling using either LV-TrkBsiRNA or AAV-TrkB.T1 enhanced cocaine seeking in WD1 tests.

### Attenuating BDNF-TrkB signaling in the NAc shell before cocaine self-administration training suppresses cocaine seeking on WD90

Next, we studied whether BDNF in the NAc shell also plays a role in the incubation of cocaine craving by using similar viral vectors to attenuate BDNF-TrkB signaling. First, rats received intracranial infusion of LV-GFP, LV-TrkBsiRNA, or LV-TrkB.T1 into the NAc shell and then were trained to self-administer cocaine for 10 d (Fig. 8*A*). Previous work showed similar behavioral effects regardless of whether BDNF-TrkB signaling was attenuated by injection of LV-TrkBsiRNA or LV-TrkB.T1 into the NAc (Bahi et al., 2008). Cue-induced cocaine seeking behavior was tested on WD1 and WD45. Similar to the lentivirus experiments in the core, we used different cohorts of rats for each WD (Cohort 4, WD1; Cohort 5, WD45). Viral injection into the NAc shell before cocaine self-administration training did not affect responding of the rats during training (Fig. 8*B*). Results of WD1 and WD45 tests indicated that all three groups exhibited incubation of cocaine craving (within group comparisons of WD1 and WD45), and no differences were observed between the three groups on either WD1 or WD45 (Fig. 8*C*, $p < 0.05$).

To determine whether BDNF in the NAc shell is involved in a late phase of incubation, we tested cocaine seeking on WD90 after knocking down BDNF-TrkB signaling in the NAc shell using AAV-TrkB.T1 (Fig. 9*A*). Consistent with previous results obtained using LV-TrkBsiRNA and LV-TrkB.T1 (Fig. 8*C*), no difference was found on WD1. However, a significant decrease (~30%) of cue-induced seeking was observed in AAV-TrkB.T1 rats compared with AAV-GFP rats on WD90 (Fig. 9*C*, $p < 0.5$), although both groups of rats showed incubation compared with their responding on WD1. Virus expression was verified after the WD90 test by FLAG or GFP immunohistochemistry (Fig. 9*D*). These results indicate that BDNF-TrkB signaling in the NAc shell is required to achieve maximal levels of incubation during prolonged withdrawal.

### Long-term elevation of BDNF in the NAc core or shell is not sufficient to increase CP-AMPAR expression

Our laboratory has previously shown that the expression of incubation of cocaine craving on WD45, after the same cocaine self-administration regimen used here, is mediated by GluA1-containing CP-AMPARs that accumulate in the NAc during withdrawal (Conrad et al., 2008). As noted in the Introduction, there is evidence that BDNF can promote synaptic delivery of CP-AMPARs in the NAc core of adult drug-naive rats (Li and Wolf, 2011a) and *in vitro* preparations from other brain regions (Caldeira et al., 2007; Li and Keifer, 2008; Li and Keifer, 2009; Keifer and Zheng, 2010; Fortin et al., 2012). These results led us to hypothesize that elevation of BDNF levels in the NAc might underlie the accumulation of CP-AMPARs during cocaine withdrawal. To test this hypothesis, we used LV-BDNF-GFP

to overexpress BDNF in the NAc core or shell in drug-naive rats and studied AMPAR expression and distribution using a $BS^3$ protein crosslinking assay. As the LV-BDNF-GFP virus has not been previously studied, we first verified viral expression *in vivo* using BDNF immunohistochemistry. As shown in Figure 10*A*, neurons expressing GFP immunoreactivity also showed intense staining for BDNF. Next, we studied the effect of long-term BDNF overexpression in the NAc core or shell on AM-PAR subunit surface expression. An increase in surface expression of GluA1 in the absence of a change in GluA2 would be suggestive of elevated CP-AMPAR levels (e.g., Conrad et al., 2008; Li and Wolf, 2011a). However, 47 d after infusion of LV-BDNF-GFP into NAc core or shell, both GluA1 and GluA2 surface expression were unchanged compared with LV-GFP rats (Fig. 10*B*, *C*, $p < 0.05$). The postinfusion time point (47 d) was chosen because it corresponds to a withdrawal time when both BDNF and CP-AMPAR levels are elevated in the NAc core during the incubation of cocaine craving (Grimm et al., 2003; Conrad et al., 2008; Wolf and Tseng, 2012).

Together, our results show that long-term overexpression of BDNF in the NAc is not sufficient to increase CP-AMPAR surface expression. This argues against our original hypothesis that cocaine-induced elevation of BDNF is responsible for the increase in CP-AMPARs. Also arguing against this hypothesis is our finding that BDNF levels in the shell subregion are not yet elevated on WD45 (Fig. 3), despite the fact that CP-AMPARs can be detected in the shell at similar withdrawal times (McCutcheon et al., 2011). However, it remains possible that viral mediated overexpression of BDNF does not reproduce the spatial and/or temporal pattern of the cocaine-induced elevation in endogenous BDNF during incubation. For example, anterograde transport of BDNF from particular brain regions may lead to a specific pattern of BDNF-TrkB signaling, which is required to influence GluA surface expression. It is also possible that elevation of BDNF is involved in CP-AMPAR expression but is not sufficient, i.e., other cocaine-induced adaptations are also necessary.

### Ampakine treatment increases BDNF protein expression in the NAc

Ampakines, positive modulators of AMPAR transmission that work by slowing deactivation and desensitization of AMPARs (Lynch and Gall, 2006), have been shown to increase BDNF protein levels in cultured hippocampus neurons as well in rat hippocampus *in vivo* (Lauterborn et al., 2000; 2003; Rex et al., 2006; Lauterborn et al., 2009). This led us to consider a novel hypothesis for the relationship between BDNF and CP-AMPARs during incubation. Rather than proposing that BDNF mediates the increase in CP-AMPARs observed during incubation, we wondered whether enhanced AMPAR transmission after prolonged withdrawal (due to high conductance CP-AMPAR accumulation) contributes to the time-dependent increase in BDNF levels in the NAc. As a first step toward exploring this hypothesis, we conducted a proof-of-principle study aimed at determining whether enhancing AMPAR transmission, using ampakines, increases BDNF expression in NAc of drug-naive rats.

Rats were treated with the ampakine CX929 (5 mg/kg, i.p.) using a repeated injection regimen previously shown to increase BDNF levels in the rat hippocampus (Rex et al., 2006). Consistent with their results, we observed a significant increase in BDNF protein levels in hippocampus after ampakine treatment (Fig. 11, $p < 0.05$). BDNF protein was also significantly elevated in the NAc (Fig. 11), supporting our hypothesis. Using tissue from the same animals, we failed to detect an increase in BDNF mRNA levels in either hippocampus (data not shown) or NAc (Fig. 11) after ampakine treatment. This was not surprising, as prior studies have shown that BDNF protein and mRNA levels in hippocampus do not always show parallel alterations after ampakine treatment (Lauterborn et al., 2000).

An alternative to the ampakine hypothesis is that BDNF protein levels increase in the NAc during incubation as a result of anterograde transport from another brain region. The mPFC is one candidate, based on a report that BDNF levels increase in the mPFC after limited access cocaine self-administration and 7 d of withdrawal (Sadri-Vakili et al., 2010). We therefore measured BDNF protein levels in mPFC on WD45, using tissue obtained previously after the same cocaine or saline self-administration regimen used herein. We also measured BDNF mRNA in mPFC tissue obtained from the same saline and cocaine rats used to measure BDNF mRNA levels in the NAc on WD45 (Fig. 5). No differences were found between groups for mPFC levels of BDNF protein [data normalized to saline controls; saline, 100 ± 9.1% ($n$ =9); cocaine, 99.4 ± 3.5% ($n$ =6); $p$ >0.05] or BDNF mRNA [saline, 100.0 ± 7.4% ($n$ = 9); cocaine, 99.7 ± 7.7% ($n$ = 8); $p$ > 0.05].

## Discussion

We investigated the role of BDNF-TrkB signaling in the incubation of cocaine craving, as well as BDNF's role in the CP-AMPAR accumulation that accompanies incubation. Levels of BDNF protein, but not mRNA, were increased in whole NAc on WD45. In studies distinguishing core from shell, we found that BDNF increased first in the core (WD45). However, BDNF was further elevated in the core and also elevated in the shell on WD90, a time point at which cell surface pTrkB was also significantly increased in whole NAc. Behavioral studies using viral vectors showed that attenuating BDNF-TrkB signaling in core before cocaine self-administration training enhanced cocaine seeking during early withdrawal (WD1), whereas similar attenuation in shell suppressed cocaine seeking after prolonged withdrawal (WD90). Finally, in studies designed to explore the relationship between BDNF and CP-AMPAR plasticity, we found no evidence of increased CP-AMPAR surface expression after chronic elevation of BDNF levels in core or shell. However, enhancing AMPAR transmission with an ampakine increased NAc BDNF levels.

### Possible mechanisms underlying increased BDNF protein levels during incubation

Although cocaine exposure can increase BDNF mRNA in the NAc (Le Foll et al., 2005; Filip et al., 2006; Fumagalli et al., 2007; Graham et al., 2007; Huang et al., 2011), our results show that BDNF protein levels in the NAc increase during incubation in the absence of changes in mRNA levels. Dissociation between cocaine's effects on BDNF mRNA and protein levels has been reported previously (Fumagalli et al., 2007). We also failed to detect changes in BDNF exon IV levels, which are altered after other cocaine regimens (Liu et al., 2006; Sadri-Vakili et al., 2010). These results suggest that mechanisms other than increased mRNA levels must explain increased BDNF protein during incubation. One possibility is anterograde transport from regions, such as the mPFC or VTA, which project to NAc and express high BDNF levels (Altar et al., 1997; Conner et al., 1997; Altar and DiStefano, 1998; Lessmann et al., 2003). To address this, we measured BDNF mRNA and protein in mPFC on WD45 after cocaine self-administration and failed to find any difference between saline and cocaine groups. This argues against anterograde transport from mPFC but does not rule out transport from other regions, most notably the VTA, in which BDNF has been implicated in incubation of cocaine craving (Grimm et al., 2003; Lu et al., 2004a). However, additional possibilities exist. Translation of existing BDNF mRNA in the NAc could be enhanced (e.g., resulting from changes in abundance of long 3′UTR BDNF mRNA) (Lau et al., 2010). In addition, BDNF mRNA can be stabilized by $Ca^{2+}$ influx (Fukuchi et al., 2005), and $Ca^{2+}$influx should be increased once CP-AMPARs are in the synapse. Finally, processing of pro-BDNF into mature BDNF can also be increased in a $Ca^{2+}$-dependent manner (Gualandris et al., 1996).

### Activation of TrkB receptors in the NAc after prolonged withdrawal

Prolonged exposure to BDNF leads to downregulation of TrkB receptors *in vitro* (Sommerfeld et al., 2000), making us wonder whether TrkB receptor expression in the NAc changes in response to BDNF elevation during incubation. By biotinylating NAc tissue at different withdrawal times, we found no difference between cocaine and saline groups in cell surface or total levels of TrkB or pTrkB (Thr 706/606; phosphorylation at this site reflects TrkB activation) in whole NAc on WD25 and WD48, even though BDNF levels are increased in the core by WD45. However, cell surface pTrkB was increased on WD90, the time when BDNF levels in core are further elevated and BDNF elevation in shell is also observed. The reason for the delay in increased surface expression of pTrkB is unclear. However, the rate of BDNF application affects subsequent signaling (Ji et al., 2010). A gradual increase in endogenous BDNF during incubation may elicit a slow activation of TrkB (contrasting with rapid activation after acute exogenous BDNF application; Li and Wolf, 2011a). It should be noted that we did not measure TrkB in core and shell separately, but other results indicate the possibility of different TrkB regulation in these subregions (Toda et al., 2002; Graham et al., 2007; Graham et al., 2009).

### Core-shell differences in the role of BDNF during incubation

As discussed above, BDNF levels increase first in the core and later in shell during incubation, adding to evidence that cocaine differently affects BDNF expression in core versus shell (Filip et al., 2006; Graham et al., 2007; Huang et al., 2011). Before our work, however, it was unclear whether BDNF in core and shell contribute differently to cocaine seeking. Graham et al. (2007) showed that daily delivery of BDNF into the shell facilitated cocaine reward after 11–15 d of withdrawal from cocaine self-administration, whereas delivery of BDNF blocking-antibody produced the opposite effect. Although analogous studies in the core were not performed, a series of studies provide indirect support for an opposite effect of BDNF in core (Berglind et al., 2007, 2009; Whitfield et al., 2011). They showed that intra-PFC infusion of BDNF, immediately after completion of cocaine self-administration training, suppressed cocaine seeking 22 h later. This depended on TrkB activation in the mPFC. However, BDNF levels in NAc were also elevated (presumably through anterograde transport) and cocaine-induced adaptations in the NAc were normalized. These studies did not distinguish core from shell. However, the dorsomedial region of PFC injected with BDNF projects primarily to the core (Pierce et al., 1998). Thus, the intra-PFC infusion of BDNF may have elevated BDNF predominantly in core, and this may have contributed to the ability of intra-PFC BDNF to suppress cocaine seeking.

To investigate the possibility that BDNF in core and shell play opposite roles in incubation of cocaine seeking, we used viral vectors to attenuate BDNF-TrkB signaling before cocaine self-administration. When BDNF-TrkB signaling was attenuated in the core, we observed enhanced seeking on WD1, but no difference from controls at later withdrawal times (WD30 or WD90). Because the cocaine-induced increase in endogenous BDNF does not occur until well after WD1 (Grimm et al., 2003), these results suggest that basal levels of BDNF transmission in the NAc core normally exert a suppressive effect on cue-induced cocaine seeking during early withdrawal. The direction of the effect is consistent with our interpretation of the results of intra-PFC BDNF infusion mentioned above (Berglind et al., 2007). On the other hand, after attenuation of BDNF-TrkB signaling in shell, cue-induced cocaine seeking was unaltered on WD1 or WD30 but significantly attenuated on WD90, the same withdrawal time at which we observed elevated BDNF levels in the shell. Indeed, in rats treated to knock down TrkB signaling in the shell, the level of responding on WD90 (Fig. 9*C*) was very similar to that observed on WD45 (Fig. 8*C*), supporting the idea that attenuation of BDNF-TrkB signaling in the NAc shell prevents the enhancement of responding that normally occurs between WD45 and WD90. Together, these results suggest

that withdrawal-dependent elevation of BDNF-TrkB signaling in the NAc shell contributes to incubation during late withdrawal. This is consistent with evidence that BDNF in the shell facilitates cocaine seeking (Graham et al., 2007), although different cocaine regimens preclude a direct comparison. To our knowledge, our study is the first to investigate the role of endogenous BDNF in core versus shell during incubation. Our results suggest that BDNF's role is not only distinct in these subregions, but also in different timeframes during withdrawal. Adding to the complexity, BDNF levels also increase in dorsal striatum during extended access cocaine self-administration, promoting cocaine seeking (Im et al., 2010).

### Possible relationship between BDNF and CP-AMPARs during incubation

Using a protein crosslinking assay, we found that chronic BDNF elevation using viral vectors failed to affect GluA1 surface expression in the NAc. Because CP-AMPARs accumulating during incubation are largely homomeric GluA1 (Conrad et al., 2008), this suggests that increased BDNF levels during incubation are not sufficient to account for CP-AMPAR accumulation. However, we showed that an AMPAR potentiator (ampakine CX929) increased BDNF protein in the NAc, supporting the alternative hypothesis that enhanced NAc AMPAR transmission during incubation led to the increase in BDNF. The time course of both effects supports this hypothesis (Grimm et al., 2003; Wolf and Tseng, 2012; present results). Therefore, future studies should explore the possibility that CP-AMPAR accumulation plays a causal role in elevation of BDNF levels in the NAc during incubation, although other possibilities mentioned above (e.g., anterograde transport of BDNF to the NAc) should also be investigated.

In conclusion, BDNF levels increase in both the NAc core and shell during withdrawal from extended access cocaine self-administration, but this occurs with different time courses and has different functional consequences in the two subregions. These findings help to reconcile seemingly contradictory reports about BDNF's role in cocaine seeking. In addition, our results support the novel hypothesis that the gradual increase in BDNF levels in the NAc during incubation occurs as a result of increased AMPAR transmission, providing a new direction for future studies. The clinical implication of our results is that reducing BDNF signaling might decrease cue-induced cocaine craving at long withdrawal times and thereby help to maintain abstinence.

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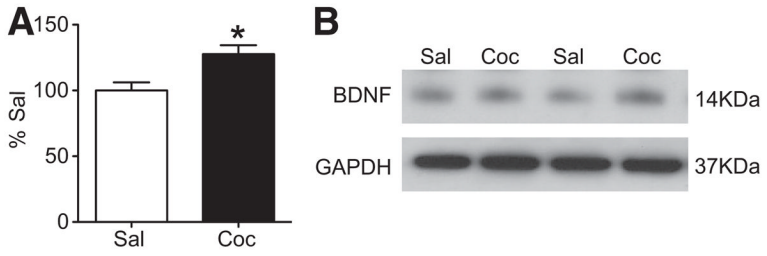


**Figure 1.**

BDNF protein levels are increased in the NAc 45 d after discontinuing cocaine self-administration training. ***A***, Western blot analysis indicated a significant increase in BDNF protein levels (*$p < 0.05$, $t_{(13)} = 3.015$, *t* test) in whole NAc of rats that self-administered cocaine (6 h/d for 10 d; $n = 8$) compared with rats that self-administered saline ($n = 7$). ***B***, Representative blots comparing cocaine and saline groups. Data (mean ± SEM) are expressed as percentage of mean values in the saline group.

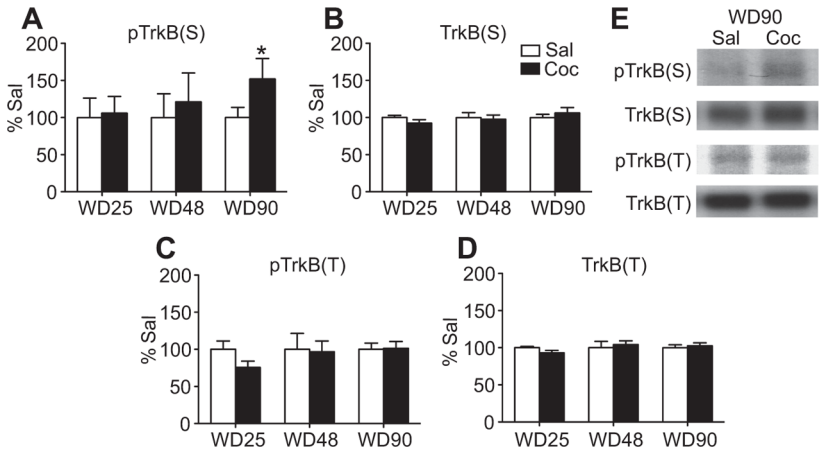


**Figure 2.**

Cocaine self-administration leads to a significant increase in surface expression of phosphorylated TrkB (pTrkB; Tyr706/707) in whole NAc on WD90. ***A***, Whole NAc was obtained on WD25, WD48, or WD90 after saline or cocaine self-administration and biotinylated. Surface-expressed pTrkB [pTrkB( *S*)] was significantly increased in the NAc of the cocaine group on WD90 (saline, $n = 13$; cocaine, $n = 8$; $^{*}p<0.05$, $t_{(19)} = 1.839$, *t* test, one tail), whereas there were no differences between cocaine and saline groups on either WD25 (saline, $n= 10$; cocaine, $n= 9$) or WD48 (saline, $n= 9$; cocaine, $n= 10$). ***B***–***D***, Surface TrkB [TrkB( *S*)], total pTrkB [pTrkB( *T*)], and total TrkB [TrkB( *T*)] in the cocaine group were similar to their respective saline controls at each withdrawal time. ***E***, Representative blots fromWD90groups are shown (TrkB: 140 kDa). Data (mean ± SEM) are expressed as percentage of mean values in the saline group from the same WD.

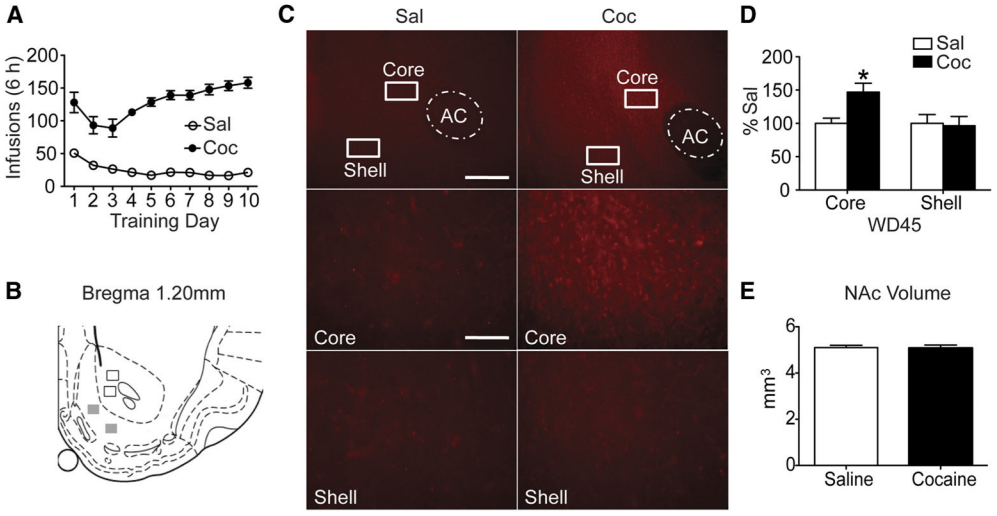


**Figure 3.**

BDNF immunoreactivity is selectively increased in the NAc core on WD45 after cocaine self-administration training. ***A***, Infusions during 10 d of saline or cocaine self-administration training (saline, $n = 13$; cocaine, $n = 11$). ***B***, Schematic diagram illustrating regions sampled to measure BDNF immunoreactivity in NAc core (open boxes) and shell (closed gray boxes). ***C***, Representative images of BDNF staining in the NAc on WD45 after saline (left panels) or cocaine self-administration (right panels). Top, Low-magnification images. Scale bar, 1000 $\mu$m. Middle and bottom, Higher-magnification images in core and shell, respectively. Scale bar, 100 $\mu$m. ***D***, A significant increase in BDNF staining was observed in the NAc core (*$p < 0.05$, $t_{(22)} = 3.117$, *t* test), but not shell, of the cocaine group on WD45 compared with the saline group. Data (mean ± SEM) are expressed as percentage of mean values in the saline group. ***E***, The volume of the NAc ($mm^3$) did not differ between saline and cocaine groups on WD45.

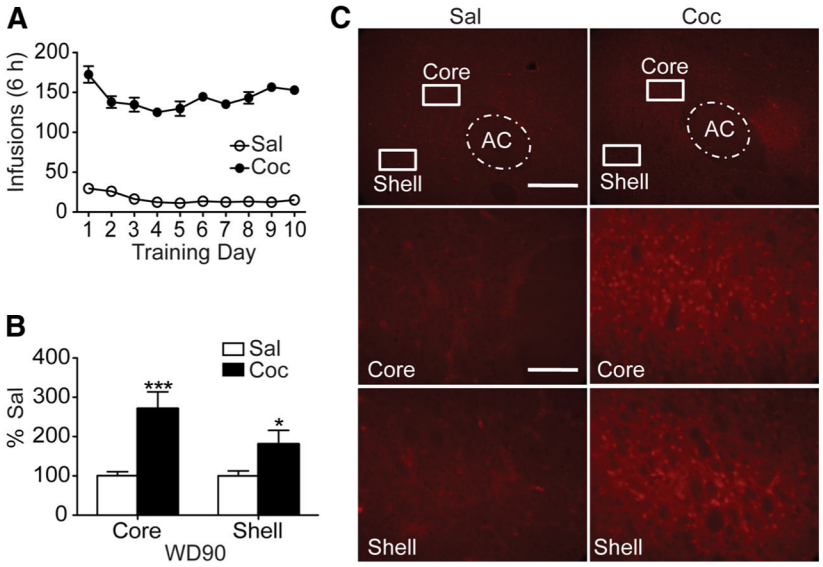


**Figure 4.**

BDNF immunoreactivity is significantly elevated in both the NAc core and shell on WD90 after cocaine self-administration training. ***A***, Infusions during 10 d of saline or cocaine self-administration training (saline, $n$ =12; cocaine, $n$ =8). ***B***, BDNF staining was significantly increased in the NAc core (***$p$ <0.0005, $t_{(15)}$ = 5.070, $t$ test; saline, $n$ = 11; cocaine, $n$ = 6) and shell (*$p$ < 0.05, $t_{(18)}$ = 2.537, $t$ test; saline, $n$ = 12; cocaine, $n$ = 8) in the cocaine group on WD90 compared with saline controls. Data (mean ± SEM) are expressed as percentage of mean values in the saline group. ***C***, Representative images of BDNF staining in the NAc on WD90 after saline (left) or cocaine self-administration (right). Top, Low-magnification images. Scale bar, 1000 $\mu$m. Middle and bottom, Higher-magnification images in core and shell, respectively. Scale bar, 100 $\mu$m.

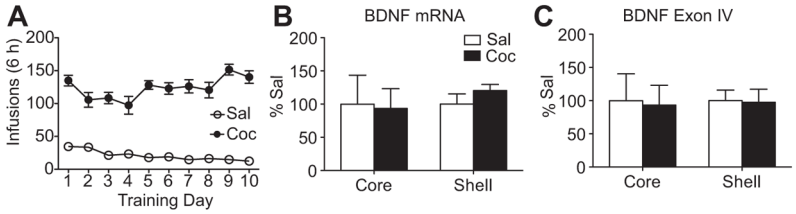


**Figure 5.**

Neither total BDNF mRNA nor BDNF exon IV levels are increased in the NAc 45 d after discontinuing cocaine self-administration training. ***A***, Training data are expressed as infusions (mean ± SEM) on each 6 h training day for rats that self-administered saline ($n$ =9) or cocaine ($n$ =8). ***B***, ***C***, Real-time PCR analysis revealed no differences in total BDNF mRNA expression or BDNF exon IV levels in either NAc core or shell of the cocaine group compared with the saline group on WD45. Data (mean ± SEM) are expressed as percentage of mean values in the saline group.

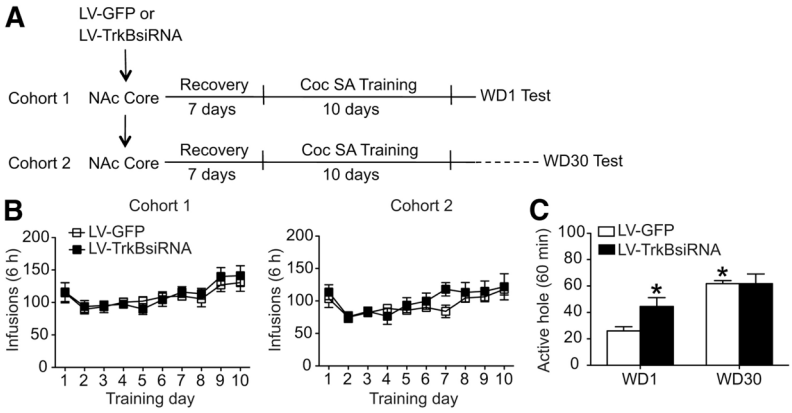


**Figure 6.**

Attenuating BDNF-TrkB signaling with LV-TrkBsiRNA in the NAc core led to enhanced cue-induced cocaine seeking on WD1. ***A***, Experimental timeline. ***B***, Cocaine self-administration training was similar in both cohorts, and no differences were found between the LV-GFP and LV-TrkBsiRNA groups. ***C***, Cue-induced cocaine seeking was significantly enhanced on WD1 in rats injected with LV-TrkBsiRNA into the NAc core before cocaine self-administration training compared with rats injected with LV-GFP (*$p < 0.05$, $t_{(11)} = 2.576$, *t* test; LV-GFP, $n = 7$; LV-TrkBsiRNA, $n = 6$). By WD30, no difference was apparent between the LV-GFP and LV-TrkBsiRNA groups (LV-GFP, $n = 6$; LV-TrkBsiRNA, $n = 8$). Whereas the LV-GFP groups exhibited incubation (significantly increased active hole nose-pokes on WD30 compared with WD1) (*$p < 0.05$, $t_{(11)} = 6.207$, *t* test), no significant difference was found between LV-TrkBsiRNA groups tested on WD1 versus WD30. Results are expressed as nose-pokes (mean ± SEM) in the previously active hole during a 60 min cocaine-seeking test in which cocaine was not available and each nose-poke into the active hole resulted in the delivery of the light cue previously paired with cocaine infusions.

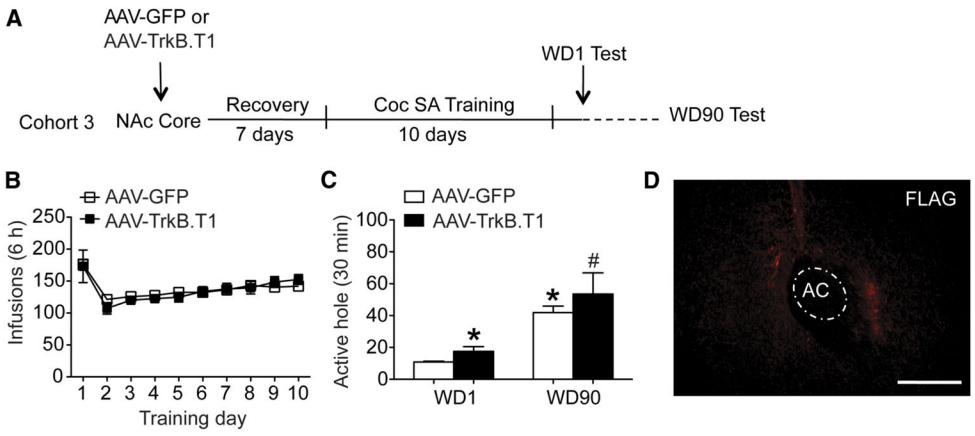


**Figure 7.**

Attenuating BDNF-TrkB signaling with AAV-TrkB.T1 in the NAc core led to enhanced cue-induced cocaine seeking on WD1. ***A***, Experimental timeline. ***B***, Cocaine self-administration training was similar between AAV-GFP ($n$ =7) and AAV-TrkB.T1 ($n$ =6) groups. ***C***, Similarly to results obtained with LV-TrkBsiRNA (Fig. 6), we observed significantly enhanced cue-induced cocaine seeking on WD1 in the AAV-TrkB.T1 group compared with the AAV-GFP group (*$p$ <0.05, $t_{(11)}$ = 2.357, $t$ test). No difference was found between the groups on WD90, but both groups showed incubation compared with their respective WD1 (AAV-GFP: *$p$ <0.05, $t_{(6)}$ = 7.704; AAV-TrkB.T1: $^{\#}p$ <0.05, $t_{(5)}$ = 2.326; one tailed, paired $t$ tests). Results are expressed as nose-pokes (mean ± SEM) in the previously active hole during a 30 min cocaine-seeking test conducted as described in the legend to Figure 6. ***D***, Representative image showing FLAG immunostaining in the NAc core after the final seeking test on WD90. Scale bar, 1000 $\mu$m.

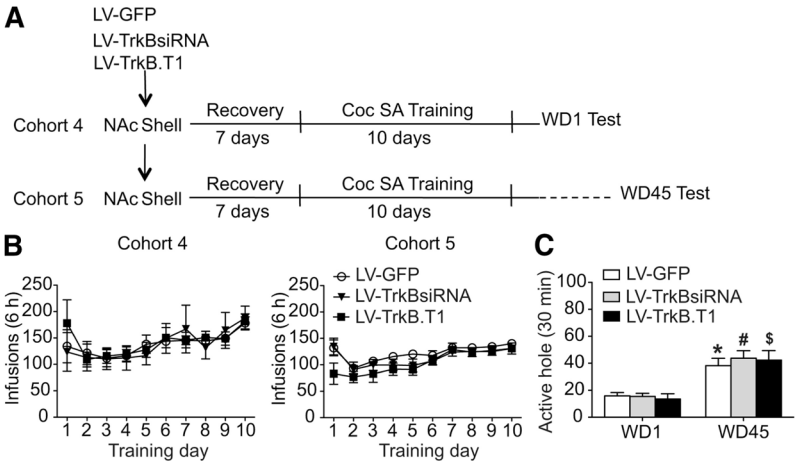


**Figure 8.**

Attenuating BDNF-TrkB signaling with LV-TrkBsiRNA or LV-TrkB.T1 in the NAc shell had no effect on cue-induced cocaine seeking on either WD1 or WD45. ***A***, Experimental timeline. ***B***, Cocaine self-administration training was similar in both cohorts, and no differences were found between LV-GFP, LV-TrkBsiRNA, and LV-TrkB.T1 groups. ***C***, No effects on cue-induced cocaine seeking were observed on either WD1 or WD45 in rats injected with LV-GFP, LV-TrkBsiRNA, or LV-TrkB.T1 in the NAc shell before cocaine self-administration training, although all three WD45 groups (LV-GFP: $n$ =9; LV-TrkBsiRNA: $n$ =9; LV-TrkB.T1: $n$ = 6) showed incubation compared with respective WD1 groups (LV-GFP: $n$ = 9; LV-TrkBsiRNA: $n$ = 9; LV-TrkB.T1: $n$ = 7). Comparison of WD1 and WD45 groups: LV-GFP, $^{*}p < 0.05$, $t_{(16)} = 3.758$; LV-TrkBsiRNA, $^{\#}p < 0.05$, $t_{(16)} = 4.617$; LV-TrkB.T1, $^{\$}p < 0.05$, $t_{(11)} = 3.716$; $t$ tests. Results are expressed as nose-pokes (mean ± SEM) in the previously active hole during a 30 min cocaine-seeking test conducted as described in the legend to Figure 6.

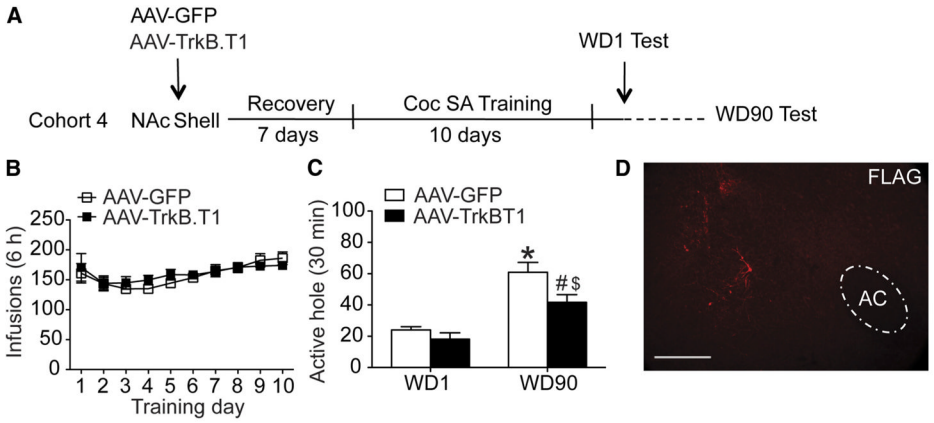


**Figure 9.**

Attenuating BDNF-TrkB signaling with AAV-TrkB.T1 in the NAc shell led to significantly decreased cue-induced cocaine seeking on WD90. ***A***, Experimental timeline. ***B***, Cocaine self-administration training was similar for AAV-GFP ($n = 7$) and AAV-TrkB.T1 ($n = 6$) groups. ***C***, On WD1, there was no difference in cue-induced cocaine seeking between AAV-GFP and AAV-TrkB.T1 groups. On WD90, although both groups showed incubation compared with their respective WD1 (AAV-GFP: $^{*}p < 0.05$, $t_{(6)} = 7.017$; AAV-TrkB.T1: $^{\#}p < 0.05$, $t_{(5)} = 4.437$; paired *t* tests), the response of the AAV-TrkB.T1 group was significantly decreased compared with the AAV-GFP group ($^{\$}p < 0.05$, $t_{(11)} = 2.333$, *t* test). ***D***, Representative image showing FLAG immunostaining in NAc shell after the behavioral test on WD90. Scale bar, 1000 $\mu$m.

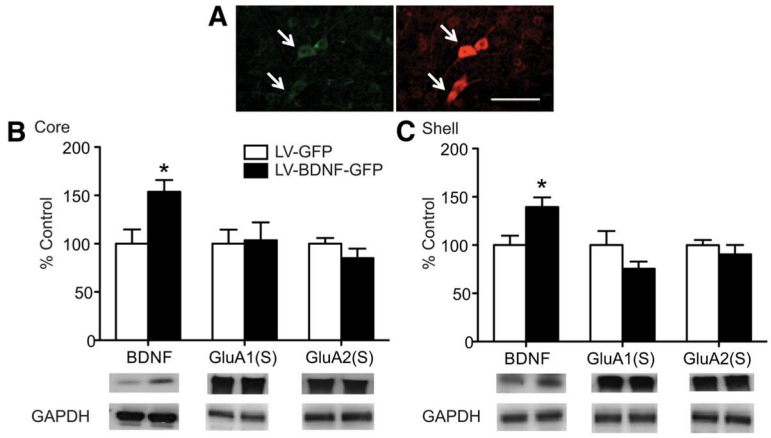


**Figure 10.**

Long-term elevation of BDNF levels with LV-BDNF-GFP had no effect on GluA1 or GluA2 surface expression in either the NAc core or shell. ***A***, Representative image of BDNF staining 3 weeks after injection of LV-BDNF-GFP into the NAc core. Intense staining for BDNF (red) was observed in infected neurons, identified based on GFP expression (green). Note that the GFP signal is weak because it was not amplified by immunostaining. These results demonstrate that LV-BDNF-GFP was effective *in vivo*. Scale bar, 100 $\mu$m. ***B***, Forty-seven days after injection of LV-GFP ($n$ =9) or LV-BDNF-GFP ($n$ =4) into the NAc core, BDNF levels in the core of the LV-BDNF-GFP group were significantly elevated compared with LV-GFP controls (*$p$ <0.05, $t_{(11)}$ = 2.242, $t$ test), but this chronic elevation of BDNF levels failed to affect GluA1 and GluA2 surface expression. ***C***, Similarly, whereas BDNF levels in the shell of the LV-BDNF-GFP group ($n$ = 8) showed a significant increase compared with the LV-GFP group ($n$ = 11; *$p$ < 0.05, $t_{(17)}$ = 2.750, $t$ test), no changes on GluA1 and GluA2 surface expression were observed. Data (mean ± SEM) are expressed as percentage of mean values in the LV-GFP group.

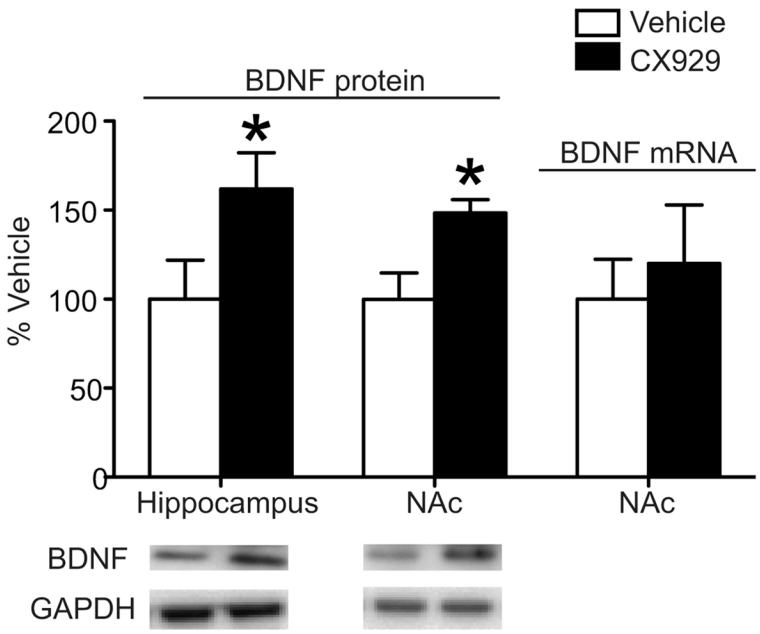


**Figure 11.**

Treatment with the ampakine CX929 in drug naive rats significantly increased BDNF protein levels in whole NAc. As a positive control, we first confirmed that BDNF protein levels in rat hippocampus were significantly elevated in the ampakine treatment group ($n$ =8) compared with the vehicle group ($n$ =8) (*$p$ =0.05, $t_{(7)}$ = 2.506, paired $t$ test), consistent with previous studies (Rex et al., 2006). Analysis of whole NAc from the same animals revealed elevated BDNF protein levels (*$p$ <0.05, $t_{(7)}$ =2.328, paired $t$ test) but no change in BDNF total mRNA levels. Data (mean ± SEM) are expressed as percentage of mean values in the vehicle group.