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ORIGINAL INVESTIGATION

Role of accumbens BDNF and TrkB in cocaine-induced psychomotor sensitization, conditioned-place preference, and reinstatement in rats

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Abstract
Background Brain-derived neurotrophic factor (BDNF) is involved in the survival and function of midbrain DA neurons. BDNF action is mediated by the TrkB receptor–tyrosine kinase, and both BDNF and TrkB transcripts are widely expressed in the rat mesolimbic pathway, including the nucleus accumbens (NAc) and the ventral tegmentum area (VTA).
Objective BDNF was previously shown to be involved in cocaine reward and relapse, as assessed in rat models. The goal of this study is to explore the role of BDNF and TrkB in the rat nucleus accumbens (NAc) in cocaine-induced psychomotor sensitization and in conditioned-place preference acquisition, expression, and reinstatement.
Materials and methods In vivo genetic manipulations of BDNF and TrkB were performed using a lentiviral gene delivery approach to over-express these genes in the NAc and siRNA-based technology to locally knockdown gene expression. Behavioral experiments consisted of locomotor activity monitoring or cocaine-induced conditioned-place preference (CPP).
Results BDNF and/or its receptor TrkB in the NAc enhance drug-induced locomotor activity and induce sensitization in rats. Furthermore, LV-BDNF- and LV-TrkB-treated rats display enhanced cocaine-induced CPP, delayed CPP-extinction upon repeated measurements, and increased CPP reinstatement. In contrast, expression of TrkT1 (truncated form of TrkB, acting as a dominant negative) inhibits these behavioral changes. This inhibition is also observed when rats are fed doxycycline (to block lentivirus-mediated gene expression) or when injected with siRNAs-expressing lentiviruses against TrkB. In addition, we investigate the establishment, maintenance, extinction, and reinstatement of cocaine-induced CPP. We show that BDNF and TrkB-induced CPP takes place during the learning period (conditioning), whereas extinction leads to the loss of CPP. Extinction is delayed when rats are injected LV-BDNF or LV-TrkB, and in turn, priming injections of 2 mg/kg of cocaine reinstates it.
Conclusions These results demonstrate the crucial function of BDNF—through its receptor TrkB—in the enhancement of locomotor activity, sensitization, conditioned-place preference, CPP-reinstatement, and rewarding effects of cocaine in the mesolimbic dopaminergic pathway.

Keywords siRNA · RNA interference · BDNF · TrkB · Cocaine · Addiction · Lentivirus · CPP

Abbreviations

BDNF	brain-derived neurotrophic factor
DA	dopamine

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GFP	green fluorescent protein
HEK293T	human embryonic kidney 293T cells
NAc	nucleus accumbens
NGF	nerve growth factor
qRT-PCR	quantitative real-time polymerase chain reaction
shRNA	short hairpin RNA
siRNA	small interference RNA
TH	tyrosine hydroxylase
Trk	receptor–tyrosine kinase
uPA	urokinase-type plasminogen activator
VTA	ventral tegmental area

Introduction

Psychostimulant exposure induces transient increases in brain-derived neurotrophic factor (BDNF) signaling and increases expression of its receptor TrkB in the dorsal striatum (Meredith et al. 2002; Meredith and Steiner 2006). Cocaine induces TrkB-mediated signaling in the nucleus accumbens (NAc; Berhow et al. 1995). Furthermore, acute cocaine administration increases striatal expression of a specific BDNF4 splice variant up to fivefold, not observed upon chronic administration, suggesting a role of specific BDNF promoter regions and regulatory sequences in stimulatory-induced alterations in BDNF expression (Liu et al. 2006; Pu et al. 2006). Administration of BDNF prevents biochemical and morphological changes associated with chronically administered cocaine and morphine, whose rewarding effects are mediated by the activation of dopamine neurons (DA) in the nucleus accumbens (NAc). BDNF has been implicated in mediating cue-induced cocaine craving and was found to enhance responding for cues associated with natural rewards (Bolaños and Nestler 2004), possibly through sustained increases in BDNF levels within the VTA, NAc, and amygdala (Grimm et al. 2003). Furthermore, BDNF injections into the VTA or NAc enhances cocaine sensitization and conditioned reinforcement (Horger et al. 1999). In addition, BDNF is responsible for inducing normal expression of D3 dopamine receptors in the NAc shell (Guillin et al. 2001), regulating cellular signaling pathways activated by cocaine, and preventing drug-induced morphological changes in the DA system. Recent studies, using localized inducible BDNF knockout in mice, showed 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 (Graham et al. 2007). In addition, BDNF and NT3 potently influence behavioral sensitization to cocaine and the regulation of dopaminergic transmission to the NAc (Martin-Iverson and Altar 1996; Pierce et al. 1999). Behavioral studies have shown that heterozygous BDNF knockout throughout the brain delays the development of cocaine sensitization (Horger et al. 1999; Messer et al. 2000) and attenuates the rewarding effects of cocaine in conditioned-place preference (Hall et al. 2003). As BDNF is implicated in synaptic plasticity, primarily through its receptor TrkB (Bibel and Barde 2000; Bonhoeffer 1996; Kryl et al. 1999; Patapoutian and Reichardt 2001; Thoenen 2000), BDNF-mediated neuroadaptations in mesolimbic areas are therefore probably involved in the persistent cocaine seeking induced by exposure to drug cues after withdrawal (Lu et al. 2004).

In this report, we evaluate the role of BDNF and its receptors TrkB or TrkT1 in behavioral adaptations to cocaine by means of lentivirus-mediated gene transfer. Lentiviral vectors are known to maintain ectopic gene expression for a long period of time and to infect neurons efficiently. Therefore, our approach aims at locally modifying gene expression levels, overexpressing or silencing BDNF or TrkB in the NAc. Lentiviruses expressing BDNF, TrkB, or TrkT1 or lentiviruses expressing TrkB-specific siRNAs were stereotaxically injected into the NAc, and behavioral changes in response to cocaine were monitored. We show that BDNF and TrkB activation significantly contributes to cocaine-induced locomotor activity and conditioned-place preference (CPP), delayed extinction, and increased CPP reinstatement, and we discuss the mechanisms implicated in this adaptation.

Materials and methods

Lentiviral construction

LV-BDNF

The rat BDNF cDNA was amplified by reverse transcription using specific primers and Superscript II RNaseH Reverse Transcriptase according to the manufacture's instructions (Invitrogen, Switzerland). The total RNA was prepared from rat NAc of cocaine-treated animals. The cDNA was then polymerase chain reaction (PCR) amplified and tagged with the two following primers: 5′-CGC GGG ATC CAT GAC CAT CCT TTT CCT T-3′ and 5′-GCC GCT CGA G**CT ACA GGT CCT CCT CTG AGA TCA GCT TCT G**TC TTC CCC TTT TAA T-3′ (restriction sites are underlined). The forward primer contains a *BamHI* restriction site followed by the 5′ rat BDNF cDNA-specific sequence; the reverse primer contains the 3′ rat BDNF cDNA-specific sequence, a Myc tag (sequence in bold), a stop codon, and an *XhoI* restriction site.

LV-TrkB and LV-TrkT1

The rat TrkB and its truncated form TrkT1 were amplified by PCR using specific primers and plasmids holding these cDNAs (pEF/BOS-TrkB-Flag and pEF/BOS-TrkT1-Flag, respectively, kindly provided by Prof. E. Castren, University of Helsinki, Finland). The forward primer used for both genes was 5′-CGC GGG ATC CA TGT CGC CCT GGC CGA GG-3′. The TrkB-Flag reverse primer: 5′-GCC GCT CGA GCT AGC CTA GGA TGT CCA G-3′. The TrkT1 reverse primer: 5′-GCC GCT CGA GCT ACC CAT CCA GGG GGA T-3′. The forward primer contains a *BamHI* restriction site followed by the 5′ rat TrkB/T1 cDNA-specific sequence, the reverse primers an *XhoI* restriction site (restriction sites are underlined). The PCR products (BDNF, TrkB and TrkT1) were digested with *BamHI*/*XhoI* and cloned into pTK431 previously digested with the same enzymes. The resulting vectors (pTK431-BDNF, pTK431-TrkB and pTK431-TrkT1) were then sequenced to check for mutation absence, amplified, and used for lentiviral production as described below. The green fluorescent protein (GFP) expression vector pTK433 (Bahi et al. 2004a, b, 2005a, b, 2006) was used as a control vector.

LV-siRNAs

To knockdown TrkB and TrkT1 expression in vitro and in vivo, three targets were designed according to the rat TrkB mRNA sequence. The following targets within the TrkB sequence were selected, based on Hannon's design criterion (http://katahdin.cshl.org:9331/RNAi/html/rnai.html): first target, bp 5–27; second target, bp 2,438–2,460; third target, bp 1,500–1,522. To each oligo, an *XhoI* restriction site was added at 3′ and a U6-3′-specific 15-mer at 5′. Using the pSilencer 1.0-U6 (Ambion, UK) as a template and a U6 promoter-specific forward primer containing a *BamHI* restriction site (GCG GAT CCC GCT CTA GAA CTA GTG C), each siRNA target was added to the mouse U6 promoter by PCR. The PCR conditions were highly stringent to avoid mutations within the targets. The following PCR program was performed: 120 s at 94°C (initial denaturation) followed by 94°C for 45 s, 64°C for 45 s, and 72°C for 45 s, repeated for 35 cycles. The PCR reaction contained 4% dimethyl sulfoxide (Sigma, Switzerland). The PCR product was digested with *BamHI* and *XhoI*, cloned into similar sites into pTK431 and sequenced to verify the integrity of each construct.

The vector plasmids (either pTK433-GFP, pTK431-BDNF, pTK431-TrkB, pTK431-TrkT1, or pTK431-U6-Trk-siRNAs), together with the packaging construct plasmid pΔNRF and the envelope plasmid pMDG-VSV-G, were co-transfected into HEK293T cells to produce the viral particles (Bahi et al. 2004a, b, 2005a, b, 2006). The viral titers were determined by p24 antigen measurements (KPL, USA) after concentration by mean of ultracentrifugation. For the in vivo experiments, the different viral stocks were matched for viral particle content and used at 0.2 mg/mL of p24.

In vitro BDNF, TrkB, and TrkT1 transcript uantification assays

The efficiency of the LV-BDNF, LV-TrkB, and LV-TrkT1 was tested in vitro by infection of HEK293T cells. HEK293T cells were plated at 1×10^5 per well in six-well plates. The next day, lentivirus stocks were mixed with 10 μg/mL Polybrene (Sigma, Switzerland), incubated for 30 min at room temperature, added to the cells, and incubated at 37°C with or without 30 ng/mL doxycycline (Sigma, Switzerland). After 48 h, the medium was replaced with normal growth medium supplemented with 10% fetal calf serum and 1× penicillin/streptomycin (with or without 30 ng/mL doxycycline), and cells were left for a further 48 h. Cells were then collected and used for total RNA isolation (for real time PCR). Infections with the different lentiviruses were carried out as follows:

(1) *In vitro doxycycline regulation*: cells were infected using 0, 2, 4, or 8 μL of the LV-BDNF, LV-TrkB, or LV-TrkT1 stock. In addition, each well was co-infected with 4 μL of LV-CD81 and 4 μL of LV-uPA (Bahi et al. 2004a, b, 2005a, b, 2006), doxycycline regulatable lentiviruses used as controls under each condition. One part of the cells was incubated in the presence of 30 ng/mL doxycycline and the other part without doxycycline.

(2) *In vitro silencing of TrkB/TrkT1*: cells were infected with 4 μL each of LV-TrkB, LV-TrkT1, LV-CD81, and LV-uPA stocks, either alone or together with 2 μL of either, respectively, LV-Trk-siRNA1, LV-Trk-siRNA2, or LV-Trk-siRNA3, or with 2 μL of all three targets together (mix of LV-Trk-siRNA1, LV-Trk-siRNA2, and LV-Trk-siRNA3). After 96 h, cells were collected for RNA extraction, mRNA was quantified by quantitative real-time (qRT)-PCR and normalized against GAPDH (Bahi et al. 2005a, b).

RT-PCR and quantification of BDNF, TrkB, and TrkT1 transcripts expressed in the NAc

For qRT-PCR, primer sets for rat, BDNF, TrkB, TrkT1, GAPDH, beta-actin, uPA, and CD81 were designed to amplify 100- to 200-bp products, using PRIMER3 software: (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

The following specific primer pairs were used: BDNF: 5′-GGT TCG AGA GGT CTG ACG AC-3′ and 5′-CAA AGG CAC TTG ACT GCT GA-3′; TrkB: 5′-CCT CGT CGG AGA AGA TCA AG-3′ and 5′-CGT GGT ACT CCG TGT GAT GT-3′; TrkT1: 5′-AGC AGC CCT GGT ATC AGC TA-3′; GAPDH: 5′-ATG ACT CTA CCC ACG GCA AG-3′ and 5′-CAT ACT CAG CAC CAG CAT CAC-3′; beta-actin: 5′-AGC CAT GTA CGT AGC CAT CC-3′ and 5′-CTC TCA GCT GTG GTG GTG AA-3′; uPA: 5′-CAG ATC CGA TGC TCT TAG CC-3′ and 5′-TAG AGC CTT CTG GCC ACA CT-3′; CD81: 5′-TGA TCC TGT TTG CCT GTG AG-3′ and 5′-CAG TTG AGC GTC TCA TGG AA-3′. GAPDH was used as endogenous control for normalization. Total RNA was extracted from the HEK293T cells (for in vitro quantification) and from the NAc of brains of cocaine-treated rats (for in vivo quantification) using TRIzol Reagent (Invitrogen, Switzerland) including an RNase-free DNase step according to the manufacturer's instructions. RNA was quantified by spectrophotometry and its integrity verified by agarose gel electrophoresis as visualized with ethidium bromide staining. First-strand cDNA was generated from 2 μg of total RNA and Oligo($dT_{12–18}$) primer with the Superscript II RNaseH Reverse Transcriptase kit according to the manufacturer's instructions (Invitrogen, Switzerland) in a total volume of 20 μL. The reaction product was used for quantitative RT-PCR using the RT-PCR iCycler according to the manufacturer's instructions (Bio-Rad, Switzerland). Briefly, cDNA preparation (5 μL), 0.5 μM of forward and reverse primers, and 10 μL of IQ SYBR Green Supermix (Bio-Rad) in a total volume of 20 μL was applied, and PCR was performed as follows: 3 min at 95°C (initial denaturation), 20°C/s temperature transition rate up to 95°C for 45 s then 45 s at 64°C, repeated 40 times (amplification). The PCR reaction was evaluated by melting curve analysis and by checking the PCR products on 2% agarose gel.

The PCR cycle number at which each assay target reached the threshold detection line was determined ("threshold cycles", Ct value). The Ct of each gene was normalized against that of GAPDH. To determine the linearity and detection limit of the assay, cDNA samples were amplified for successive tenfold dilutions in a series of RT-PCRs, using duplicate assays on each dilution, so that the correlation coefficient could be calculated from the standard curve of Ct values. Comparisons were made between cocaine and saline groups, and significance was calculated using two-tailed Student's *t* test, and the level of statistical significance was set at $p<0.05$. Data were expressed as mean±SEM. The ΔCt for each candidate was calculated as ΔCt=[Ct(candidate)−Ct(GAPDH)]. The relative abundance of each target in each protocol can be calculated as the ratio between treated and untreated samples (Bahi et al. 2004a, b, 2005a, b, 2006).

Animal handling and drug delivery protocols

Animals used in these experiments were male Wistar rats weighing 225–250 g. All animal experiments were carried out in accordance with the guidelines and regulations for Animal Experimentation, BAG, Bern, Switzerland. The animals were housed three per cage in clear plastic cages with wire grid lids. Access to food and water was unrestricted. The rats were kept in the animal facility maintained on a 12-h light/12-h dark cycle (lights off at 7 A.M.).

Stereotaxic surgery Stereotaxic surgery and injection of the lentiviral vectors were performed according to previous publications (Bahi et al. 2004a, b, 2005a, b). Briefly, rats ($n=9$ per group) were bilaterally injected into the NAc with a total of 4 μL of concentrated lentiviral stock (0.2 mg/mL of p24, corresponding to 8×10^9 IU/mL). Seven groups ($n=9$ per group) were prepared as follows: LV-GFP; LV-BDNF; LV-TrkB; LV-BDNF+LV-TrkB; LV-TrkT1; LV-TrkB+LV-siRNAs; LV-BDNF+LV-TrkB+LV-Trk-siRNAs. All injections were performed bilaterally at the following co-ordinates, as calculated from the bregma and the dura mera: anterior +1.4, lateral ±1.6, ventral −6.8 (Paxinos and Watson 1998). After surgery, animals were left for recovery for 7 days.

Experiment 1: LV–BDNF- and LV-TrkB-induced locomotor activity upon cocaine treatment

One week after surgery, locomotor activity was monitored in MED-OFA-RS cages (MED Associates, USA) during the dark cycle in daily sessions over 15 days, according to previously published procedures (Bahi et al. 2004a, b, 2005a, b, 2006). The rat was injected with 0.9% saline solution and placed into the activity-monitoring cage for a 30-min baseline. After a 30-min period, the session automatically paused, and during this interval, each subject received cocaine–HCl (15 mg/kg, i.p.) and was then placed back into the locomotor activity-monitoring cage for a further 60 min. Animals were fed 5% sucrose in drinking water for 5 days (*Session A, days 1–5*), then 0.02% doxycycline and 5% sucrose in water for a further 7 days (*Session B, days 6–12*), then again 5% sucrose for seven more days (*Session C, days 13–19*).

At the end of each behavioral session, three animals were killed by decapitation to assess the efficiency of the BDNF, TrkB, and TrkT1 mRNAs expression in vivo. Brains ($n=3$) were dissected out, the NAc region was isolated, and total RNA was extracted using TRIzol Reagent according to the manufacturer's guidance (Invitrogen, Switzerland). The isolated total RNA was used for transcript quantification by means of qRT-PCR.

Experiment 2: Cocaine-induced conditioned-place preference

Conditioned-place preference was performed as previously described (Mueller and Stewart 2000). Briefly, in the preconditioning period, the rat was allowed to move freely between two boxes (consisting of either wire grid or mesh floor) daily for 20 min for 3 days (days 1–3). On day 3, the time spent in each chamber was monitored and used to assess unconditioned preferences. Before conditioning, the rats were divided into two groups (n=18 per group). One group of rats received water supplemented with 5% sucrose and 0.02% doxycycline in drinking water during conditioning, the other group was fed only 5% sucrose. During the conditioning phase, the rat was injected with cocaine (5 or 20 mg/kg, i.p.; n=9 per group) on days 4, 6, and 8 and immediately confined into the mesh floor box for 20 min. On days 5, 7, and 9, the rat was injected with saline (1 ml/kg) and placed into the wire grid floor chamber for 20 min. During the conditioning phase, chambers were blocked by a guillotine door and were never communicating. On day 10, the post-conditioning "CPP" test was performed without drug treatment. Rats were placed between the two chambers with the guillotine door removed and allowed free access to the entire set-up. The time spent in each chamber was measured for 20 min. Drug-induced-place preference was expressed by post versus pre, which was calculated as: [(post-value)−(pre-value)], where post- and pre-values were the difference in time spent at the drug-conditioning site during post-conditioning and preconditioning periods, respectively.

Experiment 3: extinction by repeated testing

After conditioning and following the initial CPP test, rats underwent 20 min tests daily for 20 days. No injections were performed during this prolonged period of withdrawal from cocaine.

Experiment 4: reinstatement by priming using low dose of cocaine

The day after the last extinction trial, rats received a priming injection of saline (1 ml/kg), followed 24 h later, by a priming injection of cocaine (2 mg/kg, i.p.) and were placed between the two boxes with access to the entire set up for 20 min.

Experiment 5: maintenance of the CPP

After CPP, test rats (n=9 per group) underwent a 5-week period of withdrawal from cocaine. During this time, animals were not injected and not tested but were kept in their home cages. After this period, rats were placed in a set up between the two boxes with free access to both compartments and measured for 20 min.

Immunohistochemistry

Brain sections were prepared for immunohistochemistry according to previously published procedures (Bahi et al. 2004a, b, 2005b, 2006). Briefly, rat brains were rapidly removed and frozen in isopentane at −30°C for 3 min and kept at –25°C. Coronal sections were cut at 14 μm in a cryostat (Leitz), placed on gelatinized glass slides, and air-dried at room temperature for 20 min. Antigens were localized using the avidin–biotin–peroxidase technique. Slices were fixed in 4% p-formaldehyde for 15 min and washed three times with 1× phosphate-buffered saline (PBS). Endogenous peroxidase activity was quenched with 2% hydrogen peroxide in water (H_2O_2) for 40 min at room temperature. Nonspecific binding was blocked for 30 min at RT in 1× PBS containing 1% bovine serum albumin, 1% Triton X-100, and 3% normal goat serum. Sections were then rinsed and incubated overnight either with mouse monoclonal anti-Myc tag antibody (Abcam, UK; 1:500) to check for BDNF expression or with rabbit polyclonal anti-Flag tag antibody (Abcam, UK; 1:500) to check for TrkB expression. Antibodies were diluted in 1× PBS containing 1% Triton X-100 and 1% normal goat serum. Sections were then washed three times with 1× PBS and incubated for 45 min at room temperature with the biotinylated secondary antibodies, either goat anti-mouse immunoglobulin G for LV-BDNF slices (Vector Laboratories, USA; 1:500) or goat anti-rabbit immunoglobulin G for LV-TrkB slices (Sigma, Switzerland; 1:1,000). Sections were rinsed three times for 5 min in 1× PBS at room temperature, followed by incubation in avidin–biotin complex (Vector Laboratories) in 1× PBS solution. After three rinses in 1× PBS, all sections were developed in 0.025% 3,3′-diaminobenzidine-HCl (DAB) plus 0.02% H_2O_2 for 10–15 min. Sections were then dehydrated, mounted in Eukitt permanent medium (VWR International, Switzerland) and examined with a Zeiss light microscope.

Statistical analysis

All data were expressed as means±SEM and analyzed using SPSS v11 software. The general design of locomotor activity ("Experiment 1") was a seven-level between-subjects factor (LV-GFP, LV-BDNF, LV-TrkB, LV-BDNF+LV-TrkB, LV-TrkT1, LV-TrkB+LV-siRNAs, and LV-BDNF+LV-TrkB+LV-siRNAs)×repeated measures on the same subject during 19 days. Drug condition (saline vs. cocaine)

and doxycycline treatment (session A vs. session B vs. session C) were added as within-subjects factors. For conditioned-place preference ("experiment 2"), extinction ("experiment 3"), reinstatement ("experiment 4") and maintenance of the CPP after withdrawal ("experiment 5"), a three-level between-subjects factor (LV-GFP, LV-BDNF and LV-TrkB)×repeated measures on the same subject during 20 days design was made (within-subjects factor for extinction). Cocaine treatment during conditioning (5 vs. 20 mg/kg) and doxycycline regimen were considered as between-subjects factors. Statistical data were analyzed using an analysis of variance (ANOVA). First, an over-all *f* test analysis was performed to identify significant differences among any of the group means. In case of statistically significant *f* ratio scores ($p<0.05$), a second analysis using Tukey test has been carried out, where sets of two groups at a time were compared, to specifically determine significant differences. Acceptable significance level was set at $p<0.05$.

Results

Lentivirus expression in HEK293T cells and doxycycline regulation (data not shown)

Tetracycline-regulatable lentiviruses expressing BDNF (LV-BDNF) or its receptors TrkB or TrkT1 (LV-TrkB or LV-TrkT1) were generated (see "Materials and methods" section). HEK293T cells were infected with different concentrations of each lentivirus. Transcript levels were measured by means of qRT-PCR and normalized against β-actin used as endogenous control. Infection of HEK293T cells with LV-BDNF induced a titer-dependent expression of BDNF-specific mRNA. Interestingly, expression of BDNF also induced a titer-dependent up-regulation of its receptor TrkB, but not its inactive TrkT1 receptor. Treatment of infected HEK293T cells with doxycycline (30 ng/mL) blocked BDNF expression in LV-BDNF-infected cells (as the virus is tetracycline-regulatable) and also produced a down-regulation of its corresponding TrkB receptor back to basal levels (changes are highly significant, $p<0.01$). Under these conditions, TrkT1 levels remain unchanged at very low initial levels of expression. Infection of HEK293T cells with LV-TrkB or of LV-TrkT1 also induced a titer-dependent expression of their mRNA, (but no significant expression of corresponding neurotrophins), which could be blocked by 30 ng/mL doxycycline. Infection with various titers of LV-BDNF, LV-TrkB, or LV-TrkT1 did not affect the expression of β-actin. Co-infection of various titers of LV-BDNF with another regulatable lentivirus (e.g., LV-CD81 or LV-urokinase) did not affect expression of the control gene and its doxycycline-mediated regulation. Similar results were found with LV-TrkB or LV-TrkT1.

Three siRNAs-expressing lentiviruses, LV-siRNA1, LV-siRNA2, and LV-siRNA3, targeted against TrkB, have been generated and tested. HEK293T cells coinfected with LV-TrkB and LV-siRNA1, LV-siRNA2, or LV-siRNA3 displayed a 52%, 30%, and 65% reduction of TrkB mRNA, respectively, and when co-infected with a mix of all three silencers (LV-siRNAs), >93% silencing of TrkB mRNA could be achieved ($p<0.01$ compared to control cells). LV-siRNA2 and LV-siRNA3 had no effects on TrkT1 mRNA expression in HEK293T cells infected with LV-TrkT1, whereas LV-siRNA1 produced ca. <45% of TrkT1. These siRNAs-expressing lentiviruses did not affect expression of other mRNAs, e.g., endogenous β-actin (data not shown).

Experiment 1: cocaine-induced locomotor activity mediated by overexpression of BDNF and TrkB

Seven groups of lentivirus-infected animals (see "Materials and methods" section, $n=9$ per group) were used for assessing the levels of BDNF or its receptors expression in the NAc, as measured by qRT-PCR (Figs. 1 and 2). First, behavioral evaluation was performed in three 5-day sessions: session A (no doxycycline, enabling full ectopic gene expression), session B (animals fed doxycycline, inducing ectopic gene suppression), and then session C (no doxycycline, recapitulating both gene expression of session A and initially observed behavior). Statistical analysis revealed significant interactions between all the factors: doxycycline × lentivirus ($F_{(12,264)}=154.44$; $p<0.001$), doxycycline × cocaine ($F_{(2,264)}=664.26$; $p<0.001$), cocaine × lentivirus ($F_{(6,132)}=674.36$; $p<0.001$), and doxycycline × cocaine × lentivirus ($F_{(12,264)}=125.74$; $p<0.005$). Chronic cocaine administration was continued daily throughout each session A, B, and C. This protocol enables for behavioral evaluation of effects of local gene expression changes on the very same animals (Bahi et al. 2004a, b, 2005a, b, 2006). A seven-level between-subjects comparison revealed a significant effect of groups, indicating that lentiviral injection has an effect on locomotor activity ($F_{(39,320)}=2898.32$; $p<0.0001$)

When rats were injected with LV-GFP, there was an interaction between cocaine × doxycycline ($F_{(2,264)}=197.14$; $p<0.001$); within-subjects comparison indicated a significant effect of the drug, showing that cocaine induces a 6.9-fold increase in locomotor activity over saline (Fig. 1A and A'; saline vs cocaine $F_{(2,24)}=18.98$; $p<0.01$). Treatment with doxycycline slightly reduced this activity ratio to 5.7-fold.

By contrast, when rats were injected with LV-BDNF (cocaine × doxycycline ($F_{(2,264)}=433.38$; $p<0.001$), cocaine induced a 13.6-fold increase in locomotor activity, but this activation was only 7.5-fold when animals were fed doxycycline in drinking water ($F_{(2,375)}=13.699$; $p<0.001$), turning off expression of ectopic BDNF (Fig. 1B'; within-

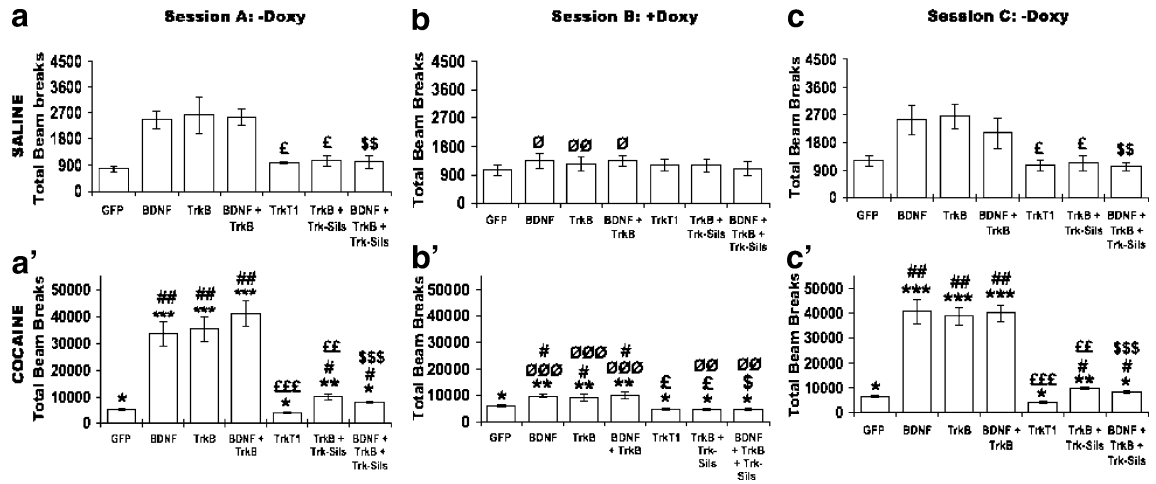

Fig. 1 *BDNF and TrkB induced locomotor activity after saline and cocaine injections.* Locomotor activity was monitored daily as rats (n=9 per group) were chronically injected, first with saline ("habituation period"), then, 30 min later, with cocaine–HCl (15 mg/kg, i.p.). The total horizontal activity is expressed as distance traveled in cm (mean±SEM) during the three 5-days sessions. In the first session (*a* and *a′*/-doxycycline**),** rats (n=9 per group) were fed 5% sucrose, then switched to doxycycline regimen during the second session (*b* and *b′*/+ doxycycline) and, finally, changed back to normal regimen, with doxycycline-free 5% sucrose for the last session (*c* and *c′*/− doxycycline). At the end of each session, three rats per group were killed *p<0.05, **p<0.01, and ***p<0.005 vs. saline-treated animals "baseline"; #p<0.05, ##p<0.01 and ###p< 0.005 vs. LV-GFP-infected animals; £ p<0.05, ££ p<0.01, and £££ p<0.005 vs. LV-TrkB-infected animals; $$p$<0.05, $$$p$<0.01 and $$$$p$<0.005 vs. LV-BDNF+LV-TrkB infected animals; Ø p<0.05, ØØ p<0.01 and ØØØ p<0.005 vs. water fed animals

subjects analysis saline vs. cocaine $F_{(2,24)}$=893.71; p<0.0001). In addition, in animals injected with LV-TrkB (cocaine × doxycycline ($F_{(2,264)}$=501.11; p<0.001), the locomotor activity was enhanced by 13.9-fold, but only by approximately 7.5-fold when fed doxycycline (Fig. 1B', $F_{(2,24)}$=893.71; p< 0.0001). When the expression of ectopic BDNF or TrkB genes was turned on again—upon removing doxycycline from the drinking water (Fig. 1C')—cocaine again re-induced hyperlocomotion and reinstated initially observed levels of ca. 16.8- and 14.9-fold increase in locomotor activity, respectively (Fig. 1C'; doxycycline treatments $F_{(2,24)}$=893.71; p< 0.001). These behavioral activities were reflected in mRNA expression in the NAc (Fig. 2A).

When LV-BDNF is co-injected with LV-TrkB (cocaine × doxycycline ($F_{(2,264)}$=549.24; p<0.001), cocaine-induced hyperlocomotion is highest, ca. 16-fold higher over saline (within-subjects comparison saline vs. cocaine $F_{(2,24)}$= 1,494.86; p<0.0001), but only ca. 7.4-fold when animals were fed doxycycline (Fig. 1A' and 1B'; p<0.001). Removal of doxycycline restored cocaine-induced hyperlocomotion to its highest level, ca. 20.0-fold over saline ($F_{(2,24)}$=1494.86; p<0.001 and p>0.1666 compared to sessions B and C, respectively). These effects were also reflected in the doxycycline-dependent expression of BDNF and TrkB mRNAs (Fig. 2A).

When rats were injected with LV-TrkT1 (cocaine x doxycycline ($F_{(2,264)}$=14.06; p<0.05), the dominant-negative form of the Trk receptor, cocaine, induced a slight hyperlocomotor activity (Fig. 1; 3.9-fold higher than saline in session A, p<0.001), and doxycycline had a significant

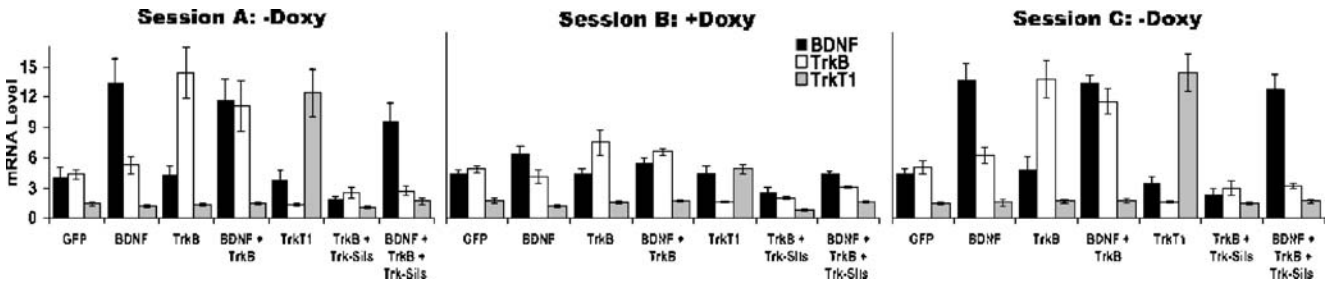

Fig. 2 BDNF, TrkB, and TrkT1 mRNA quantification after locomotor activity monitoring and doxycycline regulation of transcripts in vivo. Rats (n=9) were stereotaxically injected into the NAc with the lentiviral vectors mentioned on the *X*-axis. Animals were chronically injected cocaine (15 mg/kg i.p.) under different regimen, i.e., 5 days without doxycycline (session A); followed by 5 days under doxycycline (session B) and, finally, by 5 days without doxycycline (session C), and locomotor activity was measured daily. At the end of each session, three rats per group were sacrificed by decapitation, brains were removed, and various regions including the NAc were dissected out. Total RNA was extracted, reverse transcribed, and used for qRT-PCR using specific primers for BDNF, TrkB and TrkT1 mRNAs. Data are presented as means±SEM

behavioral effect (Fig. 1B; within-subjects comparison of doxycycline treatments $F_{(2,24)}$=72.40; p<0.0001), and it affected the expression level of TrkT1 mRNA (Fig. 2A). No difference was observed between sessions A and C ($F_{(2,24)}$=72.40; p>0.938). Overexpression of TrkT1 did not affect the expression of BDNF or TrkB mRNA (Fig. 2A). Animals co-injected with LV-TrkT1 and LV-BDNF also induced a low response to cocaine, similar to GFP-treated animals, which was almost unchanged by doxycycline (data not shown).

In vivo silencing of TrkB The effects of silencing TrkB on BDNF signaling in the NAc was also tested on separate groups (Figs. 1 and 2). Animals co-injected with LV-GFP and LV-TrkB-siRNAs (cocaine × doxycycline ($F_{(2,264)}$= 330.33; p<0.001) showed slightly lower levels in cocaine-mediated hyperlocomotion over LV-GFP controls, probably due to inhibition of endogenous TrkB (data not shown). However, when animals were co-injected with LV-TrkB and LV-TrkB-siRNAs (cocaine × doxycycline; $F_{(2,264)}$= 197.14; p<0.001), cocaine induced ca. a 9.6-fold increase in hyperlocomotor activity over saline (Fig. 1; within-subjects comparisons saline vs. cocaine $F_{(2,24)}$=1022.03; p<0.0001) that could be blocked by doxycycline reversibly (Fig. 1; within-subjects comparisons of doxycycline treatment: sessions A and C vs. session B: $F_{(2,24)}$=1022.03; p< 0.0001; session A vs. session C: p>0.056). This cocaine-mediated increased hyperlocomotion is much smaller than in the absence of the LV-TrkB-siRNAs (p<0.01) because the siRNA-expressing lentivirus also suppresses the expression of the endogenous TrkB (Fig. 2A) and part of the observed hyperlocomotion was mediated by cocaine up-regulation of TrkB in the NAc. Doxycycline reversibly blocked BDNF- or TrkB-mediated hyperlocomotion (Fig. 1B' and C'). An interesting observation must also be noted, namely, the fact that silencing TrkB also induced a down-regulation of the endogenous BDNF expression in the NAc (compare BDNF mRNA expression, Fig. 2).

When LV-TrkB-siRNAs was co-injected with LV-TrkB together with LV-BDNF, similar behavioral effects were observed (cocaine × doxycycline ($F_{(2,264)}$=177.86; p<0.001), (Fig. 1; within subjects comparison saline vs. cocaine $F_{(2,24)}$= 206.74; p<0.0001). The effects were of similar amplitude and were doxycycline regulated in a reversible way (Fig. 1; within-subjects comparisons of doxycycline treatment: sessions A and C vs. sessions B: $F_{(2,24)}$=206.74; p<0.01; session A vs. session C: p>0.924). However, when co-injected with LV-TrkT1, slightly lower levels in cocaine-mediated hyperlocomotion over LV-GFP controls were observed because of inhibition of endogenous TrkB, but this was not affected by doxycycline.

Experiment 2: LV-BDNF- and LV-TrkB-induced conditioned-place preference

Animals (n=9 per group) were injected with either LV-GFP, LV-BDNF, or LV-TrkB into the NAc and trained for conditioned-place preference using 5 or 20 mg/kg of cocaine. The pre-exposure test showed that animals spent an equal amount of time in the two different chambers. Repeated ANOVA for both chambers (wire grid, floor mesh) revealed no significant effect (between-subjects factor comparisons, wire grid vs. floor mesh: $F_{(11,60)}$= 0.733; p>0.396); thus, the test was unbiased in terms of chamber preferences of untreated animals. Nevertheless, between-subjects comparisons revealed an effect of lentiviral vector used ($F_{(11,60)}$=68.772; p<0.0001).

Figure 3 shows the results of the CPP test. Animals displayed significant place preference over the GFP-treated control and spent markedly more time into the cocaine-

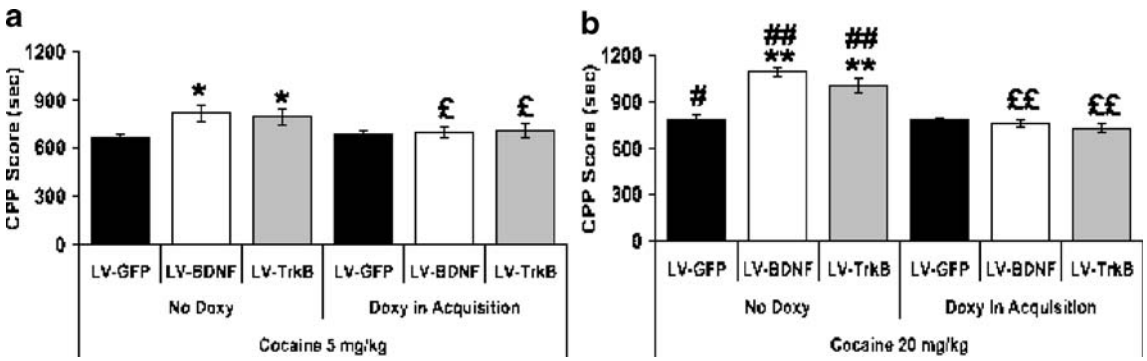

Fig. 3 LV-BDNF and LV-TrkB enhance cocaine-induced-place preference**.** Four sets of three groups of rats (n=9 per group) were injected with either LV-GFP, LV-BDNF, or LV-TrkB. Rats were then trained for conditioned-place preference using 5 mg/kg of cocaine (**a**) or 20 mg/kg of cocaine (**b**; see "Materials and Methods" section). One set of each (six groups, n=9 per group) was given 0.02% doxycycline in 5% sucrose during the conditioning period to block gene expression during learning. The other sets were fed 5% sucrose only, allowing full expression of both BDNF and TrkB. After conditioning, rats were tested at day 10, and the time spent in cocaine-paired box was monitored. Values indicate means±SEM. *p<0.05 compared to LV-GFP; #p<0.05 ##p<0.01 compared to animals injected 5 mg/kg during conditioning; £ p<0.05 ££ p<0.01 compared to rats with no doxycycline during the conditioning (learning) period

paired box ($F_{(11,60)}$=68.722; p<0.0001). The time increase spent into cocaine-paired chamber was related to the dose of drug administered. Between-subjects comparisons for cocaine dose during acquisition indicate a significant dose effect (Fig. 3A; 5 mg/kg vs. 20 mg/kg $F_{(1,70)}$=24.955, p< 0.001), corresponding to ca. 24% increase at low dose (Fig. 3A; within subjects comparisons, saline vs. cocaine at 5 mg/kg: $F_{(5,30)}$=14.39; p<0.005) and 53% increase at higher dose (Fig. 3B; saline vs. cocaine at 20 mg/kg: $F_{(5,30)}$= 106.38; p<0.0001). During conditioning (days 3–10), some groups were fed 0.02% doxycycline in 5% sucrose to block ectopic gene expression. Between-subjects comparisons for doxycycline feeding during acquisition indicated a strong and significant effect ($F_{(1,70)}$=24.704, p<0.001). Under these conditions, no place preference was observed, compared to controls ($F_{(2,15)}$=0.22; p>0.842), even at high dose of cocaine ($F_{(2,15)}$=0.68; p>0.491).

Experiment 3: extinction by repeated testing

In another series of experiments, animals underwent a prolonged period of withdrawal from cocaine for 20 days without injections, following the establishment of CPP (Fig. 4). Under these conditions, time spent in cocaine-paired box was gradually diminishing over testing days, and within-subjects comparisons of extinction revealed significant differences (extinction session 1 to 20: $F_{(11,60)}$= 76.447; p<0.001). Animals treated with LV-BDNF or LV-TrkB (Fig. 4B and C) clearly displayed marked delayed extinction over LV-GFP control animals (Fig. 4A; between-subjects comparisons, LV-BDNF, and LV.TrkB vs. LV-GFP: $F_{(2,15)}$=30.12; p<0.01). Again, delayed extinction was dose-dependent and was very pronounced at high dose of cocaine (between subjects factor, 5 mg/kg vs. 20 mg/kg: $F_{(5,36)}$=163.59; p<0.0001). However, if animals had been fed doxycycline during acquisition (days 3–9 of the CPP protocol), preventing ectopic gene expression, no significant difference with LV-GFP-treated animals could be observed, even at a high dose of cocaine (Fig. 4A', B' and c'; between-subjects factor, with vs. without doxycycline: $F_{(4,31)}$=1.06; p>0.394). Furthermore, at the end of the extinction process, there was no difference among all the groups (three-level between-subjects comparisons, LV-GFP vs. LV-BDNF vs. LV-TrkB: $F_{(11,60)}$=0.55; p>0.858). This observation prompted us to proceed towards priming assessments.

Experiment 4: reinstatement by priming using low dose of cocaine

At the end of the period of withdrawal from cocaine (experiment 3), animals received saline injections or cocaine priming with low dose of cocaine (2 mg/kg; Fig. 5). Under these conditions, within-subjects saline priming did not affect CPP score (Fig. 5A and B; $F_{(11,60)}$= 0.39; p>0.956). In contrast, priming with very low dose of cocaine (2 mg/kg) at the end of this 20-day withdrawal period induced full recovery of place preference for animals treated with high doses of cocaine during the conditioning period (Fig. 5B'; within subject saline vs. cocaine: $F_{(11,60)}$=

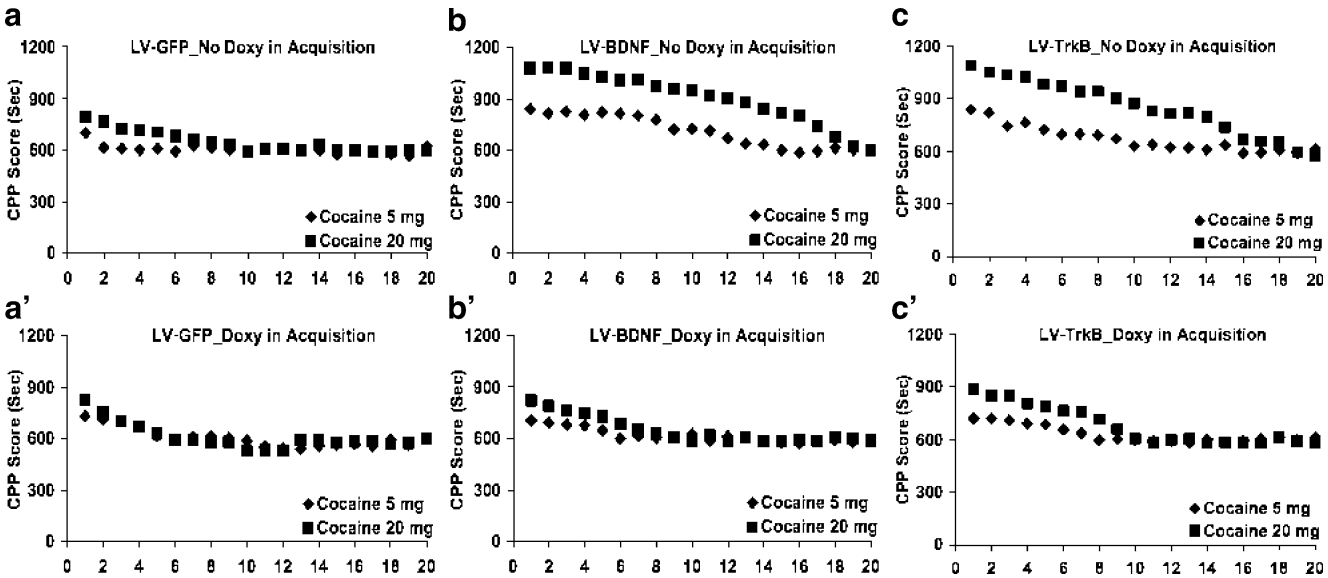

Fig. 4 *LV-BDNF and LV-TrkB-mediated delayed extinction of cocaine-induced-place preference.* Four sets of three groups of rats (n=9 per group) were injected either with LV-GFP (*a*), LV-BDNF (*b*), or LV-TrkB (*c*). Rats were trained for conditioned-place preference as in Fig. 3. During the conditioning period only, one set of each (six groups, n=9 per group) was fed 5% sucrose (*a*, *b*, and *c*), and the other sets were fed 5% sucrose supplemented with 0.02% doxycycline (*a'*, *b'* and c'). After CPP monitoring on day 10, rats were fed 5% sucrose only and were placed daily into the setup for 20 min during 20 days, but no injections were given during this prolonged period of withdrawal from cocaine. Values indicate means±SEM. The SEM bars have been omitted from the points for clarity

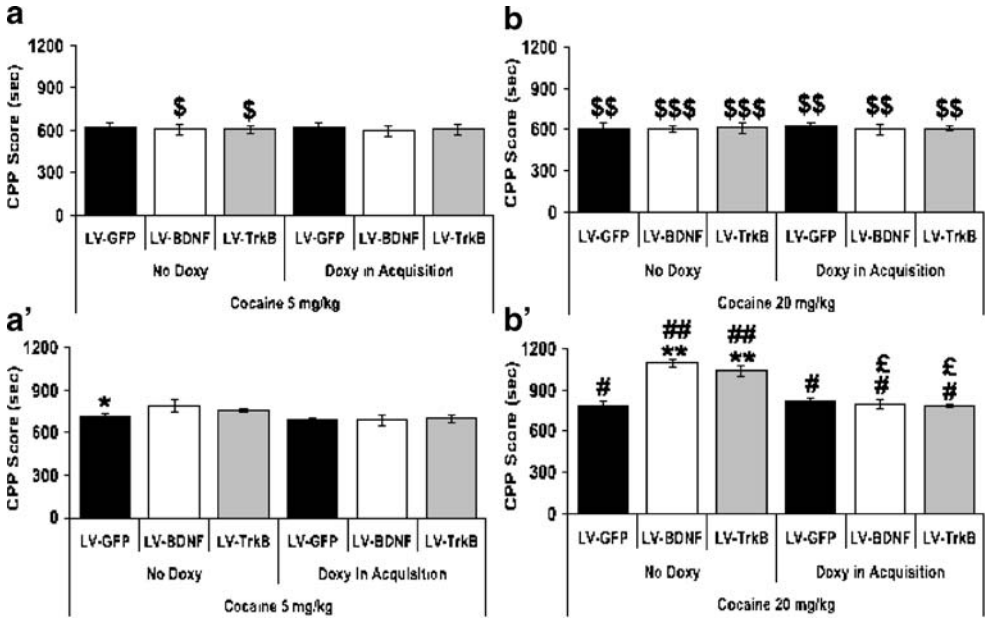

Fig. 5 *Extinction with saline pairings and reinstatement with low dose of cocaine injection.* Four sets of three groups of rats (n=9 per group) were injected with either LV-GFP, LV-BDNF, or LV-TrkB. Rats were then trained for conditioned-place preference with 5 mg/kg of cocaine (*a* and *a′*) or 20 mg/kg of cocaine (*b* and *b′*). During the conditioning period only, one set of each (six groups, n=9 per group) was fed 5% sucrose only, and the other sets were fed 5% sucrose and 0.02% doxycycline as in Fig. 4. After CPP monitoring on day 10, all animals were fed 5% sucrose only, and saline injections paired with each chamber alternately were performed once a day for 10 days. Place preference was then monitored (*a* and *b*). The next day, all animals received a priming injection of cocaine (2 mg/kg, i.p.), and place preference was immediately monitored again (a′ and b′). Values indicate means±SEM. *p<0.05 compared to LV-GFP; #p<0.05 ##p< 0.01 compared to animals injected 5 mg/kg during conditioning; £ p< 0.05 ££ p<0.01 compared to animals with no doxycycline during the conditioning (learning) period; **$** p<0.05 **$$** p<0.01 **$$$** p<0.005 compared to animals receiving 2 mg/kg of cocaine for priming

90.60; p<0.001). Between-subjects comparisons showed that animals receiving low dose of drug (5 mg/kg vs. 20 mg/kg cocaine during acquisition: $F_{(1,70)}$=44.33; p< 0.001) and no doxycycline during acquisition (with vs. without doxycycline: $F_{(1,70)}$=18.35; p<0.001) still displayed slight but significant recovery of place preference upon priming after this 20-day period (Fig. 5A'). Animals fed doxycycline during the acquisition period, i.e., when gene expression had been blocked during learning, displayed no place preference upon cocaine priming (Fig. 5A'; between-subjects comparison, 5 mg/kg during acquisition: $F_{(2,15)}$=0.299; p>0.746). In contrast, animals previously conditioned with 20 mg/kg of cocaine displayed highly significant CPP scores upon cocaine priming (Fig. 5B'; between-subjects comparison, 20 mg/kg during acquisition: $F_{(2,15)}$=130.62; p<0.001).

Experiment 5: maintenance of the CPP

In another series of experiments, animals have been trained for CPP, then kept in their home cages for 5 weeks without drug injection (Fig. 6). At the end of this period, place

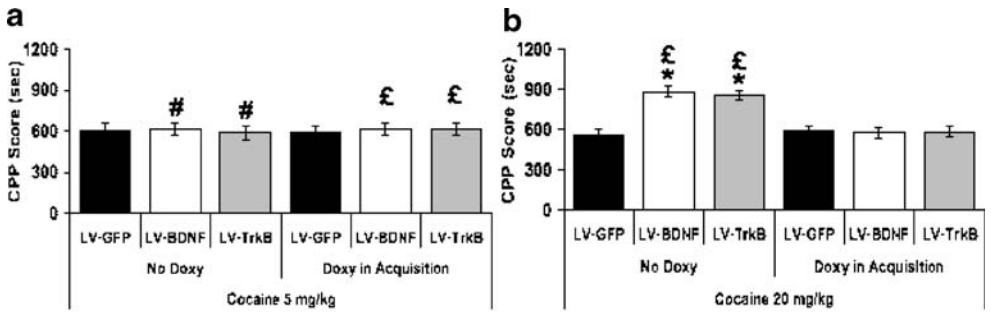

Fig. 6 *LV-BDNF and LV-TrkB maintained cocaine-induced-place preference.* Four sets of three groups of rats (n=9 per group) were injected with either LV-GFP, LV-BDNF, or LV-TrkB. Rats were then trained for conditioned-place preference, with 5 mg/kg of cocaine (**a**) or 20 mg/kg of cocaine (**b**), fed with or without doxycycline in 5% sucrose, as in Figs. 4 and 5 above. After CPP monitoring on day 10, rats were kept in their home cages for 5 weeks. Thereafter, place preference was monitored. Values indicate means ± SEM. * p<0.05 compared to LV-GFP; # p<0.05 compared to 20 mg/kg injected rats during conditioning; £ p<0.05 compared to rats with no doxycycline during the conditioning (learning) period

preference was monitored without drug injection. Interestingly, place preference was maintained upon a prolonged period of withdrawal from cocaine (three-level between-subjects factor: $F_{(2,15)}=38.33$; $p<0.001$). Between-subjects comparison of the initial CPP test showed that animals treated with high dose of cocaine spent more time in the cocaine-paired chamber when using 20 mg/kg during conditioning (Fig. 6B; between-subjects comparisons, 5 vs. 20 mg/kg: $F_{(1,70)}=7.85$; $p<0.007$). The enhanced CPP score was observed only if ectopic gene expression was possible during the learning period (between-subjects comparison of doxycycline treatment: $F_{(1,70)}=13.53$; $p<0.004$). Indeed, animals fed doxycycline during acquisition, i.e., when ectopic gene expression had been blocked at this period, displayed no place preference (Fig. 6A and B; within-subjects comparison of doxycycline treatment: $F_{(2,15)}=0.14$; $p>0.872$). CPP reinstatement did not occur in all groups that have received doxycycline during acquisition, and no statistical difference was observed between LV-GFP, LV-BDNF, or LV-TrkB-infected animals (three-level between-subjects comparison of doxycycline treatments: $F_{(5,30)}=0.76$; $p>0.585$). It should be mentioned that between-subjects comparisons of post-conditioning and reinstatement showed that there was a slight but significant difference between CPP scores during post-conditioning and reinstatement (Fig. 3 vs. Fig. 6; $F_{(23,120)}=71.066$; $p<0.005$).

At the end of behavioral analysis, animals have been sacrificed and gene expression assessed by means of immunohistochemistry (Fig. 7 and supplementary Figure S1). As expected, behavioral changes fully correlate lentivirus-mediated expression changes of BDNF or TrB. Animals infected with LV-BDNF and LV-TrkB displayed high expressions of BDNF or TrkB in the infected brain area (NAc), which was almost completely suppressed when the animals were fed doxycycline.

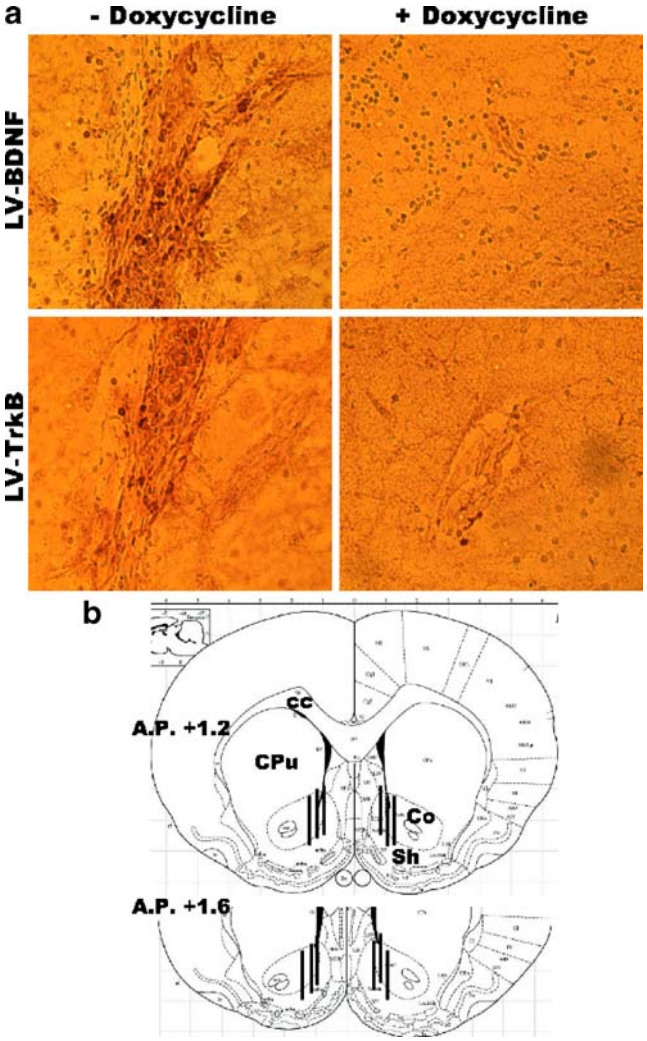

Fig. 7 BDNF and TrkB immunohistochemistry at the site of stereotaxic injection of LV-BDNF or LV-TrkB in the nucleus accumbens (NAc). **a** Rats were killed at the end of the experiment, brains were dissected out and processed for immunohistochemistry (see "Materials and Methods" section for details). Magnification ×40. **b** Topography of stereotaxic injection sites. Antero-posterior coordinates according to Paxinos and Watson (1998), are shown on the left side of each coronal section. *cc* Corpus callosum, *Co* NAc core, *CPu* caudate putamen, *Sh* NAc shell

Discussion

In this study, we have developed an approach enabling a thorough investigation of the role of BDNF in cocaine-induced behavior, in a way that is superior to previous genetic and pharmacological methods. The use of regulatable lentiviruses locally overexpressing either BDNF or its receptors TrkB or TrkT1 in combination enables to test on the very same animal the effects of very local changes in gene expression on behavior. In combination with lentiviruses locally silencing these genes, it enabled for the first time a very clear evaluation of the role of BDNF expression in the NAc in cocaine reward. Our data demonstrate that transient overexpression of BDNF and/or its receptor TrkB in the mesolimbic DA pathway induces potent and long-lasting behavioral effects. Intra-NAc BDNF or TrkB expression increased locomotor activity and resulted into the development of behavioral sensitization together with enhanced cocaine-induced place preference. In contrast, animals receiving lentiviral injection of TrkT1 or TrkB-specific siRNAs were less sensitive to the locomotor stimulant effects of cocaine than TrkB-treated animals. Furthermore, BDNF and TrkB are involved in the learning of drug preference, and this acquisition is maintained throughout after a prolonged period of withdrawal from cocaine, but only if BDNF expression is reactivated. This confirms studies showing that BDNF is important in

regulating synaptic plasticity in the brain areas that process reward information and that BDNF in the NAc promotes persistent cocaine-seeking behaviors and heightens relapse vulnerability (Schoenbaum et al. 2007).

Four major findings arise from cocaine-induced CPP experiments. First, these data show that cocaine-induced CPP was enhanced by BDNF and TrkB, and once established, CPP was maintained for at least 5 weeks compared to GFP-injected animals. Second, the conditioned-place preference could be extinguished by repeated testing for the place preference without drug exposure. However, this extinction was delayed when animals were overexpressing BDNF or TrkB in the mesolimbic DA system. Finally, and most importantly, our data show that, after extinction, a priming injection with low dose of cocaine (2 mg/kg) reinstated the cocaine-induced CPP. After a prolonged period of withdrawal from cocaine, animals given an injection of cocaine before the test session spent more time in the chamber originally paired with cocaine during the conditioning phase. Reinstatement after extinction was enhanced when animals were stereotaxically injected with LV-BDNF or LV-TrkB in the NAc. Furthermore, CPP scores were highly reduced if animals were fed doxycycline during the acquisition period; therefore, when BDNF and TrkB were inhibited during learning, animals had greater difficulties to associate cocaine injections with a specific environmental context.

Our data clearly support previous observations and further establish that BDNF modulates behavior and reward during the acquisition of cocaine CPP. BDNF and TrkB are involved in appetite behavior (Kernie et al. 2000); therefore, it is not surprising that overexpression of BDNF activates brain reward and place preference in a CPP test. Furthermore, we show that upon BDNF expression, extinction is delayed, which stresses the significant implications for BDNF signaling in cocaine reward. The rewarding effects of cocaine assessed in the CPP paradigm and open-field locomotor activity monitoring are drastically and reversibly attenuated in TrkB-silenced animals and confirm important roles for BDNF in psychostimulant actions. This agrees with observations on BDNF knockout mice (Sora et al. 2001). Heterozygous BDNF knockout mice displayed less locomotion during habituation and less locomotion after cocaine injections. Cocaine-CPP was reduced in the BDNF heterozygotes mice, which displayed no significant difference from saline control values at a dose of 10 mg/kg s.c. cocaine, although they exhibited cocaine-induced preference at a 20 mg/kg dose. However, developmentally related behavioral impairment and particularly learning deficits could not be excluded in knockout models—and indeed have been documented in BDNF+/− mice—which is not the case in the lentiviral approach proposed in this study.

The finding that CPP, once established, was maintained over time is interesting. Our findings clearly demonstrate that drug-related cues are still effective over 5 weeks, implying that BDNF and TrkB may facilitate memory consolidation. This agrees with previous reports showing that cocaine-induced CPP is maintained up to 4 weeks (Mueller and Stewart 2000). In another study, conditioned-place preference based on pairing an environment with either morphine or naloxone has been reported to occur after three pairings of subcutaneous drug injections and to be retained for at least 1 month (Mucha and Iversen 1984). In addition, our data show that a prolonged period of withdrawal from cocaine led to the reduction of the CPP score. This fully agrees with published results from other groups (Mueller and Stewart 2000), showing that once cocaine-induced CPP has been established, rats injected only with saline subsequently display significant attenuation in cocaine-induced increase in time spent, in comparison to non-extinction-treated control rats (Calcagnetti and Schechter 1993).

The changes observed in the CPP paradigm were strikingly similar to the effects of TrkB knockout on cocaine-induced locomotion (Grimm et al. 2003; Hall et al. 2003; Horger et al. 1999). Locomotor stimulating effects of cocaine were reduced but not eliminated in TrkB-silenced animals with a similar dose–effect profile. TrkB silencing produces a down-regulation of endogenous BDNF, again emphasizing the striking role of the BDNF system on cocaine-mediated behavioral changes. Decreases in cocaine efficacy with reduced BDNF or TrkB expression are consistent with observations that cocaine sensitization is delayed in the BDNF +/− mice (Hall et al. 2003) and with observations that BDNF administration delays extinction (Grimm et al. 2003) and enhances cocaine sensitization and conditioned reinforcement (Horger et al. 1999). Together, this strengthens the notion that differences in BDNF expression may underlie associations between markers near the BDNF gene locus and drug addiction and supports suggestions that human allelic variation at a locus in or near the BDNF gene could contribute to individual differences in human addiction vulnerability (Krebs et al. 2000; Uhl et al. 2001). Human genomic markers within or near the BDNF locus have been linked to or associated with substance abuse (Hall et al. 2003). Post mortem human brain specimens reveal individual differences in the levels of BDNF mRNA and in mRNA splicing patterns.

However, these previous works did not address two important questions. What is the role of BDNF in cocaine reward and relapse, and in which brain area(s) does BDNF have its effects on cocaine reward and relapse? The present study has been performed into the NAc shell, thus supporting the view that behavioral sensitization may involve BDNF or TrkB expression changes preferentially in this

brain area. In the shell, but not in the core of the NAc, the level of BDNF mRNA is significantly increased after acute and repeated cocaine treatment, and during cocaine withdrawal (Filip et al. 2006). Combined knockouts of DAT and SERT genes are required to eliminate cocaine reward (Sora et al. 2001). As BDNF effects on both systems have been identified (Hyman et al. 1991, 1994), the large effects of partial TrkB knockdown on cocaine reward mechanisms might also be largely because of actions of BDNF on both dopaminergic and serotoninergic systems (Altar et al. 1994). This may thus support the idea that both neurotransmitter functions may be critical determinants of cocaine reward (Hall et al. 2003; Uhl et al. 2002). To confirm that BDNF had a critical role in the NAc shell, as opposed to upstream or downstream structures, Graham locally injected an adeno-associated viral vector to knockdown BDNF protein production in the accumbens shell, leading to a substantial reduction in BDNF levels in this brain area (46%) and causing a downward shift in the dose–response curve, suggesting a decrease in cocaine's rewarding effects (Graham et al. 2007). Our present experiments clearly consolidate these suggestions and identifies the BDNF gene together with its active TrkB receptor because of their mutual individual variations and mutual regulatory effects in the NAc, as an essential gene for cocaine reward and cocaine addiction.

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