BDNF Overexpression in the Ventral Tegmental Area Prolongs Social Defeat Stress-induced Cross-Sensitization to Amphetamine and Increases Δ FosB Expression in Mesocorticolimbic Regions of Rats

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Social defeat stress induces persistent cross-sensitization to psychostimulants, but the molecular mechanisms underlying the development of cross-sensitization remain unclear. One candidate is brain-derived neurotrophic factor (BDNF). The present research examined whether ventral tegmental area (VTA) BDNF overexpression would prolong the time course of cross-sensitization after a single social defeat stress, which normally produces transient cross-sensitization lasting < 1 week. Δ FosB, a classic molecular marker of addiction, was also measured in mesocorticolimbic terminal regions. Separate groups of intact male Sprague-Dawley rats underwent a single episode of social defeat stress or control handling, followed by amphetamine (AMPH) challenge 3 or 14 days later. AMPH cross-sensitization was apparent 3, but not 14, days after stress. Intra-VTA infusion of adeno-associated viral (AAV-BDNF) vector resulted in a twofold increase of BDNF level in comparison to the group receiving the control virus (AAV-GFP), which lasted at least 45 days. Additionally, overexpression of BDNF in the VTA alone increased Δ FosB in the nucleus accumbens (NAc) and prefrontal cortex. Fourteen days after viral infusions, a separate group of rats underwent a single social defeat stress or control handling and were challenged with AMPH 14 and 24 days after stress. AAV-BDNF rats exposed to stress showed prolonged cross-sensitization and facilitated sensitization to the second drug challenge. Immunohistochemistry showed that the combination of virally enhanced VTA BDNF, stress, and AMPH resulted in increased Δ FosB in the NAc shell compared with the other groups. Thus, elevation of VTA BDNF prolongs cross-sensitization, facilitates sensitization, and increases Δ FosB in mesocorticolimbic terminal regions. As such, elevated VTA BDNF may be a risk factor for drug sensitivity.

Neuropsychopharmacology (2013) **38**, 2286–2296; doi:10.1038/npp.2013.130; published online 19 June 2013

Keywords: VTA; BDNF; social defeat; Δ FosB; cross-sensitization; amphetamine

INTRODUCTION

Cross-sensitization is defined as the augmented behavioral response to a drug challenge produced by intermittent exposure to an agent other than the challenge drug, such as stress or another drug (Antelman *et al*, 1980; Stewart and

Badiani, 1993). In humans, stressful life events increase vulnerability to drugs of abuse, particularly psychomotor stimulants (Sinha, 2008). Likewise, it has been demonstrated in animal models that various forms of stress can increase sensitivity and vulnerability to drugs of abuse (de Jong *et al*, 2005b; Miczek *et al*, 2004; Nikulina *et al*, 2004; Piazza *et al*, 1990; Robinson *et al*, 1985).

The mesocorticolimbic circuitry is comprised of the ventral tegmental area (VTA), where dopamine neurons are located, and its projection areas: the prefrontal cortex (PFC), nucleus accumbens (NAc), hippocampus, and amygdala (Swanson, 1982). Both stress and psychostimulants activate the mesocorticolimbic dopamine system and increase dopamine release in VTA terminal regions (Di Chiara and Imperato, 1988; Tidey and Miczek, 1996). Although aversive social events such as social stress can increase vulnerability to the development of drug sensitization, not all individuals exposed to social stress become

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Received 19 October 2012; revised 14 May 2013; accepted 14 May 2013; accepted article preview online 21 May 2013

sensitized to drugs of abuse. These differences may result from individual genetic differences.

One candidate gene for such individual differences is brain-derived neurotrophic factor (BDNF). Genetic addiction studies in humans have determined that a polymorphism in the *bdnf* gene is associated with vulnerability for polysubstance abuse (Uhl *et al*, 2001). In particular, the presence of the BDNF 66Val allele, which confers greater activity-dependent secretion of BDNF compared with the BDNF 66Met allele (Egan *et al*, 2003), is associated with greater vulnerability to drug addiction (Cheng *et al*, 2005; Flanagin *et al*, 2006; Tsai, 2007). This finding, coupled with the fact that BDNF has an important role in the survival of dopamine neurons (Baquet *et al*, 2005), activity-dependent neural plasticity (Cohen-Cory *et al*, 2010), and learning and memory (Bekinschtein *et al*, 2008), places BDNF in a good position to influence response to both stress or drugs of abuse.

Several lines of research support a role for VTA BDNF in stress- and drug-related behavior. In rodents, intermittent social defeat stress elevates BDNF expression in the VTA (Fanous *et al*, 2010) and produces lasting cross-sensitization to amphetamine (AMPH) (Nikulina *et al*, 2004). Furthermore, infusion of BDNF into the VTA increases locomotor response to cocaine (Pierce and Bari, 2001) and facilitates drug sensitization (Horger *et al*, 1999). By contrast, depletion of BDNF in the VTA prevents the social aversion induced by chronic social defeat stress and increases general social interaction (Berton *et al*, 2006; Fanous *et al*, 2011). As VTA BDNF is involved in modulating responses to both social defeat stress and psychostimulants, VTA BDNF may have a role in sensitivity to psychostimulants after social defeat stress. The present study examined the association between enhanced VTA BDNF and increased vulnerability to cross-sensitization and sensitization to AMPH in the presence of social defeat stress.

Another factor that may affect stress-induced sensitization to psychostimulants is Δ FosB, a truncated form of the FosB transcription factor. Δ FosB is considered a molecular marker for chronic stimulation of reward circuitry, stress-induced neuroplasticity, and sensitization to psychostimulants. Δ FosB, barely detectable immediately after acute stimuli, accumulates to considerable levels after repeated social defeat stress or repeated drug administration and persists due to its stability (Nestler *et al*, 1999; Nikulina *et al*, 2008; Perrotti *et al*, 2004). Δ FosB regulates the expression of many neuroplasticity-related genes in reward circuitry after chronic drug exposure (McClung *et al*, 2004), and increased Δ FosB in reward circuitry enhances the sensitivity to psychostimulants, such as cocaine (Kelz *et al*, 1999). In the current study, we characterized Δ FosB in VTA terminal regions as a molecular marker for long-term sensitization related to neuroadaptive changes.

Here we overexpressed BDNF in the VTA using an adeno-associated virus (AAV) vector to examine effects on AMPH sensitization in two non-sensitizing behavioral manipulations: (1) long-term cross-sensitization after a single episode of social defeat stress and (2) sensitization after non-sensitizing repeated dosing regimen of AMPH injections (Segal and Mandell, 1974). We chose a single social defeat paradigm because single social defeat stress-induced effects are transient (de Jong *et al*, 2005b; Miczek *et al*,

1999) in comparison to intermittent social defeat stress that result in long-lasting cross-sensitization (Covington and Miczek, 2001; Nikulina *et al*, 2004). We also investigated whether a single social defeat stress exposure in the presence of elevated VTA BDNF could induce long-lasting Δ FosB expression in mesocorticolimbic projection regions.

MATERIALS AND METHODS

Animals

Subjects were male Sprague-Dawley rats (Charles River Laboratories, Hollister, CA). Rats weighed 225–250 g upon arrival and were acclimated to laboratory conditions for 1 week before the start of experiments. All subjects were maintained under a reverse light/dark cycle (12h: 12h, lights off at 0900 hours), with unlimited access to food (Purina Rodent Diet, Brentwood, MO) and water. After the acclimation period, rats underwent stereotaxic surgery and were single-housed for the remainder of study in standard plastic cages (25 × 50 × 20 cm³). Male Long-Evans rats (Charles River Laboratories), termed 'residents,' were pair-housed with females in larger (37 × 50 × 20 cm³) cages. Residents were screened for aggressive behavior as described previously (Nikulina *et al*, 2004). All experimental procedures were approved by the Arizona State University Institutional Animal Care and Use Committee and conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996). All efforts were used to minimize suffering and to limit the number of animals used.

Viral Vector

Pseudotyped AAV2/10 vectors were used in all the experiments. The AAV2/10 rep/cap plasmid provided the AAV2 replicase and AAV10 capsid genes (Gao *et al*, 2002), while adenoviral helper functions were supplied by the pHelper plasmid (Stratagene, La Jolla, CA). The AAV plasmids contain a transgene cassette, which consists of the cytomegalovirus promoter, and either the gene for rat BDNF fused to enhanced green fluorescent protein or GFP alone (GFP gift from R. Klein), followed by a combined intron/polyadenylation signal derived from SV40. These elements are flanked by two AAV2 inverted terminal repeats. A standard triple transfection protocol was used to generate the helper-free pseudotyped AAV2/10 vectors as previously described (Xiao *et al*, 1998). Briefly, the three plasmids were co-transfected into HEK-293 cells (Stratagene, La Jolla, CA) via calcium phosphate precipitation. Cells were harvested 48 h later, resuspended in DMEM, freeze-thawed with dry ice–ethanol slurry three times and centrifuged to produce a clarified cell lysate. The resulting viral stocks produced either GFP (AAV-GFP, as control virus) or BDNF-GFP (AAV-BDNF) upon infection and were stored at –80 °C before use.

Intracranial Viral Infusion

Rats were anesthetized with isofluorane and positioned in a stereotaxic frame (Leica Angle Two; Richmond, IL). Two holes were drilled at stereotaxic coordinates (AP –5.1 mm;

ML $\pm$ 2.15 mm from the bregma) on the surface of skull, and Hamilton syringes (Model 7105 KH; Reno, NV) with 24 gauge injector tips were lowered through the holes bilaterally at a 10° angle until needle tips reached the VTA (AP $-$ 5.1 mm; ML $\pm$ 0.6 mm; DV $-$ 8.8 mm from the bregma; Paxinos and Watson, 2007); 0.5 μ l of virus was bilaterally infused into each side of the VTA for 10 min at a speed of 0.05 μ l/min. After infusion, the syringes remained in the infusion sites for 5 min to prevent retrace of virus.

Confirmation of Viral Efficacy

To confirm viral efficacy in enhancing VTA BDNF at the relevant time points for the behavioral experiments, we examined VTA BDNF expression by BDNF immunohistochemistry 14 and 45 days after viral infusions. Rats received AAV-GFP or AAV-BDNF. One cohort of rats was used only to measure VTA BDNF expression 14 days after viral infusions. Measurement of VTA BDNF expression 45 days after viral infusions was performed in tissue from the rats used in Experiment 2 below.

All placements of infusion sites were confirmed based on observation of GFP expression in serially collected brain sections. The cases in which infusion sites were inaccurate were excluded from further assessment.

Experimental Design

A timeline of the experiments is presented in Figure 1. Based on the finding that VTA BDNF is elevated from 2 weeks to at least 45 days after viral infusions (described in RESULTS below), we waited at least 2 weeks after the viral infusion to commence any experimental procedures.

Experiment 1: Time course of a single social defeat stress-induced cross-sensitization. Rats were randomly assigned to four groups, exposed to either a single social

defeat stress or handling procedure, then received d-amphetamine sulfate (1.0 mg/kg, i.p.; Sigma-Aldrich; St Louis, MO) either 3 days ($n=6$ each) or 14 days ($n=4$ and 5, respectively) after defeat or the handling procedure (Figure 1a). Following AMPH challenge, locomotor activity was tracked and assessed.

Experiment 2: Molecular alterations induced by VTA BDNF overexpression. Rats received bilateral intra-VTA infusion of either AAV-GFP ($n=4$) or AAV-BDNF ($n=7$). Two weeks after the surgery, rats were handled, followed 2 weeks later by 0.9% saline (1.0 ml/kg, i.p.) injection. Brain tissue was collected and processed 45 days after viral infusion surgery (Figure 1b). BDNF in the VTA and Δ FosB expression in the PFC and NAc were examined.

Experiment 3: Effect of single social defeat stress and VTA BDNF overexpression on behavioral sensitization to AMPH and molecular alterations. Rats were randomly assigned to four groups based on two experimental factors: virus (AAV-GFP vs AAV-BDNF) and behavioral treatment (handling vs single social defeat). The groups were: AAV-GFP-handled ($n=7$), AAV-GFP-stressed ($n=7$), AAV-BDNF-handled ($n=8$), and AAV-BDNF-stressed ($n=7$). The stressed groups underwent a single social defeat stress exposure 2 weeks after the surgery, at which time the handled groups were handled, followed by two AMPH (1.0 mg/kg, i.p.) challenge injections 14 and 24 days after stress. To examine whether prolonged cross-sensitization was induced by VTA BDNF overexpression in rats exposed to single social defeat stress, the first AMPH challenge was given 2 weeks after defeat or handling. Three rats were excluded from this study due to artifact during locomotor tracking. The second AMPH challenge was given 10 days later (24 days after defeat stress; one rat was excluded due to an artifact during tracking). All animals were euthanized at day 45 after surgery, whereupon brain tissue was collected and processed (Figure 1c). Besides locomotor activity, BDNF single labeling and BDNF/TH double labeling in the VTA and Δ FosB expression in the PFC and NAc were examined. The sample numbers of different immunohistochemical procedures varied due to the exclusion of samples with occasional heterogeneity of section quality.

Social Defeat Stress

Social defeat stress consisted of a short aggressive confrontation between an aggressive resident rat and an experimental intruder rat as described in detail previously (Nikulina et al, 2004). After removing the female from the resident's cage, an experimental intruder rat was placed into the home cage of a resident male rat under a stainless steel protective cage (15 $\times$ 25 $\times$ 15 cm³) for 5 min, then the protective cage was removed, and the resident displayed aggressive behavior; 'defeat' occurred when the intruder exhibited a supine posture for at least 4 s. Aggressive interactions were 2–5 min in duration. The protective cage was then replaced for an additional 15-min exposure, after which the intruder was returned to its home cage. Control rats were handled and weighed on the days their counterparts were stressed and weighed.

Figure 1 Schematic of experimental design. (a) Experiment 1: Time course of single social defeat stress-induced cross-sensitization to AMPH. (b) Experiment 2: Effect of VTA BDNF overexpression on molecular alterations in mesocorticolimbic areas. (c) Experiment 3: Effect of single social defeat stress and VTA BDNF overexpression on behavioral sensitization to AMPH and molecular alterations.

Locomotor Tracking

For 2 consecutive days immediately before AMPH challenge, rats were given a 0.9% saline injection (1.0 ml/kg, i.p.) in their home cages. Rats inside their home cages were placed into the locomotion tracking arena for 1 h to acclimate to the testing environment and injection procedure. On the drug challenge day, rats were placed into the locomotion tracking arena within their home cages, habituated to the testing environment for 30 min during which locomotion was tracked, injected with saline followed by tracking for 40 min, and then challenged with AMPH followed by tracking for 70 min. Peak locomotor activity in response to AMPH was achieved 10–50 min after challenge. Locomotor activity was assessed using Videotrack (Viewpoint Life Sciences; Montreal, Canada); total distance travelled in the form of large ambulatory movements (>10 cm) was measured in 10-min bins. The dosage of AMPH used herein mainly induces large ambulatory movements (Geyer *et al*, 1987), so total distance of such movements can be used as an indicator of the extent of psychomotor sensitization in response to AMPH.

Perfusion and Tissue Processing

Forty-five days after surgery, all rats were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and perfused transcardially with 10 ml of 10% heparin in 0.1 M phosphate-buffered saline (PBS; pH 7.4) followed by 250 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were post-fixed for 1.5 h in the same fixative at 4 °C, soaked in 12.5% sucrose, then in 25% sucrose in 0.1 M PBS at 4 °C until saturated, then stored at 4 °C before sectioning. Brains were sectioned at 20 µm in a cryostat at –22 °C and thaw-mounted onto glass slides (Superfrost Plus; Fisher; Waltham, MA). Sections were collected from +3.2 to +2.8 mm from bregma for the PFC, +1.8 to +0.8 mm from bregma for the NAc, and –4.8 to –5.6 mm from bregma for the VTA (Paxinos and Watson, 2007).

Immunohistochemistry

Sections were washed in 0.05 M potassium phosphate-buffered saline (KPBS), then blocked for 1 h in 10% normal donkey serum and 0.4% Triton X-100 in 0.05 M KPBS. Sections were then incubated with primary antibody: BDNF (AB1779SP, 1:3000 dilution; Millipore; Temecula, CA) or FosB (SC-48, 1:10 000 dilution; Santa Cruz Biotechnology; Santa Cruz, CA). The FosB antibody used here targets the N terminal of FosB protein contained in both FosB and ΔFosB. However, based on the timeline of the present research, FosB-like labeling would primarily capture accumulation of ΔFosB, because FosB expression is transient and only ΔFosB persists after stimulation (Perrotti *et al*, 2004). Following incubation with primary antibody for 48 h at 4 °C, slides were washed in 0.05 M KPBS and incubated for 1 h in biotin-conjugated goat anti-rabbit serum (1:200 dilution in blocking solution, Vectastain ABC kit; Vector Laboratories; Burlingame, CA). After washing in 0.05 M KPBS, sections were incubated with avidin-biotin-peroxidase complex (ABC kit, Vector Laboratories) for 1 h, then washed again, and developed using DAB chromogen with nickel (DAB

Peroxidase Substrate kit, Vector Laboratories). After dehydration in graded concentrations of ethanol and xylene, coverslips were applied.

To examine the cellular localization of BDNF expression in the VTA of animals that showed persistent cross-sensitization, we performed fluorescent double labeling of BDNF and tyrosine hydroxylase (TH). Primary antibodies, rabbit anti-BDNF (AB1779SP, 1:500 dilution; Millipore; Temecula, CA) and mouse anti-TH (SC-7837, 1:500 dilution, Santa Cruz Biotechnology; Santa Cruz, CA), were applied simultaneously and incubated for 48 h at 4 °C. After incubation with biotinylated goat anti-rabbit IgG (1:200 dilution, Vector Laboratories) for 1 h, Alexa Fluor 488 Anti-Rabbit conjugated streptavidin and Alexa Fluor 548 Goat Anti-Mouse (1:500 dilution, Invitrogen, San Diego, CA) were applied for 2 h. After washing with 0.05 M KPBS, coverslips were applied with ProLong Gold Antifade Reagent with DAPI (Invitrogen, San Diego, CA).

Image Analysis

Tissue sections were examined for the presence of chromogen or fluorophore reaction products using a Zeiss Axioskop microscope with ×20 or ×40 objectives; selected areas were captured and digitized using a color digital video camera (MBF Biosciences; Williston, VT) or AxioCam MR (Zeiss) interfaced to the microscope. A cell profile was considered labeled if its pixel intensity was >2 SDs greater than the background, as calculated by Stereo Investigator software (MBF Biosciences). Fluorescent-labeled cells were counted manually, first in single channels for single labeling, and then in merged channels for double labeling. At least three adjacent sections were analyzed for each brain region per animal, and labeling density was calculated by dividing the estimated total number of labeled profiles by the total area analyzed.

Statistics

In experiment 1, one-way ANOVA (between-subjects factor: handling *vs* stress) was used to analyze locomotor activity during the peak locomotor response period (10–50 min after AMPH injection). In experiment 2, one-way ANOVA was used to analyze the number of BDNF or ΔFosB-labeled cells in saline-treated animals (between-subjects factor: AAV-BDNF *vs* AAV-GFP). In experiment 3, locomotor activity and immunohistochemical data were analyzed by two-way ANOVA (between-subjects factors: viral vector (AAV-BDNF *vs* AAV-GFP) and behavioral treatment (handling *vs* stress)). Three-way mixed ANOVA with repeated measures (two between-subjects factors: viral vector (AAV-BDNF *vs* AAV-GFP) and behavioral treatment (handling *vs* stress); one within-subjects factor: number of AMPH injections (first *vs* second)) was used to analyze locomotor activity in response to the first and the second AMPH challenge. In a few cases, an optical artifact produced an unrecoverable error in locomotor tracking. Therefore, several cases were excluded due to the presence of this artifact. To assure that the same cases were assessed repeatedly across both challenge sessions by three-way ANOVA with repeated measures, samples with such artifact in either of the two AMPH challenges were excluded. Thus, the final number of

cases assessed using three-way mixed ANOVA with repeated measures is $n=6$ per group.

T-test was used for planned comparisons between AAV-BDNF-stressed and all the other groups when significant interaction was not present, and *post-hoc* Tukey's test was used in the presence of significant interaction. All data are reported as mean $\pm$ SEM. Results were considered significant if $p \leq 0.05$.

RESULTS

Experiment 1

Time Course of Single Social Stress-Induced Cross-Sensitization. To measure the time course of single social defeat stress-induced cross-sensitization in naive rats, locomotor response to AMPH challenge was evaluated in adult male Sprague-Dawley rats 3 or 14 days after exposure to a single social defeat or control handling. Rats exposed to single social defeat 3 days earlier exhibited significantly more total locomotor activity after AMPH than rats handled 3 days earlier (measured 10–50 min after AMPH challenge; $F_{(1, 11)} = 7.170$, $p = 0.023$; Figure 2a), reflecting the presence of cross-sensitization. No difference in total locomotor activity was observed between stressed and handled rats in a separate cohort that were challenged with AMPH 14 days after exposure to single social defeat stress or handling ($F_{(1, 7)} = 0.230$, $p = 0.646$; Figure 2b). This indicates that cross-sensitization after a single defeat is short-lasting and detectable 3 days, but not 14 days, later.

Experiment 2

Confirmation of viral efficacy. The viral infusion site was visualized using GFP as an indicator of transfection (Figure 3a). Based on GFP expression from serially collected sections, we observed that transfected neurons in the VTA extend approximately 300 μ m in diameter on the rostral-caudal axis, and 200 μ m in diameter on dorsal-ventral and medial-lateral axes around the infusion site. Expression of viral product at 45 days after infusion is consistent with the observation that AAV-mediated transgene expression persists 1–3 months in neurons after transfection (Klein *et al*, 1998). To compare the increase in VTA BDNF after AAV-BDNF across time points, VTA BDNF expression was normalized to that of the AAV-GFP control group at each respective time point (Figure 3c). Fourteen and 45 days after viral infusion, we observed twofold increase of BDNF in rats receiving AAV-BDNF compared with those receiving AAV-GFP: 14 days $F_{(1, 7)} = 11.105$, $p = 0.013$; Figure 3c; 45 days $F_{(1, 9)} = 7.312$, $p = 0.027$; Figure 3b–d). At both 14 and 45 days, the volume of infection was approximately 25% of the rostral VTA (bregma -4.8 to -5.6) around the infusion site (bregma -5.1).

Δ FosB expression after BDNF overexpression in the VTA of drug-naïve rats. VTA BDNF overexpression alone increased Δ FosB labeling in anterior cingulate (ACG; $F_{(1, 6)} = 9.880$, $p = 0.020$), prelimbic (PRL; $F_{(1, 6)} = 12.930$, $p = 0.007$), and infralimbic (IL; $F_{(1, 6)} = 15.620$, $p = 0.008$) regions of the PFC (Figures 4a and c) and in the NAc core ($F_{(1, 8)} = 16.108$, $p = 0.043$) and shell ($F_{(1, 8)} = 11.978$,

Figure 2 Cross-sensitization to AMPH after a single episode of social defeat stress is observed 3 days, but not 14 days, after stress. Rats were exposed to a single episode of social defeat stress or handling and then challenged with AMPH (1.0 mg/kg, i.p.) 3 days or 14 days later. Distance traveled was measured before and after saline injection and following AMPH (1.0 mg/kg, i.p.) challenge. Injection times are indicated by vertical arrows. (a) Locomotor activity in response to AMPH challenge 3 days after single social defeat stress (* $p \leq 0.05$). (b) Locomotor activity in response to AMPH challenge 14 days after single social defeat stress.

$p = 0.011$; Figure 4b and c) compared with the AAV-GFP-saline rats.

Experiment 3

Social defeat stress-induced cross-sensitization and sensitization to AMPH. To determine whether elevated VTA BDNF prolonged single social defeat stress-induced cross-sensitization, rats receiving AAV-GFP control virus or AAV-BDNF were exposed to a single episode of stress or handling followed by AMPH (1.0 mg/kg) 14 days later. The absence of stress-induced cross-sensitization to AMPH 14 days later (Experiment 1) was confirmed in rats which received AAV-GFP (one-way ANOVA, $F_{(1, 12)} = 1.873$, $p = 0.198$). By contrast, AAV-BDNF-stressed rats showed significantly greater locomotor activity in response to the first AMPH injection compared with AAV-BDNF-handled rats (one-way ANOVA, $F_{(1, 12)} = 4.730$, $p = 0.050$). Two-way ANOVA showed significant main effects of stress ($F_{(1, 23)} = 6.228$, $p = 0.020$) and AAV-BDNF ($F_{(1, 23)} = 4.464$, $p = 0.046$), but the interaction between the two factors was not significant ($F_{(1, 23)} = 0.277$, $p = 0.603$; Figure 5a). Ten days after the first AMPH challenge, a second AMPH

Figure 3 AAV-BDNF infusion into the VTA produces persistent overexpression of BDNF. (a) Brain atlas and representative image of AAV-GFP infusion site (arrow) in the VTA visualized by GFP fluorescence, bar = 200 μ m; fr: fasciculus retroflexus, SN: substantia nigra. (b) Representative photomicrographs of BDNF labeling 45 days after AAV-BDNF infusion. Bar = 100 μ m. BDNF expression near the infusion site is significantly increased after AAV-BDNF; solid arrow—BDNF-labeled neurons. (c) Number of BDNF-labeled cells at infusion site, normalized to AAV-GFP control, 14 and 45 days after infusion. (d) Number of BDNF-labeled cells at infusion site 45 days after infusion, in AAV-GFP and AAV-BDNF rats. * $p \leq 0.05$ compared with control AAV-GFP-saline rats.

challenge was given, and a significant main effect of AAV-BDNF ($F_{(1,25)} = 8.284$, $p = 0.008$) and interaction between stress and AAV-BDNF on locomotor activity was observed ($F_{(1,25)} = 5.950$, $p = 0.022$), but we observed no main effect of stress ($F_{(1,25)} = 1.587$, $p = 0.219$; Figure 5b). Additionally, AAV-BDNF-stressed rats showed significantly greater locomotor activity than the AAV-GFP-handled, AAV-GFP-stressed and AAV-BDNF-handled groups in response to the second AMPH injection (*post-hoc* multiple comparisons: $p = 0.008$, 0.001, and 0.014, respectively; Figure 5c).

To examine locomotor response to a non-sensitizing AMPH dose regimen in rats with a history of stress exposure and enhanced VTA BDNF, we compared locomotor activity in response to first and second AMPH challenges within each group (Figure 5c). Analysis of the amplitude of variation of locomotor activity between the first and the second AMPH challenge was achieved using three-way ANOVA with repeated measures. The data showed significant interaction of AAV-BDNF and the number of AMPH injections ($F_{(1,22)} = 4.503$, $p = 0.045$). Also, a significant interaction among stress, AAV-BDNF, and the number of AMPH injections ($F_{(1,22)} = 5.463$, $p = 0.029$) was observed. Furthermore, AAV-BDNF-stressed rats showed significantly augmented locomotor response to the second AMPH injection compared with the first AMPH injection (*post-hoc* multiple comparisons: $p = 0.031$), indicating facilitated sensitization after repeated dosing. Although a weak increasing trend was present in AAV-GFP-handled rats in response to multiple AMPH injections, this effect was not statistically significant ($p = 0.09$). No significant changes in locomotion after AMPH was observed

in AAV-GFP-stressed or AAV-BDNF-handled rats (*post-hoc* multiple comparisons: $p = 0.418$ and 0.241, respectively, Figure 5c).

BDNF expression in VTA dopaminergic neurons. To characterize the distribution and phenotype of BDNF-expressing neurons in the VTA, double-label fluorescent immunohistochemistry was performed on tissue from Experiment 3. Two-way ANOVA of the total number of BDNF-expressing neurons in the rostral VTA also indicated a significant main effect of AAV-BDNF ($F_{(1,22)} = 41.381$, $p = 0.001$) and an interaction between AAV-BDNF and stress approaching significance ($F_{(1,22)} = 4.055$, $p = 0.056$). Also, AAV-BDNF-stressed rats showed higher BDNF labeling across the entire rostral VTA compared with all the other groups (Figure 6a; *post-hoc* comparisons: $p \leq 0.05$ for all comparisons).

Furthermore, two-way ANOVA showed significant main effect of AAV-BDNF on BDNF/TH double-labeled cells in the rostral VTA ($F_{(1,22)} = 14.298$, $p = 0.001$); and significantly more BDNF/TH double-labeled cells were present in the rostral VTA in AAV-BDNF-stressed rats than in AAV-GFP-handled or stressed rats (*t*-test: $p = 0.018$ and 0.001, respectively; Figure 6b and c).

Δ FosB in the mesocorticolimbic projection areas. Consistent with the effect of VTA BDNF overexpression on Δ FosB expression in PRL and ACG cortices in drug-naïve rats (Experiment 2), two-way ANOVA showed a significant main effect of AAV-BDNF on Δ FosB expression

Figure 4 Persistent elevation of VTA BDNF leads to Δ FosB accumulation in the PFC and NAc. (a) Δ FosB expression in the anterior cingulate (ACG), prelimbic (PRL), and infralimbic (IL) regions of the PFC. (b) AAV-BDNF infusion significantly increased Δ FosB expression in the NAc core and shell. * $p \leq 0.05$ compared with AAV-GFP-saline rats. (c) Representative photomicrographs of Δ FosB labeling in the PFC (ACG, PRL, IL areas) and NAc (core and shell) of AAV-GFP-saline and AAV-BDNF-saline rats; bar = 50 μ m.

in both the ACG and PRL cortices in AMPH-treated rats (ACG: $F_{(1,21)} = 6.837$, $p = 0.016$; PRL: $F_{(1,21)} = 4.469$, $p = 0.047$; Table 1). In IL cortex, a main effect of stress was observed ($F_{(1,21)} = 4.941$, $p = 0.037$).

Among all the brain regions examined, the greatest induction of Δ FosB labeling was present in the NAc shell of AAV-BDNF-stressed animals (*post-hoc* multiple comparisons: $p \leq 0.01$ for all comparisons, Figures 7a and b), where we observed significant main effect of stress ($F_{(1,23)} = 26.100$, $p = 0.001$) and AAV-BDNF ($F_{(1,23)} = 39.670$, $p = 0.001$) estimated by two-way ANOVA. Also, significant interaction between these two factors ($F_{(1,23)} = 10.010$, $p = 0.004$) on Δ FosB expression was found in NAc shell. By contrast, no difference in Δ FosB cell counts was observed in the NAc core after AMPH challenge in any group (*t*-test: $p > 0.05$ for all comparisons; two-way ANOVA: behavioral treatment $F_{(1,22)} = 0.110$, $p = 0.743$; virus: $F_{(1,22)} = 0.187$, $p = 0.743$; behavioral treatment $\times$ virus: $F_{(1,22)} = 1.244$, $p = 0.277$; Table 1).

DISCUSSION

The present results demonstrate that virus-mediated enhancement of VTA BDNF expression both extends the time course of single social defeat stress-induced cross-sensitization to AMPH and facilitates sensitization to a non-sensitizing dosing regimen of AMPH in rats exposed to a single episode of social defeat stress. We also show that enhancement of VTA BDNF alone is sufficient to increase Δ FosB expression in projection areas of the VTA.

Our behavioral results are consistent with previous findings (de Jong *et al*, 2005b) that single social defeat-induced cross-sensitization to AMPH is transient, apparent at 3 days but not at 14 days after a single exposure to social defeat. It is important to note that handled rats single-housed for 14 days showed higher locomotor response to AMPH than did handled rats single-housed for 3 days. The increased locomotor response to AMPH in drug-naïve rats handled/stressed 14 days earlier is likely due to the longer period of single-housing, a form of social isolation which can elevate rewarding effects of psychostimulants (Deroche *et al*, 1994) and can amplify the physiological effect of social defeat (de Jong *et al*, 2005a). In the present study, handled/stressed rats received control virus, and non-viral-treated rats that were single-housed for 2 weeks showed similar locomotor responses to AMPH. This suggests that single-housing induces an upward shift of locomotor activity to AMPH, which is consistent across experiments. Above all, single social defeat stress-induced cross-sensitization to AMPH was not observed 14 days after stress in non-viral treated rats or in rats that received the control GFP virus. The fact that cross-sensitization to AMPH was observed 14 days after single social defeat stress in the presence of enhanced VTA BDNF suggests that BDNF prolonged single stress-induced cross-sensitization. However, such an effect should be interpreted with caution, because persistent VTA BDNF elevation also might alter single social stress-induced cross-sensitization at early time points. This possibility remains to be investigated.

Figure 5 AAV-mediated VTA BDNF overexpression extends the time course of AMPH cross-sensitization after a single social defeat stress and facilitates drug sensitization after repeated AMPH. Locomotor activity (distance in cm) over time (left) before and after saline, and following AMPH (1.0 mg/kg, i.p.) challenge ($*p \leq 0.05$ compared with all the other groups, $^{\dagger}p \leq 0.05$ compared with the first AMPH injection). Injection times are indicated by vertical arrows; response to AMPH (total distance traveled in cm) during the peak activity period 10–50 min after drug challenge. (a) Locomotor activity in response to the first AMPH challenge (14 days after stress or handling). (b) Locomotor activity in response to the second AMPH challenge (24 days after stress or handling). (c) Interaction plot of locomotor activity during the peak activity period in response to the first and the second AMPH challenge.

Figure 6 The number of BDNF expressed neurons increases in whole rostral VTA after combined VTA BDNF overexpression, single social stress, and repeated AMPH. (a) BDNF expression in whole rostral VTA, 45 days after viral infusion, with stress/handling procedure and two times AMPH challenges ($*p \leq 0.05$ compared with all the other groups), labeled with fluorophore-conjugated secondary antibodies. (b) BDNF/TH double labeling in VTA 45 days after viral infusion, with stress/handling procedure and two times AMPH challenges. (c) Representative microphotographs of fluorescent BDNF labeling (left), TH labeling (center), and BDNF/TH double-labeling (right); bar = 50 μm; filled arrow: BDNF and TH double-labeled cell; open arrow: non-dopaminergic BDNF-labeled cell.

Table 1 Numbers of Δ FosB-Positive Cells per mm^2 Present after Two AMPH Injections

Brain region	AAV-GFP-handled	AAV-GFP-stressed	AAV-BDNF-handled	AAV-BDNF-stressed
Nucleus accumbens core	419.4 $\pm$ 50.1	479.2 $\pm$ 53.8	537.5 $\pm$ 44.3	473.8 $\pm$ 95.7
Anterior cingulate cortex	303.3 $\pm$ 53.9	300.0 $\pm$ 24.0	446.7 $\pm$ 16.2 ^a	359.5 $\pm$ 47.3
Prelimbic cortex	308.3 $\pm$ 50.2	347.9 $\pm$ 20.3	380.0 $\pm$ 38.9	435.7 $\pm$ 64.3
Infralimbic cortex	262.5 $\pm$ 29.9	316.7 $\pm$ 22.9	350.0 $\pm$ 29.8	385.7 $\pm$ 61.3

^aSignificantly different from AAV-GFP-handled, *t*-test, $p \leq 0.05$

Figure 7 Δ FosB expression in the NAc shell increases only in response to combined VTA BDNF overexpression, single social stress, and repeated AMPH. (a) Number of Δ FosB-labeled cells in the NAc shell across groups (* $p \leq 0.05$ compared with all other groups). (b) Representative photomicrographs of Δ FosB labeling in the NAc shell of each group; bar = 50 μm .

Cross-sensitization and sensitization share the common molecular mechanism of enhanced dopamine transmission in mesolimbic circuitry (Imperato *et al*, 1989; Kalivas and Stewart, 1991), and both behaviors represent increased responsiveness of mesolimbic dopaminergic neurons to drugs of abuse. In addition, intermittent social defeat stress sensitizes NAc dopamine response to cocaine administration (Miczek *et al*, 2011). Our data show that AAV-mediated VTA BDNF is enhanced in DA neurons, which may directly increase DA release (Blochl and Sirrenberg, 1996) and metabolism (Altar *et al*, 1992). As such, the increased number of BDNF-expressing dopamine neurons in the rostral VTA could enhance mesocorticolimbic dopamine neurotransmission, contributing to the prolonged cross-sensitization and the sensitization to AMPH we observed. Our viral infusion resulted in increased VTA BDNF in a physiological range similar to the level of VTA BDNF in rats exposed to intermittent social defeat stress (Fanous *et al*, 2010), so it is possible that VTA BDNF overexpression induced neuroadaptive changes similar to those induced by intermittent social defeat stress to result in sensitized drug response.

The ability of VTA BDNF to directly modulate sensitization is consistent with the finding that infusions of BDNF into the VTA enhance cocaine sensitization (Horger *et al*, 1999). Increased endogenous BDNF signaling in the VTA leads to sensitized synaptic response in VTA dopamine neurons after cocaine withdrawal (Pu *et al*, 2006). BDNF in the VTA may act to titrate the effect of social defeat stress on activating mesolimbic neurochemistry by rendering this

circuit more vulnerable to stress-induced activation. This enhanced vulnerability might then cause the VTA to be more sensitive to stress or multiple drug challenges, leading to facilitated sensitization. One mechanism for facilitated sensitization may be through AMPH-induced enhancement of calcium signaling, which has been associated with the development of sensitization and produces increased BDNF in regions, such as the cortex and hippocampus (Licata *et al*, 2000; Shieh *et al*, 1998; Tao *et al*, 1998). Calcium signaling specifically in the VTA is important in the development of psychostimulant sensitization (Licata *et al*, 2000). The presence of increased VTA BDNF may therefore facilitate prolonged synaptic plasticity in the face of a stimulus that produces calcium release (but whose magnitude would not normally result in such prolonged plasticity, such as a single administration of AMPH). In this way, enhanced VTA BDNF may be considered a possible 'risk factor' for development of neuronal and behavioral drug sensitization, which may interact with factors such as stress to further increase 'risk.' This agrees with the role mesolimbic BDNF has been hypothesized to play in addictive behavior (Bolanos and Nestler, 2004).

Intermittent social defeat stress resulted in prolonged BDNF elevation in the VTA (Fanous *et al*, 2010) and Δ FosB accumulation in the projection areas of the VTA (Nikulina *et al*, 2008). In the present study, we found that VTA BDNF overexpression alone was sufficient to induce Δ FosB accumulation in the NAc and PFC. This finding indicates that persistent elevation of VTA BDNF leads to Δ FosB

accumulation, whose mechanism may be similar to Δ FosB elevation observed after repeated social defeat stress.

Δ FosB accumulation in the NAc could represent a homeostatic mechanism to balance increased VTA BDNF associated with social defeat, as Δ FosB in the NAc can enhance the resilience of animals to the aversive effects of chronic social defeat stress (Vialou *et al*, 2010). The effect of a single episode of social defeat stress alone is too transient to induce considerable Δ FosB accumulation in the NAc shell in rats receiving AAV-GFP. However, in the presence of VTA BDNF overexpression, a single defeat stress-induced neuroadaptation may persist, and transcriptional activation of Δ FosB may be prolonged in the NAc shell. The mechanism underlying increased Δ FosB accumulation in terminal regions resulting from VTA BDNF overexpression requires further investigation.

As discussed above, VTA BDNF overexpression significantly increased Δ FosB expression in the PFC and the NAc of handled drug-naïve rats. However, repeated AMPH challenges to AAV-BDNF-stressed rats induced a significant increase of Δ FosB expression in the NAc shell, but not in the NAc core and PFC, and this effect was region-specific. The explanation for this effect on Δ FosB expression is likely the differential responsiveness of different regions to stress- and drug-related stimuli. This is supported by data demonstrating different anatomical connections and biochemical characteristics of the NAc core and shell (Zahm, 2000), and as such, the subregions may mediate distinct functions related to motor and motivation (Kalivas *et al*, 1993). Furthermore, recent research indicates regionally specific effects of BDNF signaling in NAc core and shell in drug seeking (Li *et al*, 2013). Also Δ FosB modulates synaptic plasticity and reward-related behaviors in a sub-region- and cell-type-specific manner in the NAc core and shell (Grueter *et al*, 2013). Although the involvement of PFC in the sensitization to AMPH is well identified (Cador *et al*, 1999), no further Δ FosB increase was observed in AAV-BDNF-stressed rats after AMPH injections in the PFC. It is possible that Δ FosB in PFC is not susceptible to AMPH in rats with BDNF overexpression in the VTA, at least at the time point we observed. Therefore, Δ FosB expression may vary in different mesocorticolimbic regions in response to the same stimulation (drugs and stress), which may underlie why the current data reflect a distinct trend of increased Δ FosB expression only in the NAc shell.

In conclusion, rats with higher VTA BDNF levels developed prolonged AMPH cross-sensitization and sensitization to non-sensitizing dosing regimen of AMPH after social defeat stress. This was associated with increased Δ FosB expression in the NAc shell. The present findings suggest that the elevated VTA BDNF could be a risk factor that amplifies vulnerability for drug abuse triggered by socially aversive stimuli.

FUNDING AND DISCLOSURE

This work was supported by Grant numbers DA024817, DA026451, and MH073930 from the National Institute on Drug Abuse (NIDA) and the National Institute of Mental Health (NIMH). The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We gratefully acknowledge Dr Ronald Klein for his assistance with the initial viral vector construction, Dr Stephen Pratt for his assistance for statistics, and Colby Baker for his assistance with the behavioral tests in Experiment 1.

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