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METHAMPHETAMINE DIFFERENTIALLY AFFECTS BDNF AND CELL DEATH FACTORS IN ANATOMICALLY DEFINED REGIONS OF THE HIPPOCAMPUS

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Abstract—Methamphetamine exposure reduces hippocampal long-term potentiation (LTP) and neurogenesis and these alterations partially contribute to hippocampal maladaptive plasticity. The potential mechanisms underlying methamphetamine-induced maladaptive plasticity were identified in the present study. Expression of brain-derived neurotrophic factor (BDNF; a regulator of LTP and neurogenesis), and its receptor tropomyosin-related kinase B (TrkB) were studied in the dorsal and ventral hippocampal tissue lysates in rats that intravenously self-administered methamphetamine in a limited access (1 h/day) or extended access (6 h/day) paradigm for 17 days post baseline sessions. Extended access methamphetamine enhanced expression of BDNF with significant effects observed in the dorsal and ventral hippocampus. Methamphetamine-induced enhancements in BDNF expression were not associated with TrkB receptor activation as indicated by phospho (p)-TrkB-706 levels. Conversely, methamphetamine produced hypophosphorylation of N-methyl-D-aspartate (NMDA) receptor subunit 2B (GluN2B) at Tyr-1472 in the ventral hippocampus, indicating reduced receptor activation. In addition, methamphetamine enhanced expression of anti-apoptotic protein Bcl-2 and reduced pro-apoptotic protein Bax levels in the ventral hippocampus, suggesting a mechanism for reducing cell death. Analysis of Akt, a pro-survival kinase that suppresses apoptotic pathways and pAkt at Ser-473 demonstrated that extended access methamphetamine reduces Akt expression in the ventral hippocampus. These data reveal that alterations in Bcl-2 and Bax levels by methamphetamine were not associated with enhanced Akt expression. Given that hippocampal function and neurogenesis vary in a subregion-specific fashion, where dorsal hippocampus regulates spatial processing and has higher levels of neurogenesis, whereas ventral hippocampus regulates anxiety-related behaviors, these data suggest that methamphetamine self-administration initiates distinct allostatic changes in hippocampal subregions that may contribute to the altered synaptic activity in the hippocampus, which may underlie enhanced negative affective symptoms and perpetuation of the addiction cycle.

Key words: self-administration, ventral hippocampus, GluN2B, TrkB, Bcl-2, Bax.

INTRODUCTION

Methamphetamine abuse takes severe emotional and financial tolls on society, cutting across ages, races, ethnicities, and genders, and causes significant damage to self by producing cognitive impairment (Thompson et al., 2004; SAMHSA, 2008; Price et al., 2011; Weber et al., 2012). Hippocampus-dependent cognitive impairments in methamphetamine-abusing subjects can be attributed to methamphetamine-induced alterations in structural and functional plasticity of hippocampal neurons. For example, human imaging studies show reduced hippocampal volume, particularly gray matter volume, and decreased hippocampal responsiveness in chronic methamphetamine users (Thompson et al., 2004; Kim et al., 2010; Schwartz et al., 2010; Daumann et al., 2011; Nakama et al., 2011; Orikabe et al., 2011; Morales et al., 2012), indicating maladaptive hippocampal networking in methamphetamine-exposed individuals. Postmortem analyses in human brain tissue confirms that chronic methamphetamine use produces neurotoxicity in the hippocampus (Kitamura, 2009; Kitamura et al., 2010), which suggests an association between hippocampal dysfunction and toxicity in methamphetamine addicts.

The behavioral deficits observed in methamphetamine addicts have been demonstrated in preclinical models of binge methamphetamine exposure and methamphetamine self-administration with extended access to the drug. For example, these experimental paradigms produce cognitive dysfunction and memory impairments dependent on the hippocampus (Itoh et al.,

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Abbreviations: ANOVA, analysis of variance; BDNF, brain-derived neurotrophic factor; EDTA, Ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LTP, long-term potentiation; NMDA, N-methyl-D-aspartate; PVDF, polyvinylidene fluoride membranes; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TBST, Tris-buffered saline and Tween 20; TrkB, tropomyosin-related kinase B.

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1984; Yoshikawa et al., 1991; Yamamoto, 1997; Friedman et al., 1998; Rogers et al., 2008; Recinto et al., 2012). Neurodegeneration and neurotoxicity in the hippocampus is also observed in animal models of binge methamphetamine exposure and methamphetamine self-administration, suggesting a positive correlation between methamphetamine-induced toxicity and methamphetamine-induced behavioral deficits (Commins and Seiden, 1986; Eisch et al., 1996; Schmued and Bowyer, 1997; Mandyam et al., 2008). Additional mechanistic studies show that methamphetamine exposure alters the functional and structural plasticity of hippocampal neurons. For example, acute and systemic methamphetamine treatment reduces long-term potentiation (LTP) of CA1 pyramidal neurons through activation of D1 receptors and increases baseline excitatory synaptic transmission (Swant et al., 2010). Alternatively, self-administration of methamphetamine produces robust and long-lasting morphological changes in CA1 neurons (Onaivi et al., 2002; Crombag et al., 2005; Swant et al., 2010). Acute methamphetamine exposure reduces excitability of dentate gyrus neurons, whereas repeated exposure to methamphetamine increases excitability of these neurons (Criado et al., 2000). Furthermore, emerging studies in adult rats self-administering methamphetamine under extended access conditions (6 h of drug access per day) demonstrate that methamphetamine self-administration reduces dentate gyrus neurogenesis, and these effects were relative to the amount of methamphetamine consumed (Mandyam et al., 2008; Yuan et al., 2011). Withdrawal from methamphetamine self-administration produces compensatory changes in dentate gyrus neurogenesis (i.e., the enhanced survival of newly born neurons born after methamphetamine self-administration), and the hypothesis is that these changes in the hippocampus during withdrawal may regulate relapse to methamphetamine seeking (Recinto et al., 2012). These studies demonstrate that methamphetamine exposure (via either experimenter-delivered or self-administration paradigms) produces synaptic maladaptation in the hippocampus that may mediate some of the addiction behaviors dependent on the hippocampus.

In this context, brain-derived neurotrophic factor (BDNF), via interactions with its receptor tropomyosin-related kinase B (TrkB), regulates the function of neurons within the mesolimbic dopamine system and other reward regions to modulate the motivation to take drugs, while BDNF also independently affects hippocampal neurogenesis (Lee et al., 2000; Pierce and Bari, 2001; Katoh-Semba et al., 2002; Bolanos and Nestler, 2004; Scharfman et al., 2005; Donovan et al., 2008; Russo et al., 2010). Enhanced BDNF levels in the dentate gyrus positively correlate with increased number of newly born neurons in the dentate gyrus in non-pathological conditions; however, enhancing BDNF in an ischemic brain reduces ischemia-induced neurogenesis (Lee et al., 2000; Katoh-Semba et al., 2002; Larsson et al., 2002; Scharfman et al., 2005), suggesting a critical relationship between the neurogenic niche, BDNF, and neurogenesis in the hippocampus. In the context of addiction, cocaine exposure produces a widespread, but transient, induction of BDNF protein in the nucleus accumbens, prefrontal cortex, ventral tegmental area, and the central and basolateral nuclei of the amygdala (Grimm et al., 2003; Le Foll et al., 2005; Graham et al., 2007), whereas fewer studies have been conducted to examine BDNF protein levels after exposure to amphetamines. For example, BDNF protein expression in the hippocampus is enhanced in juvenile animals after forced administration of neurotoxic doses of amphetamines and the findings demonstrate that these changes in the neurotrophin may relate to the cognitive deficits in methamphetamine experienced animals (Skelton et al., 2007; Grace et al., 2008). In contrast to these studies using noncontingent methamphetamine injections, there are no published studies on the effect of methamphetamine self-administration on hippocampal BDNF levels, and therefore, was the focus of the current study. We also investigated the receptor systems (TrkB, N-methyl-D-aspartate (NMDA) receptor subunit 2B (GluN2B)), cell death factors (Bcl2 and Bax) and downstream signaling proteins (Akt, a pro-survival kinase) modulated by BDNF (Lin et al., 1998; Chao, 2003; Almeida et al., 2005).

EXPERIMENTAL PROCEDURES

Animals

Surgical and experimental procedures were carried out in strict adherence to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication number 85-23, revised 1996) and approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute. Nineteen adult, male Wistar rats (Charles River), weighing 200–250 g at the start of the experiment, were housed two per cage in a temperature-controlled vivarium under a reverse light/dark cycle (lights off 8:00 AM–8:00 PM) for at least 1 week.

Surgery

Twenty-two rats underwent surgery for catheter implantation for intravenous self-administration. Rats were anesthetized with 2–3% of isoflurane mixed in oxygen and implanted with a sterilized silastic catheter (0.64 ID × 1.19 OD mm; Dow Corning Co., Midland, MI, USA) into the right jugular vein under aseptic conditions. The distal end of the catheter was threaded under the skin to the back of the rat and exited the skin via a metal guide cannula (22G, Plastic One, Inc., Roanoke, VA, USA). Immediately after surgery, Flunixin® (2.5 mg/kg, s.c.; Bimeda – MTC Animal Health Inc., Oakbrook Terrace, IL, USA) was given as analgesic. The rats were subjected to antibiotic therapy with Timentin® (20 mg, i.v, once daily; SmithKline Beecham, Brentford, London, UK) during 10 days after the surgeries, and sulfamethoxazoletrimethoprim oral suspension (TMS, Hi-Tech Pharmacal Co., Inc., Amityville, NY, USA) in the drinking water (0.48 mg/ml) when health complications appeared during the study. Catheters were flushed daily with heparinized saline (30 USP units/ml) and tested eventually for patency using methohexital sodium (Brevital®, 10 mg/ml, 2 mg/rat; King Pharmaceutical Inc., Bristol, TN, USA). Eight rats did not experience methamphetamine. Three of the eight rats were trained to

self-administer saline (under FR1 schedule with each active lever delivering 90–100 μl of sterile saline) and experienced saline self-administration for 1 h for the same number of sessions as methamphetamine rats. Fourteen rats experienced methamphetamine self-administration.

Baseline training sessions and maintenance on an extended access schedule

Four to five days after surgery rats ($n = 14$) were trained to press a lever according to an FR1 schedule of methamphetamine reinforcement (0.05 mg/kg/injection of methamphetamine hydrochloride, generously provided by the National Institute on Drug Abuse) in operant boxes (Med Associates, St Albans, VT, USA) under baseline (acquisition) conditions (1 h access per day for 6–7 days). During daily sessions, a response on the active lever resulted in a 4-s infusion (90–100 μl volume), followed by a 20-s time-out period to prevent overdose. Each infusion was paired for 4 s with white stimulus light over the active lever (conditioned stimulus [CS]). Response during the time-out or on the inactive lever was recorded but resulted in no programed consequences. All animals were housed on a reverse cycle (lights off at 8 am) and were transferred from their home cages to their operant chambers between 9 and 10 am. Training on the first and second days was initiated with two–three priming (non-contingent) infusions of methamphetamine during the first 10 min. Rats were allowed to respond for the remaining 50 min without any additional priming. Acquiring methamphetamine self-administration was defined as maintenance of a similar number of infusions over three consecutive days during baseline training sessions. All animals acquired methamphetamine self-administration (data not shown). After baseline (acquisition) training, the rats were subjected to a short (ShA, $n = 8$, 1 h per day under an FR1 schedule) or long (LgA, $n = 6$, 6 h per day under an FR1 schedule) access schedule of methamphetamine reinforcement. Methamphetamine self-administration was performed for 17 days.

Western blotting

Procedures optimized for measuring levels of both phosphoproteins and total proteins were employed (Graham et al., 2007; Orio et al., 2009; Edwards et al., 2013). Methamphetamine-naive rats ($n = 8$), ShA ($n = 8$) and LgA ($n = 6$) rats were killed via rapid decapitation under light isoflurane anesthesia 16–20 h after the last self-administration session. Brains were quickly removed and flash-frozen. Tissue punches enriched in the dorsal hippocampus (−3.12 to −4.44 mm from bregma) or ventral hippocampus (−5.40 to −6.12 mm from bregma; Fig. 1b) from 500-μm-thick sections were homogenized on ice by sonication in buffer (320 mM sucrose, 5 mM HEPES, 1 mM EGTA, 1 mM EDTA, 1% sodium dodecyl sulfate (SDS), with Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktails II and III diluted 1:100; Sigma, St. Louis, MO, USA), heated at 100 °C for 5 min, and stored at −80 °C until determination of protein concentration by a detergent-compatible Lowry method (Bio-Rad, Hercules, CA, USA).

Mature BDNF protein levels were determined in 20-μg protein samples (mixed (1:1) with a Tricine sample buffer containing β-mercaptoethanol) subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) (15% acrylamide) using a Tris–Tricine–SDS buffer (Bio-Rad, Hercules, CA, USA), followed by electrophoretic transfer to polyvinylidene fluoride membranes (PVDF pore size 0.2 μm). TrkB, pTrkB, GluN2B, pGluN2B, Bcl-2 and Bax protein levels were determined in 20–30-μg protein samples (mixed (1:1) with a Laemmli sample buffer containing β-mercaptoethanol) subjected to SDS–PAGE (8–12% acrylamide) using a Tris–Glycine–SDS buffer (Bio-Rad, Hercules, CA, USA), followed by electrophoretic transfer to PVDF membranes. Blots were blocked with 5% milk (w/v) in Tris-buffered saline and Tween 20 (TBST) (25 mM Tris–HCl (pH 7.4), 150 mM NaCl and 0.1% Tween 20 (v/v)) for 1 h at room temperature and were incubated with the primary antibody for 16–20 h at 4 °C: antibody to BDNF (1:200, Santa Cruz cat. no. sc-546, predicted molecular weight 14 kDa, observed band between 15 and 20 kDa), TrkB (1:200, Santa Cruz cat. no. sc-8316, predicted molecular weight 95–145 kDa, observed band ~130 kDa), pTrkB Tyr-706 (1:200, Santa Cruz cat. no. sc-8316, predicted molecular weight 95–145 kDa, observed band ~95 kDa), GluN2B (1:200, Santa Cruz cat. no. sc-9057, predicted molecular weight 178 kDa, observed band ~180 kDa), antibody to pGluN2B Tyr-1472 (1:200, Cell Signaling cat. no. 4208S, predicted molecular weight 190 kDa, observed band ~180 kDa), Bcl-2 (1:500, R&D Systems cat. no. MAB8272, predicted molecular weight 24 kDa, observed band ~25 kDa), Bax (1:500, Santa Cruz cat. no. sc-493, predicted molecular weight 23 kDa, observed band ~20 kDa), antibody to Akt (1:500, Cell Signaling cat. no. 4691S, predicted molecular weight 60 kDa, observed band ~60 kDa), antibody to pAkt Ser-473 (1:500, Cell Signaling cat. no. 4060S, predicted molecular weight 60 kDa, observed band ~60 kDa). Blots were then washed three times for 15 min in TBST, and then incubated for 1 h at room temperature (24 °C), with horseradish peroxide-conjugated goat antibody to rabbit (1:2000, Bio-Rad, Hercules, CA, USA) in TBST. After another three washes for 15 min with TBST, immunoreactivity was detected using SuperSignal West Dura chemiluminescence detection reagent (Thermo Scientific, Waltham, MA, USA) and collected using HyBlot CL Autoradiography film (Denville Scientific, South Plainfield, NJ, USA) and a Kodak film processor. Net intensity values were determined using the Image Studio Lite (version 3.1). For normalization purposes, membranes were incubated with 0.125% coomassie stain for 5 min and washed three times for 5–10 min in destain solution.

Statistical analysis

The methamphetamine and saline self-administration data are expressed as active lever presses and methamphetamine data are also expressed as mean mg/kg methamphetamine self-administered per session. The effect of session duration on methamphetamine

self-administration during the 1-h session, 6-h session and during the first hour of the 6-h session was examined over the 17 escalation sessions using a two-way repeated-measures analysis of variance (ANOVA; session duration × daily session) followed by Fisher's Least Significant Difference (LSD) post hoc test or Student–Newman–Keuls post hoc test. The pattern of responding for methamphetamine is expressed as the mean mg/kg per hour over 6-h sessions in LgA rats and were compared between the first and >10th escalation sessions. Differences in the rate of responding between the first and other escalation sessions were evaluated using the paired *t*-test. Differences in the rate of responding between the first and other saline sessions were evaluated using a one-way repeated-measures ANOVA. Western blotting analysis was conducted separately for four groups, nonsaline controls, saline controls, limited access and extended access methamphetamine. Because protein levels were not significantly different between nonsaline and saline controls they were combined and were analyzed as methamphetamine naïve controls. Differences in density of proteins were analyzed by split-plot ANOVAs with methamphetamine access as a between-subject factor and hippocampal subregion as a within-subject factor. Following significant omnibus effects ($p < 0.05$), pairwise comparisons used Student's *t*-tests for split-plot ANOVAs. Data are expressed as mean ± SEM and were analyzed using SPSS or GraphPad Prism. Values of $p < 0.05$ were considered statistically significant. Graphs were generated using GraphPad Prism 5.0 software.

RESULTS

Extended access to methamphetamine self-administration resulted in escalation of methamphetamine intake

Rats experienced saline or methamphetamine for 17 days (Fig. 1a). A repeated measures ANOVA did not detect a change in saline intake in control animals (Fig. 1c). A repeated measures two-way ANOVA detected a significant Days of Self-Administration × Methamphetamine Access interaction (Fig. 1c, d, $F_{16,192} = 6.323$, $p < 0.0001$) and an increase in methamphetamine intake across 17 sessions when analyzing both the first hour of the session (Fig. 1d, $F_{16,80} = 2.684$, $p < 0.01$) and the total 6-h session ($F_{16,80} = 4.136$, $p < 0.0001$) in LgA animals. Post hoc analysis revealed a significant increase in methamphetamine intake during the 6 h session in LgA animals during Days 13–17 compared with Days 1–2. A repeated measures ANOVA did not detect a change in methamphetamine intake for ShA animals (Fig. 1c, d).

Limited and extended access methamphetamine self-administration increase BDNF expression but do not alter expression of TrkB or the phosphorylation of TrkB at Tyr-706

To determine whether limited access and extended access methamphetamine self-administration alters BDNF expression in the dorsal and ventral hippocampus, Western blot analyses were conducted on hippocampal protein lysates for methamphetamine naïve control

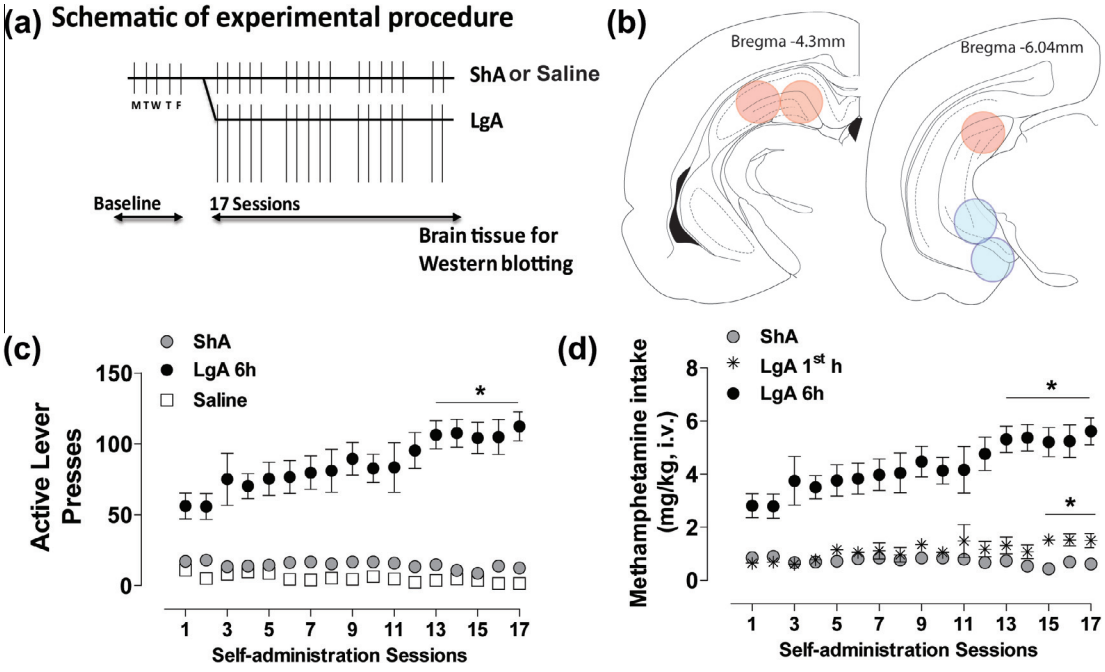

Fig. 1. Rats that underwent extended access to methamphetamine self-administration escalated methamphetamine intake. (a) Schematic showing baseline training followed by 17 sessions of either 1 h/day saline ($n = 3$), 1 h/day (ShA, $n = 8$; short vertical lines) or 6 h/day (LgA, $n = 6$; long vertical lines) methamphetamine self-administration. (b) Schematic of brain regions indicating area of tissue punches used for Western blot analysis. Red circles, dorsal hippocampus; blue circles, ventral hippocampus. (c and d) Active lever presses for saline and methamphetamine (c) and methamphetamine intake in mg/kg per session from ShA and LgA animals (d). The first hour intake is also indicated for LgA animals. $^{*}p < 0.01$ compared with initial sessions in the same animals by repeated measures two-way ANOVA followed by followed by Student–Newman–Keuls post hoc test. Data are represented as mean ± SEM. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

animals, LgA, and ShA animals sacrificed 16–20 h after the last methamphetamine self-administration session. Protein levels of BDNF (mature form) were analyzed in the dorsal and ventral hippocampus-enriched regions in control, LgA and ShA animals (Fig. 1b). Data were subjected to an ANOVA to examine the effects of treatment (control vs. methamphetamine groups) across hippocampal subregion (dorsal vs. ventral). An ANOVA detected a significant main effect of methamphetamine access on BDNF expression ($F_{2,38} = 3.7$, $p = 0.03$), indicating that methamphetamine experience enhanced BDNF protein expression in the hippocampus. Unpaired-*t*-test demonstrated significant increases in BDNF protein levels in the LgA animals in the dorsal and ventral hippocampus compared with drug naïve controls (p's = 0.02; Fig. 2).

Additionally, we determined whether methamphetamine self-administration altered total TrkB receptor expression and pTrkB expression in the dorsal and ventral hippocampus. Protein levels of TrkB (total and pTrkB at Tyr-706) were analyzed in the dorsal and ventral hippocampus-enriched regions in drug naïve controls, LgA and ShA animals. Data were subjected to ANOVAs to examine the effects of treatment (control vs. methamphetamine groups) across hippocampal subregion (dorsal vs. ventral) on total TrkB and pTrkB/TrkB expression. An ANOVA did not detect a significant main effect of methamphetamine or hippocampal region on total TrkB and pTrkB/TrkB expression (Fig. 3).

Extended access methamphetamine self-administration results in hypophosphorylation of GluN2B at Tyr-1472 in the ventral hippocampus

To determine whether methamphetamine self-administration altered total GluN2B receptor expression and pGluN2B expression in the dorsal and ventral hippocampus, protein levels of GluN2B (total and pGluN2B at Tyr-1472) were analyzed in the dorsal and ventral hippocampus-enriched regions in drug naïve control, LgA and ShA animals. Data were subjected to ANOVAs to examine the effects of treatment (control vs. methamphetamine groups) across hippocampal subregion (dorsal vs. ventral) on total and pGluN2B/GluN2B expression. An ANOVA did not detect a significant main effect of methamphetamine or hippocampal region on total GluN2B expression. An ANOVA showed a significant main effect of methamphetamine access on pGluN2B/GluN2B ratio ($F_{2,38} = 3.2$, $p = 0.05$). Unpaired-*t*-test demonstrated a significant reduction in pGluN2B/GluN2B ratio in the ventral hippocampus in LgA animals compared with drug naïve controls ($p = 0.008$; Fig. 4).

Both limited and extended access methamphetamine self-administration increased anti-apoptotic protein levels and reduced pro-apoptotic protein levels in the ventral hippocampus.

We next determined whether methamphetamine self-administration alters expression of pro-apoptotic and anti-apoptotic factors in the dorsal and ventral hippocampus. Protein levels of Bcl-2 (anti-apoptotic) and Bax (pro-apoptotic) were analyzed in the dorsal and ventral hippocampus-enriched regions in controls, LgA and ShA animals. Data were subjected to two-way ANOVAs to examine the effects of treatment (control vs. methamphetamine groups) across hippocampal subregion (dorsal vs. ventral) on pro- and anti-cell death factor expression. An ANOVA detected a significant effect of hippocampal subregion on Bcl-2 expression ($F_{1,34} = 4.8$, $p = 0.03$; Fig. 5). Unpaired-*t*-test detected an increase in Bcl-2 protein levels in the ventral hippocampus of LgA rats ($p = 0.04$) compared with controls. An ANOVA detected a significant access × hippocampal subregion interaction ($F_{2,34} = 6.1$, $p = 0.001$), effect of hippocampal subregion ($F_{1,34} = 24.7$, $p < 0.001$), and effect of methamphetamine ($F_{2,34} = 4.4$, $p = 0.02$; Fig. 5) on

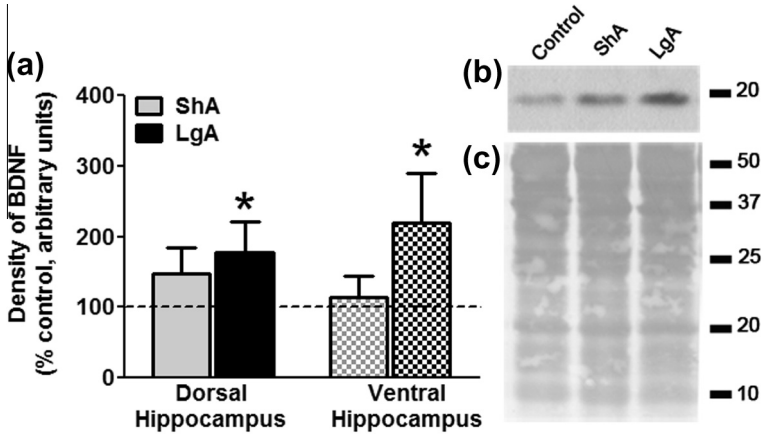

Fig. 2. Methamphetamine self-administration enhances BDNF expression in the dorsal and ventral hippocampus. (a) Quantitative analysis of BDNF expression (mature form) by Western blot analysis of dorsal and ventral hippocampus-enriched protein lysates. (b) Qualitative representation of BDNF immunoreactive bands used for quantitative data analysis from dorsal hippocampus; (c) corresponding Coomassie staining of the membrane is shown as loading control. Molecular weights of proteins are indicated in kDa. $^{*}p < 0.05$ compared to naïve controls. Data are represented as mean ± SEM.

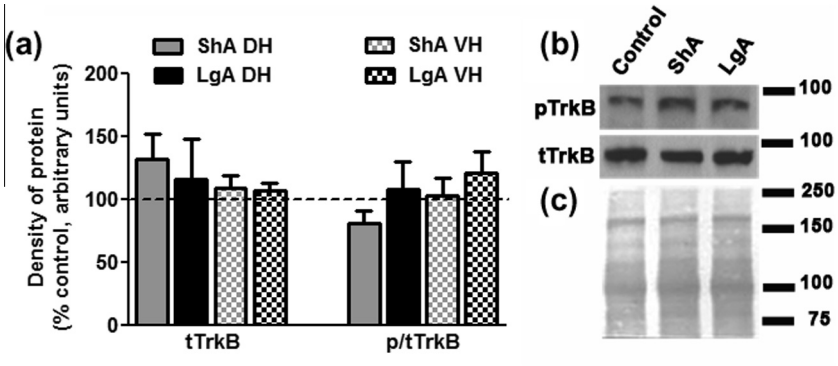

Fig. 3. Methamphetamine self-administration does not alter TrkB expression or pTrkB levels in the hippocampus. (a) Quantitative analysis of TrkB and pTrkB/TrkB expression by Western blot analysis of dorsal and ventral hippocampus-enriched protein lysates. (b) Qualitative representation of phospho (p)TrkB and total (t)TrkB immunoreactive bands used for quantitative data analysis from dorsal hippocampus; (c) corresponding Coomassie staining of the membrane is shown as loading control. Molecular weights of proteins are indicated in kDa.

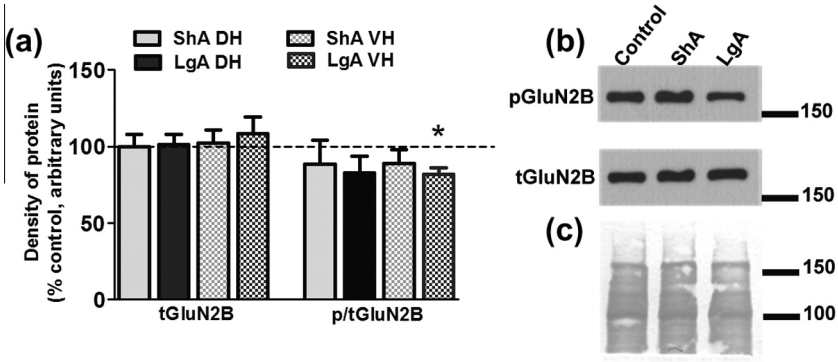

Fig. 4. Methamphetamine self-administration produces hypophosphorylation of GluN2B receptors at Tyr-1472 in the ventral hippocampus. (a) Quantitative analysis of total (t)GluN2B and phospho (p)GluN2B/tGluN2B expression by Western blot analysis of dorsal and ventral hippocampus-enriched protein lysates. (b) Qualitative representation of pGluN2B and tGluN2B immunoreactive bands used for quantitative data analysis. (c) Corresponding Coomassie staining of the membrane is shown as loading control. Molecular weights of proteins are indicated in kDa. $^{*}p < 0.05$ compared to naïve controls. Data are represented as mean ± SEM.

Bax expression. Post hoc analysis revealed a significant decrease in Bax protein levels in the ventral hippocampus of LgA animals ($p < 0.0001$) and ShA animals ($p = 0.01$) compared with controls.

Extended access methamphetamine self-administration reduces the expression of Akt in the ventral hippocampus

To determine whether methamphetamine self-administration altered total Akt expression and pAkt expression in the ventral hippocampus, protein levels of Akt (total and pAkt at Ser-473) were analyzed in the ventral hippocampus-enriched regions in drug naïve control, LgA and ShA animals. Data were subjected to one-way ANOVAs to examine the effects of treatment (control vs. methamphetamine groups) on total and pAkt/tAkt expression. An ANOVA showed a significant main effect of methamphetamine access on tAkt ($F_{2,21} = 4.29$, $p = 0.02$). Dunnett's multiple comparison test demonstrated a significant reduction in tAkt in the ventral hippocampus in LgA animals compared with drug naïve controls ($p < 0.05$; Fig. 6). An ANOVA did not detect a significant main effect of methamphetamine on pAkt or p/tAkt expression.

DISCUSSION

Functional dissociation exists along the dorsal–ventral gradient in the rat hippocampus. For example, the ventral hippocampus when compared with the dorsal hippocampus has greater output connections with the prefrontal cortex and bed nucleus of the stria terminalis, and also has extensive reciprocal connections with the amygdala (Henke, 1990; Pitkanen et al., 2000; Ishikawa and Nakamura, 2006), suggesting that neuroadaptations in the ventral hippocampus may be strongly associated with negative affective symptoms that promote drug dependence (Koob, 2003). Indeed, partial lesion and regional inactivation studies have proposed that the dorsal hippocampus is vital for spatial learning, whereas the ventral hippocampus is involved in regulating fear and anxiety (Moser et al., 1995; Kjelstrup et al., 2002; Bannerman et al., 2004; Pothuizen et al., 2004; Pentkowski et al., 2006). In the context of drug-seeking behaviors, the dorsal hippocampus is particularly critical for acquisition of drug memories and reconsolidation of reactivated drug-related associative memories (Meyers et al., 2006; Wells et al., 2011), suggesting that the act of drug taking and seeking produces neuroadaptive changes in the dorsal hippocampus that allow for

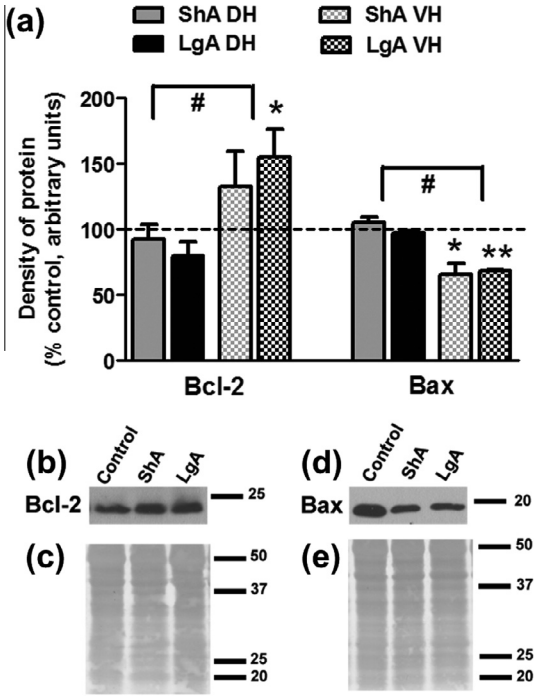

Fig. 5. Methamphetamine self-administration regulates cell death factor expression in the ventral hippocampus. (a) Quantitative analysis of Bcl-2 and Bax expression by Western blot analysis of dorsal and ventral hippocampus-enriched protein lysates. (b and c) Qualitative representation of Bcl-2 immunoreactive bands used for quantitative data analysis (b); corresponding Coomassie staining of the membrane used as loading control (c). (d and e) Qualitative representation of Bax immunoreactive bands used for quantitative data analysis (d); corresponding Coomassie staining of the membrane used as loading control (e). Molecular weights of proteins are indicated in kDa. $^{*}p < 0.05$ compared to naïve controls; $^{**}p < 0.01$ compared to naïve controls; $^{\#}p < 0.01$ main effect of hippocampal subregion. Data are represented as mean ± SEM.

long-term memory storage of drug-context memories, and allow the recall of associative memories during relapse. The ventral hippocampus, however, is important for reinstatement of drug-seeking behavior triggered by drug contexts, drug cues, or the drug itself (Rogers and See, 2007; Lasseter et al., 2010; Deschaux et al., 2012), suggesting that neuroadaptive changes in the ventral hippocampus contribute to the motivational state that drives drug-seeking behavior during abstinence. Given the distinct, yet supporting roles of the dorsal and ventral hippocampal regions in addiction, we examined the specific proteins implicated in hippocampal neuroplasticity in dorsal and ventral hippocampus-enriched tissue lysates.

Synaptic plasticity, and consequently memory formation, requires new protein synthesis (Kandel, 2001; Bruel-Jungerman et al., 2007; Costa-Mattioli et al., 2009). Endogenous pro-BDNF is the precursor for mature BDNF and undergoes intracellular processing to be rapidly converted to the mature form, indicating that pro-BDNF is a transient biosynthetic intermediate (Matsumoto et al., 2008). BDNF, in its mature form is a prominent neurotrophin, mediating many enduring changes in neuroplasticity and synaptogenesis; it has been shown to play a key role as mediator of activity-induced LTP in the hippocampus (Bramham and Messaoudi, 2005; Lu et al., 2008; Minichiello, 2009; Park and Poo, 2013; Leal et al., 2014). Upon high-frequency stimulation, endogenous hippocampal BDNF (is generated and) is secreted from axon terminals (presynaptic site) and dendrites (postsynaptic site) of glutamatergic principal neurons (hippocampal granule cells and pyramidal cells; (Lu, 2003)), in a calcium-dependent and GluN2B-dependent manner (Hartmann et al., 2001; Balkowiec and Katz, 2002; Gartner and Staiger, 2002; Aicardi et al., 2004). Emerging evidence demonstrates that elevated endogenous BDNF is also required for the maintenance of high frequency stimulation-associated late-phase hippocampal LTP (Lu et al., 2008), suggesting that BDNF levels are low under normal conditions and are elevated by stimuli that enhance hippocampal activity. In this context, chronic methamphetamine exposure enhances baseline hippocampal CA1 activity, increases excitability of dentate gyrus neurons, and increases paired pulse facilitation, and these alterations result in occlusion of hippocampal LTP development (Criado et al., 2000; Onaivi et al., 2002; Ishikawa et al., 2005; Hori et al., 2010; Swant et al., 2010; North et al., 2013). Therefore, it can be hypothesized that methamphetamine induces the same neurobiological changes in the hippocampus that are required for generation and maintenance of hippocampal synaptic activity and additional activation as a consequence of tetanic stimulations in

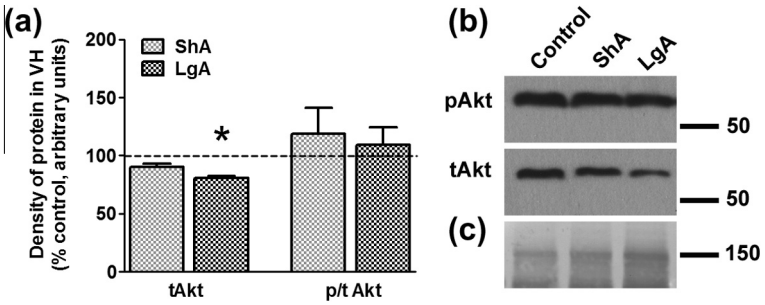

Fig. 6. Methamphetamine self-administration reduces Akt levels in the ventral hippocampus. (a) Quantitative analysis of total (t)Akt and phospho (p)Akt/tAkt expression by Western blot analysis of ventral hippocampus-enriched protein lysates. (b) Qualitative representation of pAkt and tAkt immunoreactive bands used for quantitative data analysis. (c) Corresponding Coomassie staining of the membrane is shown as loading control. Molecular weights of proteins are indicated in kDa. $^{*}p < 0.05$ compared to naïve controls. Data are represented as mean ± SEM.

the hippocampus of methamphetamine experienced animals may not produce LTP (Hope, 1998; Schulz et al., 1999; Onaivi et al., 2002).

In the context of the above hypothesis, our findings demonstrate that extended access methamphetamine self-administration enhances BDNF expression in the dorsal and ventral hippocampus that may result in a modification of the characteristics of synaptic transmission in the dorsal and ventral hippocampus (Scharfman et al., 2005). This finding is consistent with previous studies in amphetamine-experienced animals, in which drug exposure (experimenter-delivered) significantly enhanced BDNF protein levels in the hippocampus (Skelton et al., 2007; Grace et al., 2008), but significantly extend the findings with methamphetamine self-administration, a paradigm that produces much lower levels of drug intake compared with high-dose experimenter-delivered paradigms. Upon release into the synaptic cleft, BDNF can bind to its receptor TrkB, localized at both pre- and postsynaptic sites of glutamatergic synapses in the hippocampus, and BDNF binding to TrkB induces receptor dimerization and phosphorylation at the autophosphorylation site Tyr-706 (Drake et al., 1999; Chao, 2003). Further analysis revealed that enhanced BDNF expression in the hippocampus in extended access animals did not concurrently alter expression of total TrkB or pTrkB-706. While the lack of effect of methamphetamine on total TrkB is consistent with previous studies using amphetamine treatments (Meredith et al., 2002; McGinty et al., 2011), a possible explanation for no effect on pTrkB-706 could be that BDNF-mediated TrkB activation occurs in a time-dependent manner following BDNF release. For example, TrkB phosphorylation was not evident 15 min after a single amphetamine injection but was detected 2 h later, suggesting that there is a delicate relationship between BDNF release and TrkB activation (McGinty et al., 2011). Thus, it is possible that TrkB activation occurs at a different time point compared with the one used for the current study and should be further investigated. It is also possible that neuroadaptations have occurred in the hippocampus following methamphetamine self-administration, such that the typical neuronal activity-induced secretion of pro-BDNF and conversion of pro-BDNF to mature BDNF, and TrkB phosphorylation via BDNF release is hampered and therefore hinders hippocampal synaptic activity (LTP) that is dependent on TrkB activation (Aloyz et al., 1999; Binder et al., 1999; Pang et al., 2004; Nagappan and Lu, 2005).

BDNF release in the hippocampus also enhances tyrosine phosphorylation of GluN2B subunits at Tyr-1472 of the NMDA receptor via TrkB-mediated phosphorylation, and this constitutes a critical step in mediating BDNF stimulation of synaptic transmission (Lin et al., 1998; Caldeira et al., 2007). Furthermore, the increase in GluN2B tyrosine phosphorylation elicited by BDNF is comparable to that seen after hippocampal LTP induction (Rosenblum et al., 1996; Rostas et al., 1996), suggesting that BDNF-stimulated phosphorylation of GluN2B may provide a mechanism for trophin modulation of hippocampal LTP. The present results demonstrate that extended access methamphetamine self-administration reduces phosphorylation of GluN2B at Tyr-1472 in the ventral hippocampus. Functional changes in NMDARs are significantly linked with phosphorylation and dephosphorylation of GluN2B subunits (Ali and Salter, 2001; Nakazawa et al., 2001, 2006), with phosphorylation of GluN2B at Tyr-1472 regulating internalization of NMDARs via clathrin-mediated endocytosis, such that phosphorylation suppresses NMDAR endocytosis and dephosphorylation enhances NMDAR endocytosis (Lavezzari et al., 2003; Prybylowski et al., 2005). Dephosphorylation of GluN2B by methamphetamine may thus prevent neuronal activity-induced potentiation of LTP in the hippocampus. Our observations add to the growing body of evidence indicating that modification of neuronal networks in the hippocampus by methamphetamine may play a role in altering hippocampal plasticity, which may partly underlie dysfunctional hippocampus-dependent behaviors.

Emerging evidence supports the beneficial role of BDNF against excitotoxicity-induced cell death in the hippocampus (Almeida et al., 2005). For example, BDNF prevents nitrous oxide (NO)-induced cell death in hippocampal neurons (Mattson et al., 1995; Kume et al., 1997), and these effects are mediated via BDNF-induced activation of extracellular signal-regulated kinase (ERK) 1/2 and phosphatidylinositol-3-kinase (PI3K)/Akt pathways (Skaper et al., 1998; Hetman et al., 1999; Han and Holtzman, 2000; Almeida et al., 2005). Notably, hypophosphorylation of GluN2B at Tyr-1472 also reduces excitotoxicity-induced cell death in cortical neurons and these effects could be mediated via Akt activation (Chalecka-Franaszek and Chuang, 1999; Hashimoto et al., 2002). Furthermore, Akt activation inhibits neuronal apoptosis by reducing the expression of pro-apoptotic protein Bax and enhancing the expression of anti-apoptotic protein Bcl-2 (Datta et al., 1997; del Peso et al., 1997), suggesting that Akt activation by BDNF is critical for BDNF-induced increases in Bcl-2 and reduction in Bax (Almeida et al., 2005). In this context, methamphetamine has been reported to increase Akt activity in cultured hippocampal neurons and reduce apoptosis in these neurons (Rau et al., 2011), suggesting enhancement of neuronal survival in the hippocampus. Our findings extend this *in vitro* study to demonstrate that limited and extended access methamphetamine self-administration increases Bcl-2 and reduces Bax expression in the ventral hippocampus, suggesting enhancement of survival of hippocampal neurons in the ventral hippocampus. These data also suggest that alterations in cell death proteins by methamphetamine are not predicted by the amount of methamphetamine consumed. Analysis of Akt expression and Akt activity via pAkt expression suggests that methamphetamine self-administration induced increases in Bcl-2 and reduction in Bax expression in the ventral hippocampus did not correlate with increases in Akt expression, whereas, these changes in the extended access animals were associated with reduced Akt expression. One of the most compelling explanations for the differences observed in our study versus the *in vitro* study is that we examined the effects of methamphetamine

under self-administration conditions. It is intriguing to consider the possibility that the effects of methamphetamine on Akt expression observed here are neurotoxic and are not associated with neuroprotection. Indeed, the Akt signaling cascade via phosphorylation at Ser-473 has been identified as being crucial to the induction of protein synthesis-dependent synaptic plasticity required for hippocampus-dependent learning and memory processes (Opazo et al., 2003; Shehadah et al., 2010; Zhang et al., 2010), and a reduction in Akt expression in extended access animals suggests reduction in synaptic plasticity (Swant et al., 2010). Therefore, the ability of methamphetamine to enhance BDNF expression, produce hypophosphorylation of GluN2B, together with increases in Bcl-2 and reductions in Bax and Akt, suggests that methamphetamine-induced activation of signaling in the hippocampus may function to transduce the BDNF-initiated survival signal in the ventral hippocampal neurons to assist with maladaptive hippocampal plasticity that contributes to the cognitive and motivational states that drive methamphetamine addiction. Future mechanistic studies with these candidate proteins will determine their involvement in the behavioral deficits that are associated with extended access methamphetamine self-administration.

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