0026-895X/06/7006-2064–2074$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 70:2064–2074, 2006

Vol. 70, No. 6
23051/3152657
Printed in U.S.A.

Methamphetamine-Induced Sensitization Differentially Alters pCREB and ΔFosB throughout the Limbic Circuit of the Mammalian Brain

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Received January 29, 2006; accepted September 1, 2006

ABSTRACT

Enhancements in behavior that accompany repeated, intermittent administration of abused drugs (sensitization) endure long after drug administration has ceased. Such persistence reflects changes in intracellular signaling cascades and associated gene transcription factors in brain regions that are engaged by abused drugs. This process is not characterized for the most potent psychomotor stimulant, methamphetamine. Using motor behavior as an index of brain state in rats, we verified that five once-daily injections of 2.5 mg/kg methamphetamine induced behavioral sensitization that was demonstrated (expressed) 3 and 14 days later. Using immunoblot procedures, limbic brain regions implicated in behavioral sensitization were assayed for extracellular signal-regulated kinase and its phosphorylated form (pERK/ERK, a signal transduction kinase), cAMP response element binding protein and its phosphorylated form (pCREB/CREB, a constitutively expressed transcriptional regulator), and ΔFosB (a long-lasting transcription factor). pERK, ERK, and CREB levels were not changed for any region assayed. In the ventral tegmental area, pCREB and ΔFosB also were not changed. pCREB (activated CREB) was elevated in the frontal cortex at 3 days withdrawal, but not at 14 days. pCREB levels were *decreased* at 14 days withdrawal in the nucleus accumbens and ventral pallidum. Accumbal and pallidal levels of ΔFosB were *increased* at 3 days withdrawal, and this increase persisted to 14 days in the pallidum. Thus, only the ventral pallidum showed changes in molecular processes that consistently correlated with motor sensitization, revealing that this region may be associated with this enduring behavioral phenotype initiated by methamphetamine. The present findings expand our understanding of the neuroanatomical and molecular substrates that may play a role in the persistence of drug-induced sensitization.

Repeated administration of psychostimulants to humans (Sax and Strakowski, 2001) and rats (Robinson and Berridge, 1993; Stewart and Badiani, 1993) enhances the motor and subjective effects of these drugs (termed sensitization). Depending on the treatment paradigm, this enhancement can persist for years in humans (Sax and Strakowski, 2001) and for months in rats (Paulson et al., 1991). Psychostimulants increase monoamine levels in the limbic circuit of the brain. Regions in this circuit, including the ventral tegmental area (VTA), frontal cortex (FCtx), nucleus accumbens (NAc) and ventral pallidum (VP), are critically involved in drug-induced sensitization in rats (Vezina, 1993; Perugini and Vezina, 1994; Wolf et al., 1995; Johnson and Napier, 2000; Chen et al., 2001). Brain imaging studies of drug-withdrawn human addicts point to adaptations in the limbic circuit in processes that sustain drug abuse behavior (Miller and Goldsmith, 2001). Therefore, persistent changes in the limbic circuit in rats sensitized to psychostimulants is proposed to model the neural adaptations underlying psychostimulant addiction in humans (Robinson and Berridge, 2000).

Changes in expression level and/or function of brain proteins that are involved in signal transduction/gene transcription contribute to neuronal adaptations that accompany sensitization. These include extracellular signal-regulated kinase (ERK), cAMP response element-binding protein (CREB), and ΔFosB. Activated ERK (i.e., phosphorylated or

Research was supported by the Ralph and Marian Falk Medical Research Trust (to J.M. and T.C.N.) and the Irene Whitney Foundation (to T.C.N.) through the Neuroscience and Aging Institute at Loyola University Chicago Medical Center, and a United States Public Health Service grant DA503195 (to T.C.N.).

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Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.106.023051.

ABBREVIATIONS: VTA, ventral tegmental area; FCtx, frontal cortex; NAc, nucleus accumbens; VP, ventral pallidum; ERK, extracellular signal-regulated kinase; CREB, cAMP response element-binding protein; pERK, phosphorylated (activated) form of ERK; Cdk5, cyclin-dependent kinase 5; PKA, protein kinase A; ANOVA, analysis of variance; rmANOVA, repeated-measures analysis of variance; pCREB, phosphorylated (activated) form of CREB; mANOVA, multiple analysis of variance; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid.

pERK) is a well studied regulator of numerous forms of neuronal plasticity, and increases in pERK are associated with sensitization to cocaine or amphetamine (see Licata and Pierce, 2003). CREB activation (i.e., Ser133 phosphorylation) is mediated by a number of kinases, including pERK (Xing et al., 1996). The role of pCREB in sensitization is not completely understood, but it transcribes a host of time-related and region/cell-specific gene expression programs that regulate long-term changes in synaptic efficacy (Carlezon et al., 2005; Olson et al., 2005). ΔFosB is a highly stable isoform of the FosB immediate early gene family of proteins and accumulates in the nucleus accumbens with repeated treatments with cocaine and amphetamine, and levels remain elevated weeks after treatment termination (Hope et al., 1994; McClung et al., 2004). ΔFosB is a transcriptional regulator of several genes implicated in the effects of stimulants (McClung et al., 2004) and ΔFosB-mediated changes in brain gene expression may underpin the capacity of repeated psychostimulant exposure to cause the profound and persistent enhancements of behavior characteristic of sensitization.

The current study focused on molecular events that accompany stimulant-induced changes in brain and behavior. New insights into the phenomenon are provided on several fronts. First, we describe sensitization induced by methamphetamine. Thus far, the focus has been on cocaine and amphetamine. This is a significant paucity, for methamphetamine is the most addicting of the stimulants, distinguishing itself in its monoamine releasing properties (Rothman and Baumann, 2003; Steketee, 2003), behavioral features in rodents (Steketee, 2003), neurotoxicity (White, 2002), and capacity to induce mood disorders in humans (Copeland and Sorensen, 2001). Second, we assayed markers for signal transduction/gene expression in a rat model of the persistent effects of repeated drug use in humans. Thus, we could behaviorally validate the idea that the methamphetamine treatment paradigm employed induced brain sensitization that persisted to the time periods selected for harvesting the brain tissue. Third, to better emulate the drug-abstinent addict, we assayed brain regions taken from drug-free rats after sensitization to methamphetamine was established. Fourth, we evaluated the VP. Numerous reports demonstrate cocaine- or amphetamine-induced changes in signaling proteins in the frontal cortex (FCtx), ventral tegmental area (VTA), or nucleus accumbens (NAc). Each of these regions projects to the ventral palladum (VP), and it is well known that the VP is an output regulator for limbic function (see Napier, 1993). We recently reported that sensitization to methamphetamine alters neuronal spiking in the VP (McDaid et al., 2006) and that sensitization to opiates alters VP expression of CREB and ΔFosB (McDaid et al., 2005). The current study ascertained whether methamphetamine altered these signaling proteins in the VP. Fifth, although ERK, CREB, and ΔFosB may represent components in a common signaling cascade, additional regulators are also involved (e.g., Ca^{2+}, CdK5, PKA; see White and Kalivas, 1998), and the persistence of any drug-induced change in activation/expression differs among these proteins (White and Kalivas, 1998; Licata and Pierce, 2003; McClung et al., 2004; Carlezon et al., 2005). Because various behaviors seen at particular times after the drug experience are associated with particular brain regions, these processes are likely to differ among various brain regions. Taking these considerations into account, we tested the hypothesis that the in vivo regulation of ERK, CREB and ΔFosB in methamphetamine-sensitized rats differs at different times after sensitization has developed and among FCtx, VTA, NAc, and VP.

Materials and Methods

Animals. Male Sprague-Dawley rats, weighing 280 to 340 g (Harlan Laboratories Inc., Indianapolis, IN) were housed in pairs in an environmentally controlled vivarium (12 h light/dark cycle; temperature maintained at 23–25°C) with continuous access to standard laboratory rat chow and water. The rats were allowed to acclimate to these vivarium conditions for at least 1 week before behavioral testing. The room in which the rats were housed was also where behavioral evaluations were conducted. All protocols involving animals were approved by the Loyola University Medical Center Institutional Animal Care and Use Committee, in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health.

Treatment Protocols and Behavioral Assessments.

General Description and Rationale for Treatment Protocols. Methamphetamine (Sigma Chemical Co. St. Louis, MO) was administered subcutaneously (s.c.) as 2.5 mg of base/1 ml of 0.9% saline solution vehicle/kilogram of body weight once daily for 5 days. (Some rats also received an acute challenge of 1.0 mg/ml/kg s.c. 3 or 14 days later; see below). This repeated treatment paradigm was selected for the following reasons: 1) Seven- to 10-once daily injections of similarly moderate doses of methamphetamine (1–4 mg/kg) to rats were previously reported to induce motor sensitization that persists for several weeks (Higashi et al., 1989; Hamamura et al., 1991; Ohmori et al., 1995; Ito et al., 1997; Akiyama et al., 1998; Szumlinski et al., 2000). A pilot study demonstrated that sensitization did not develop during five once-daily treatments with 0.3 mg/kg s.c. methamphetamine. [For example, motor activity (total beam breaks; $n = 6$ rats) for the 90 min after the first and fifth methamphetamine treatment were (mean ± S.E.M.) 600 ± 185 and 468 ± 170, respectively. A paired t test was not significant.] 2) In an another pilot study, we determined that glial fibrillary acidic protein-like immunoreactivity was not altered in the striatum of rats killed 3 or 60 days after a 5-day treatment with 2.5 mg/kg methamphetamine (data not shown). Because glial fibrillary acidic protein increases as a consequence of reactive gliosis, this observation suggests that the treatment protocol was not neurotoxic. Thus, we believed that five once-daily injections of 2.5 mg/kg s.c. would induce persistent brain and behavioral sensitization, without overt toxicity.

Rats were either tested behaviorally or killed at 3 or 14 days after terminating the repeated treatment. These withdrawal times were selected based on the following electrophysiological studies in rats sensitized to psychostimulants: 1) neuronal activity in VTA and FCtx is altered at 3 days (but not after 14 days) of withdrawal (Wolf et al., 1993; White et al., 1995; Zhang et al., 1997; Peterson et al., 2000), and 2) in the NAc and VP, changes in spiking rates occur by 1 week of withdrawal and persist for up to 1 month (Henry and White, 1991; White et al., 1995; Brady et al., 2005; McDaid et al., 2006).

Protocols Used for Evaluating Motor Responses to Methamphetamine. Methamphetamine-induced motor effects were used to verify that the treatment protocol employed in the present study lead to sensitization. The protocol consisted of four phases:

Acclimation. For three days, the rats were weighed, placed in the test box for 30 min, administered sterile saline (1 ml/kg s.c.), and left in the test box for 90 min. This aids in acclimating the rats to the testing procedures so that the behavioral response to methamphetamine does not reflect novelty to the test environment. Motor activity was quantified for both the pre- and postinjection periods via five sets of infrared photocell beams set along the longitudinal axis and 3 cm up from the floor (Applied Concepts, Ann Arbor, MI). The number of photobeam breaks was tallied by a computer in 5 min bins, and

two parameters were monitored: "Crossings" equaled the number of times the rat traveled from one end of the test box to the other; "Beam breaks" equaled the total number of times the photo cell beams were disrupted, independent of sequence.

Repeated Treatment. For the subsequent 5 days, methamphetamine (2.5 mg/kg s.c.) or saline was injected once daily. On the second, third, and fourth days of the repeated treatment, the injection was in the home cage. On the first and fifth days, the rats were acclimated to the test box for 30 min, given their respective injections, and left in the test box for 90 min thereafter. Motor activity was quantified for both the pre- and postinjection periods.

Withdrawal. Methamphetamine (or saline) was withheld for 3 or 14 days. To assure that procedural novelty did not contribute to motor scores obtained with the longer withdrawal period, after 10 or 11 days withdrawal, the rats were reacclimated daily to the protocol, as was done for the initial acclimation, and we verified that motor scores were similar for the two acclimation sessions (Paired t test, $p > 0.05$).

Short-Term Challenge. Three or 14 days after cessation of repeated treatment (methamphetamine or saline) rats were given 1 mg/kg s.c. methamphetamine and motor function was assessed for 90 min. Moderate doses of stimulants enhance locomotion (resulting in higher photobeam counts). Locomotion is reduced as stereotypic behaviors ensue, which occur with higher stimulant doses or if the brain becomes sensitized. Thus, to aid in the interpretation of the photobeam scores, two trained observers also qualitatively scored the behaviors. Assessments were tallied for a 1-min period every 10 min, starting 10 min after methamphetamine injection. The number of rears/wall climbs and total time spent in rearing behavior were quantified. A rear was counted when the rat raised both front paws from the cage floor, balanced on its hind legs with or without placing forepaws on the cage walls, and then returned the forepaws to the floor. Rearing time was the total time the rats spent in the rearing position. In addition, categorical stereotypy scores were assigned. Scoring for stereotypy was based on the following scale: 1, asleep; 2, inactive, awake but resting quietly; 3, slow active with periodic sniffing, infrequent locomotion and rearing/wall-climbing; 4, intermittent grooming with occasional locomotion and rearing/wall-climbing; 5, investigational (more frequent) sniffing, locomotion, rearing/wall climbing, typically without a repetitive pattern; 6, faster, nonstereotyped sniffing, locomotion with frequent rearing/wall climbing, head-bobbing with sniffing; 7, a repeated pattern of more frequent head-bobbing with sniffing at floor, walls or in air, interrupted by rapid "bouts" of locomotion in a repeating pattern (often rearing/wall-climbing, head-bobbing with sniffing occurred in a corner of the box, then the rat would travel to the next corner and repeat the behavioral pattern); 8, fast, constant stereotypy with prominent sniffing and head-bobbing, the frequency of rearing and wall-climbing decreasing but the time spent in each rear increased; 9, fast perseverative stereotypy, which was limited almost exclusively to continuous head-bobbing and sniffing in one corner of the box.

Treatment Protocols for Rats Used in Protein Assays. Rats were subjected to the same methamphetamine or saline treatments employed for the behavioral assessments, but all of the injections were conducted in the home cage and motor activity was not assessed. This procedural difference did not influence the sensitization process, based on the following: 1) our prior work revealed that the pretreatment acclimation procedure employed is sufficient to remove any contribution of injection procedure context on the measured drug-induced effects (Johnson and Napier, 2000). 2) We conducted a pilot study on the effect of environmental context on pretreatment injection procedures. The study revealed that rats injected in the home cage on days 1 to 5 ($n = 6$) versus rats injected in the home cage on days 2 to 4 and in the test box on days 1 and 5 ($n = 6$) expressed similar sensitized motor responses to a subsequent acute methamphetamine challenge after 3 days of withdrawal; both of these treatment groups were different from saline-treated rats ($n = 9$). For example, an analysis of a peak response time (i.e., 30-min after short-term challenge) for number of crossings using an ANOVA revealed the expected methamphetamine versus saline repeated pretreatment effect [$F(2) = 8.3$, $p = 0.002$] and a post hoc Newman-Keuls demonstrated a significant difference ($p < 0.05$) between saline pretreated rats and both sets of methamphetamine pretreated rats, but not between the two methamphetamine pretreatment groups.

Behavioral Data Summary and Analysis. The time course of the behavioral response to methamphetamine or saline was compared for the first and last repeated treatment day (i.e., *development* of sensitization) using a two-way repeated measures ANOVA (rmANOVA) with a post hoc Newman-Keuls test. Response to the postwithdrawal acute challenge (i.e., *expression* of sensitization) was similarly analyzed with a two-way rmANOVA with post hoc Newman-Keuls test comparing the repeated pretreatment groups. Evaluations were considered significant at $p < 0.05$. Unless stated otherwise, data are presented as mean ± S.E.M.

Immunoblot Protocols for Protein Assays. Three- or 14-days after the last repeated methamphetamine or saline treatments, the rats were killed by decapitation; their brains were removed in less than 45 s and cooled rapidly in ice-cold saline for approximately 30 s. The NAc, FCtx, VP, and VTA were dissected out (see Fig. 4); average dissection times were 2 min for the first section (i.e., NAc) and 5 min for the last section (i.e., the VTA). The tissues were quick-frozen on dry ice, weighed, and then stored at −80°C. Whole-cell homogenates for each region (none were pooled) were prepared by either sonication alone or a combination of Dounce homogenization and sonication in a volume that was 20 μl × milligrams of tissue weight with a homogenization buffer (25 mM HEPES-Tris, pH 7.4 at 25°C) containing 1 mM EGTA, 1 mM EDTA, 100 nM okadaic acid, 1 mM sodium orthovanadate, 100 μM phenylmethylsulfonyl fluoride, and 10 μg/ml of aprotinin, leupeptin, and pepstatin). Tissue homogenate protein concentration was determined (protein dye reagent; Bio-Rad Laboratories, Hercules CA) (Bradford, 1976); 20-μg protein samples (premixed with SDS sample buffer) were loaded into individual lanes of a 4 to 12% Bis-Tris gel (Invitrogen, Carlsbad, CA) or 10% SDS gel (Protean III system; Bio-Rad), and electrophoresed at 165 V for approximately 1 h. Samples from each brain region were run on separate gels. NAc and VP tissues harvested from morphine-pretreated rats were also assayed, and these results were published elsewhere (McDaid et al., 2005) using the same saline controls as in the present study. FCtx and VTA samples (from only methamphetamine- or saline-pretreated) were also run on separate gels. Two lanes of each gel were spared for loading of molecular weight marker proteins (SeeBlue and MagicMark; Invitrogen). Proteins were electrophoretically transferred onto a polyvinylidene difluoride or nitrocellulose membrane at 24 V for 1 h. Nonspecific protein binding sites on the membrane were blocked by incubation at room temperature for 1 h in blocking buffer (Tris-buffered saline: 25 mM Tris-HCl, pH 7.4, and 140 mM NaCl) containing 0.1% Tween 20 and 5% nonfat dry milk. Membranes were incubated overnight at 4°C in fresh blocking solution containing the desired primary antibody: 1:2000 rabbit polyclonal or mouse monoclonal anti-phospho (Ser133) CREB (pCREB), 1:2000 rabbit anti-CREB (all from Cell Signaling Technology, Danvers, MA); 1:2000 rabbit anti-FosB (raised against the N-terminal region of FosB), 1:30,000 rabbit anti-actin (both from Santa Cruz Biotechnology, Santa Cruz, CA); 1:2000 mouse anti-phospho (Thr202/Tyr204) p44/42 MAPK (pERK1/2) and 1:2000 rabbit anti-p44/42 MAPK (ERK1/2) (both from Cell Signaling Technology). After three 20-min washes with Tris-buffered saline containing 0.1% Tween 20 (TBST), the membranes were incubated in a blocking buffer with alkaline-phosphatase (goat anti-rabbit; Promega, Madison, WI) or horseradish peroxidase-conjugated (goat anti-rabbit or rabbit anti-mouse; Jackson ImmunoResearch Laboratories, West Grove, PA) secondary antibody (1:20,000) for 1 h at room temperature. After subsequent washes, membranes were treated with a chemiluminescent substrate (ImmunStar; Bio-Rad). To visualize the immunoreactive bands, the chemiluminescent membranes were ex-

posed to light-sensitive film (Kodak BioMax light; Eastman Kodak Co., Rochester, NY). pCREB blots for NAc and VP tissue obtained using a rabbit polyclonal antibody were stripped using blot stripping buffer (2% SDS and 62.5 mM Tris, pH 6.8, with 100 mM β-mercaptoethanol) in a shaking water bath for 35 min at 52.8°C. Subsequent CREB primary omit controls (blots were not incubated with CREB primary antibody) obtained after probing and developing for pCREB using a mouse monoclonal antibody did not reveal residual pCREB signal. Thus, stripping was omitted from the protocol, and further pCREB blots were washed thoroughly with TBST before incubation with CREB antibody. A similar control was conducted for ERK blots previously probed for pERK, and although some residual binding was observed, statistical comparisons of pERK/ERK ratios between the two methods for FCtx harvested after 3 days of withdrawal were not significant [Student's $t(9) = 0.26$; $p = 0.8$]. Thus, blots were not stripped between pERK and ERK assays. For loading controls, blots were stripped (as described above) and re-probed for actin.

Western Blotting Data Summary and Analysis. Quantification of maximum pixel density and molecular weights of each band of interest was carried out using UN-SCAN-IT software (Silk Scientific Corporation, Orem, UT). Optical density of bands from saline-pretreated rats was averaged for each gel, and this mean was used as the control value for that gel. The density of each band on the gel was calculated as a percentage of this control, and the values analyzed are the average of two experimental runs. Qualitative assessments of actin indicated similar loading, and this was validated with representative samples in which similar ($p > 0.05$) protein ratios (described below) were obtained with and without normalizing to actin. Therefore, individual pCREB or pERK values were divided by their respective sample CREB or ERK values to obtain pCREB/CREB and pERK/ERK ratio values for each tissue sample and this value was averaged across gels for each treatment group. Values >2 S.D. outside the mean were considered outliers and were not included in the final statistical evaluations. Statistical evaluations were carried out using a two way ANOVA (mANOVA) with post hoc Newman-Keuls test to compare between the two withdrawal times as well as between the two pretreatment groups. Evaluations were considered significant at $p < 0.05$. Data are presented as mean ± S.E.M.

Results

Behavioral Verification of Brain Sensitization. As expected, a single injection of 2.5 mg/kg methamphetamine (i.e., day 1 of the repeated treatment, data not shown) induced a multimodal effect on motor behavior that consisted of an increase in locomotion at times of lower brain concentrations (i.e., 20–30 min after injection test time); this hyperlocomotor effect was replaced by more circumscribed stereotypic behaviors as the drug was absorbed into the brain (i.e., 30–40 min after injection test time). Comparisons for scores between treatment days 1 and 5 (for rats subsequently tested after 14-day withdrawal) illustrate that sensitization was being induced during the 5-day treatment period: For crossings, there was a repeated treatment day effect [$F(1,16) = 8.75$, $p = 0.009$], an effect of test time]$F(11,176) = 23.02$, $p < 0.0001$], and a treatment day-test time interaction] $F(1,11) = 9.46$, $p < 0.0001$]. For total beam breaks, there was a repeated treatment day effect [$F(1,16) = 16.91$, $p = 0.0008$], an effect of test time [$F(11,176) = 8.4$, $p < 0.0001$], and a treatment day-test time interaction [$F(11,176) = 6.56$, $p < 0.0001$].

Particularly relevant to the present study is the demonstration that behavioral sensitization induced by five repeated injections of 2.5 mg/kg methamphetamine *persisted* after the repeated-injection protocol ended. This was demonstrated by assessing the *expression* of enhanced motor responding to an acute challenge of methamphetamine (1.0 mg/kg s.c.) after 3 or 14 days of drug abstinence. For crossings, there was an effect of test time [$F(11,231) = 10.2$, $p < 0.0001$] and a pretreatment-test time interaction [$F(11,231) = 4.2$, $p < 0.0001$]. For total beam breaks, there was an effect of repeated pretreatment [$F(2,21) = 12$, $p = 0.0003$], test time [$F(11,231) = 25.84$, $p < 0.0001$], and pretreatment-test time interaction [$F(11,231) = 4.16$, $p < 0.0001$]. Post hoc Newman-Keuls test revealed that the response pattern differed between the saline and repeated-methamphetamine treatment histories at several time points after the acute challenge (Fig. 1, A and B). Rats with a saline pretreatment history demonstrated the typical enhanced motor effects of a single low-dose injection to stimulants; it peaked between 30 and 40 min then subsided. Rats with a methamphetamine pretreatment history showed motor enhancements with a more rapid onset; peak effects occurred in the first 10 to 15 min with a profound reduction in locomotion by 30 min. In the 3-day withdrawal rats, responding returned to a hypermotoric state by 60 min, whereas those with the

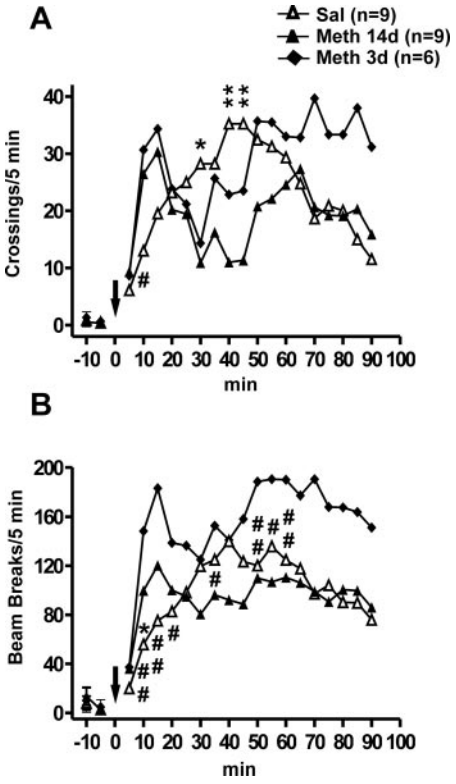

Fig. 1. Motor sensitization induced by repeated methamphetamine persists for up to 14 days. Key illustrates the repeated treatment; Sal, saline; Meth, methamphetamine. Line graphs show motor scores for test session time (min) before and after Meth acute challenge of 1.0 mg/kg s.c., administered 3 or 14 days after the repeated treatment. Error bars were omitted to enhance clarity. Arrows indicate when the rats were removed from the test box to receive the acute challenge injection. With a two-way rmANOVA, for crossings (A), there was no repeated pretreatment effect ($p = 0.13$), but significance was obtained for test time ($p < 0.0001$) and a pretreatment-test time interaction ($p < 0.0001$). For total beam breaks (B), there was an effect of repeated pretreatment ($p = 0.0003$), test time ($p < 0.0001$) and pretreatment test time interaction ($p < 0.0001$). Indicated are results of post hoc Newman-Keuls evaluations at each measured time point: Sal versus 3 day withdrawal from Meth (Meth 3d), #, $p < 0.05$; ##, $p < 0.01$. Sal versus 14 day withdrawal from Meth (Meth 14d), *, $p < 0.05$; **, $p < 0.01$.

14-day withdrawal tended to normalize during the later test time periods. Hypermotor behaviors are a hallmark of stimulant-induced response as the drug moves into and then out of the sensitized brain, and it was hypothesized that the locomotor decreases between these hypermotoric states reflect the rat engaging in stereotypic behaviors that preclude locomotion. To test this hypothesis, two trained observers scored the rats' stereotypic behaviors during the same time that beam breaks were being counted for rats subjected to the 14-day withdrawal period (Fig. 2C). Because rearing/wall climbing is known to accompany stimulant-induced hypermotoric states, these behaviors also were assessed (Fig. 2, A and B). A rmANOVA revealed an effect of test time [$F(8,128) = 2.20$, $p = 0.03$] and a repeated pretreatment-test time interaction [$F(8,128) = 9.36$, $p < 0.0001$]. For time spent in rearing, there was a test time [$F(8,128) = 4.26$, $p = 0.0001$] and a pretreatment time interaction [$F(8,128) = 5.98$, $p < 0.0001$]. Similar to the time frame of behavioral suppression assessed by the photo beam breaks, post hoc Newman-Keuls tests revealed a decrease in observationally determined rearing behavior 30 to 40 min after acute challenge (Fig. 2, A and B). For stereotypy scores, there was an effect of pretreatment [$F(1,16) = 153.87$, $p < 0.0001$], test time [$F(8,128) = 44.30$, $p < 0.0001$], and a pretreatment-test time interaction [$F(8,128) = 2.50$, $p = 0.01$]. Stereotypic behaviors were greater in the methamphetamine-pretreated rats for each 10-min assessment of the period after acute challenge test; a slight peak occurred at 30 to 40 min (Fig. 2C) when the rats were heavily engaged in head-bobbing and sniffing at the side or corner of the box.

The enhancement in motor responding to an acute challenge validated the idea that the state of the animals' brains (i.e., the capacity to respond to an acute stimulus) was altered by the repeated methamphetamine treatment paradigm, and that this alteration persisted for at least 14 days. It is noteworthy that, as illustrated in Fig. 3, each of the rats subjected to this repeated methamphetamine regimen developed motor sensitization, demonstrating that this is a reliable and consistent treatment paradigm to evaluate biochemical markers of this phenomenon in the brain.

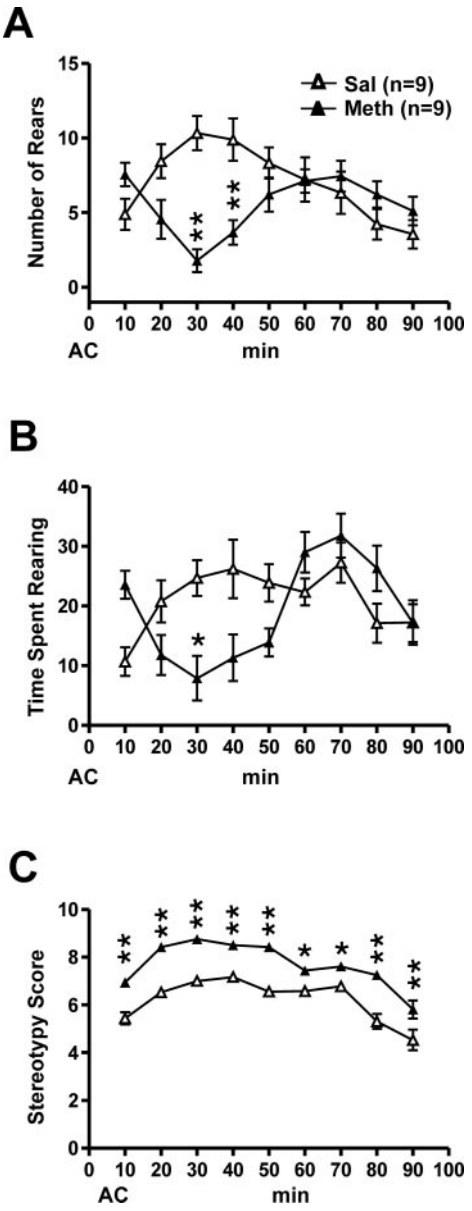

Fig. 2. Observer assessments of motor responding to a methamphetamine challenge given 14 days after repeated treatments with either methamphetamine (Meth) or saline (Sal−). An acute challenge of 1.0 mg/kg s.c. Meth was administered at 0 min and the number of rears (A), and time spent in rearing (B) were quantified. In addition, a categorical stereotypy score was assigned (C) for 1 min every 10 min thereafter for the 90-min test session. For number of rears, a rmANOVA revealed an effect of test time ($p = 0.03$) and a repeated pretreatment test time interaction ($p < 0.0001$). Likewise, for time spent in rearing there was a test time ($p = 0.0001$) and a pretreatment test time interaction ($p < 0.0001$). For stereotypy scores, there was an effect of pretreatment ($p < 0.0001$), test time ($p < 0.0001$), and a pretreatment time interaction ($p = 0.01$). S.E.M. for stereotypy scores is smaller than the symbol size used and thus could not be illustrated. Asterisks indicate results of post hoc Newman-Keuls evaluations at each measured time point; *, $p < 0.05$; **, $p < 0.01$. Taken together, the graphs show that the response profiles during peak brain concentrations of s.c. administered Meth (i.e., 20–50 min after injection; see Segal and Kuczenski, 1997) demonstrate motor sensitization characterized by intense, perseverative, stereotypic behaviors that interfered with the ability of the rats to engage in rearing behaviors.

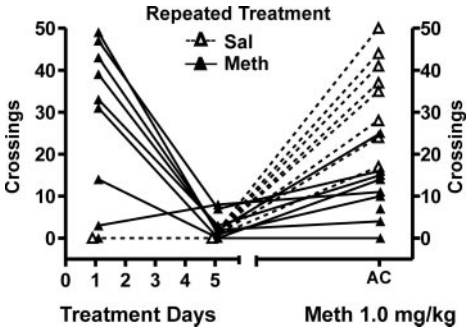

Fig. 3. With the treatment protocol used, each methamphetamine-treated rat developed a sensitized motor response to the psychostimulant. Key illustrates the repeated treatment. Sal, saline; Meth, methamphetamine. Shown are individual crossing scores for the same rats whose responses were averaged for Figs. 1 and 2. Scores illustrate total number of crossings for a 5-min period 25 min after injection on treatment days 1 and 5 (left side of graph), and 45 min after a Meth acute challenge (AC) given 14 days later (right side of graph). The 25- and 45-min post injection time periods represent the peak response time for the first 2.5 mg/kg s.c. Meth (data not shown) and for 1.0 mg/kg s.c. Meth AC in rats repeatedly pretreated with Sal (see Fig. 1), respectively. The decrease in crossings after the fifth daily 2.5 mg/kg Meth injection was due to a substantial increase in stereotypic behaviors (data not shown). Crossings were not exhibited in any of the Sal-treated rats on days 1 or 5, but after the 1 mg/kg Meth AC, each of these rats demonstrated robust crossing behaviors.

Immunoblotting. Brain tissues (refer to Fig. 4) were obtained from rats at 3 or 14 days after cessation of repeated methamphetamine (2.5 mg/kg s.c.) or saline treatments. To be able to determine whether the methamphetamine history altered the basal state of the brain, there was no acute challenge. Figure 5 illustrates the banding patterns obtained by the selected antibodies. Bands for ERK1 and pERK1 were identified at 44 kDa, and at 42 kDa for ERK2 and pERK2. For pCREB, the band quantified was approximately 43 kDa, another band at a lower molecular mass, which may be that of ATF-1 (according to the antibody manufacturer) was not quantified. The band quantified for CREB also was 43kDa. For blots obtained using the FosB antibody, one distinct band was observed between 35 and 40 kDa, which corresponds to the 37-kDa molecular mass reported for ΔFosB (McClung et al., 2004). This contrasts the report of two bands in this mass range reported by Muller and Unterwald (2005) with the same FosB antibody used in this study.

There was no effect of repeated methamphetamine on levels of pERK2, ERK2, or the pERK2/ERK2 ratio in any of the brain regions assayed at either 3 or 14 days of withdrawal. Levels of pERK1/ERK1 were not quantified because this isoform of ERK covaries with pERK2/ERK2 (Lu et al., 2005).

CREB levels also remained stable for all brain regions assayed (Figs. 6–8), and pCREB was not altered in the VTA. In contrast, pCREB levels were dynamic in the forebrain, showing regionally unique changes related to withdrawal time. For FCtx pCREB, mANOVA revealed an effect of repeated treatment [$F(1,10) = 7.25$, $p = 0.02$], withdrawal time [$F(1,10) = 7.87$, $p = 0.02$], and a treatment-withdrawal time interaction [$F(1,10) = 7.69$, $p = 0.02$]. Likewise, the pCREB/CREB ratio showed repeated treatment [$F(1,10) = 6.06$, $p = 0.03$], withdrawal time [$F(1,10) = 15.1$, $p = 0.003$], and treatment-withdrawal time interaction [$F(1,10) = 13.26$, $p = 0.005$]. This trend reflected robust changes at the short withdrawal time; a post hoc Newman-Keuls test was significant between treatment groups for both pCREB and pCREB/CREB at the 3-day withdrawal time, but not at 14 days (Fig. 6). In contrast to pCREB elevations seen in the FCtx, withdrawal from repeated methamphetamine *decreased* pCREB in subcortical forebrain regions (Figs. 7 and 8). mANOVA evaluations of the NAc revealed pretreatment effects for pCREB [$F(1,19) = 11.09$, $p = 0.004$] and the pCREB/CREB [$F(1,19) = 13.25$, $p = 0.002$]. Likewise, the VP also reflected an effect of repeated treatment on pCREB levels [$F(1,19) = 15.2$, $p = 0.001$] whereas the pCREB/CREB ratio showed an effect of time [$F(1,21) = 8.53$, $p = 0.008$] and a treatment time interaction]$F(1,22) = 8.37$, $p = 0.009$]. The decrease trend occurred at both withdrawal times for these regions; thus, there was no significant difference for withdrawal time (mANOVA, $p > 0.05$). A post hoc Newman-Keuls test revealed that the decrease in levels of pCREB and pCREB/CREB reached statistical significance for the NAc and VP in methamphetamine-treated rats only at 14 days of withdrawal (Figs. 7 and 8). Given that levels of CREB were not altered by the methamphetamine treatment, the FCtx, Nac, and VP changes in pCREB levels and pCREB/CREB are not due to changes in expression levels of CREB but probably

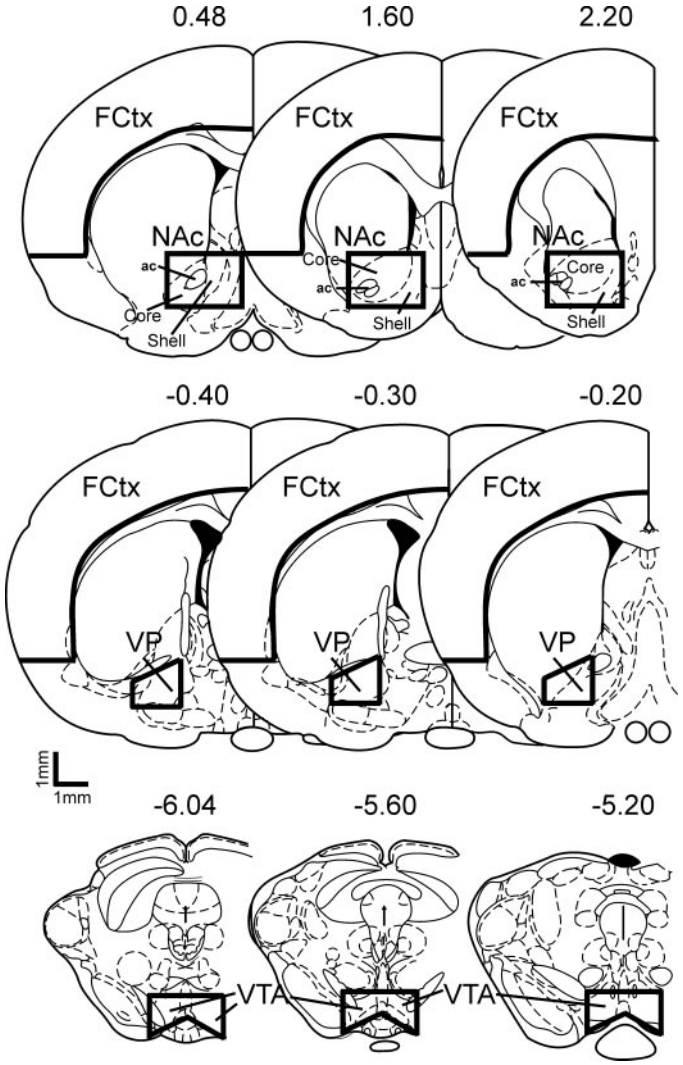

Fig. 4. Anatomical illustration of the brain regions used for immunoblotting. Sections were redrawn from Paxinos and Watson (1998) and the numbers above each section illustrate the mm distance from Bregma. FCtx, frontal cortex; NAc, nucleus accumbens (including the core and shell subregions); VP, ventral pallidum; VTA, ventral tegmental area.

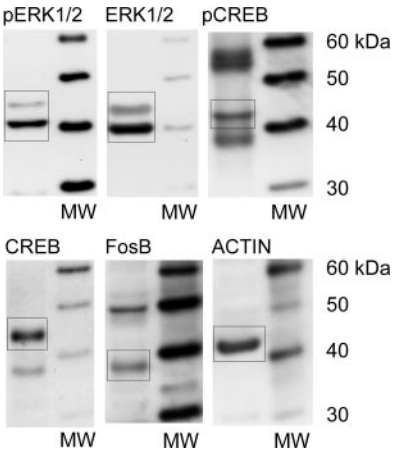

Fig. 5. Representative immunoblots. Illustrated are two lanes of each blot containing samples of rat nucleus accumbens with the bands of interest enclosed in boxes. A molecular mass (MW) marker in the adjacent lane of each blot represents molecular masses from 30 to 60 kDa (kDa). For pERK, the upper and lower bands represent pERK1 and pERK2, respectively, and for ERK, the upper and lower bands represent ERK1 and ERK2, respectively. For the FosB blot, the band corresponding to a molecular mass of 37 to 38 kDa is ΔFosB; this was the only band visible within this molecular mass range.

reflect changes in the activity of kinases and/or phosphatases that target CREB. However, the kinase responsible for this effect at the withdrawal time assayed remains unclear; at least one candidate, pERK2, was not altered by the stimulant.

The repeated methamphetamine treatment regimen increased ΔFosB levels in the VP and NAc (Fig. 9), but levels in the FCtx or VTA were unchanged. For the NAc, a mANOVA revealed a repeated treatment effect [$F(1,19) = 8.53$, $p = 0.009$] and a post hoc Newman-Keuls was significant at 3 days of withdrawal from repeated methamphetamine ($p < 0.05$). For the VP, there was a repeated treatment effect [$F(1,18) = 19.45$, $p = 0.0003$], and post hoc Newman-Keuls test showed that the methamphetamine-induced increase in levels of ΔFosB at both 3 and 14 days withdrawal was significant. Thus, like pCREB, the role of ΔFosB in the persistence of methamphetamine-induced motor sensitization probably differs among brain regions, and post-treatment withdrawal time. To indicate that the observed changes in molecular markers did not simply reflect the general effects of treating rats with methamphetamine and testing for motor function, we assayed pCREB and CREB for VP and NAc and ΔFosB for VP that was harvested 14 days after treating a separate group of rats once daily with a lower methamphetamine dose (1.0 mg/kg or saline) for only 3 days. During the repeated treatment, motor activity of these rats was assessed in three-dimensional space by automated activity systems (AccuScan Instruments, Inc., Columbus, OH). Analysis of horizontal activity (Beam Breaks) revealed a significant effect of test time [$F(17,306) = 12.05$, $p < 0.0001$], but no repeated treatment day effect [$F(1,18) = 2.15$, $p = 0.16$] or treatment day-test time interaction [$F(17,306) = 1.5$, $p = 0.09$], suggesting that motor sensitization did not develop. Paralleling the lack of motor sensitization, pCREB/CREB

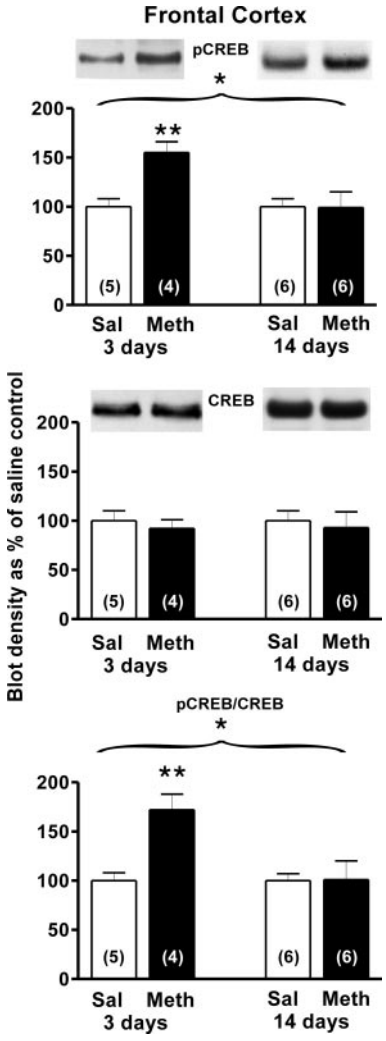

Fig. 6. pCREB and the ratio of pCREB to CREB is increased in the frontal cortex of methamphetamine-sensitized rats after 3 days of withdrawal. Immunoblots above graphs illustrate pCREB or CREB bands of cortical tissue from the same treatment group/withdrawal time as each bar. Asterisks above graphs indicate significance using a mANOVA. There was an effect of repeated treatment ($p = 0.02$), withdrawal time ($p = 0.02$), and a treatment withdrawal time interaction ($p = 0.02$). Likewise, pCREB/CREB showed an effect of pretreatment ($p = 0.03$), withdrawal time ($p = 0.003$), and pretreatment withdrawal time interaction ($p = 0.005$). Asterisks above individual bars indicate significant difference between pretreatment groups using a post hoc Newman-Keuls. *, $p < 0.05$; **, $p < 0.01$.

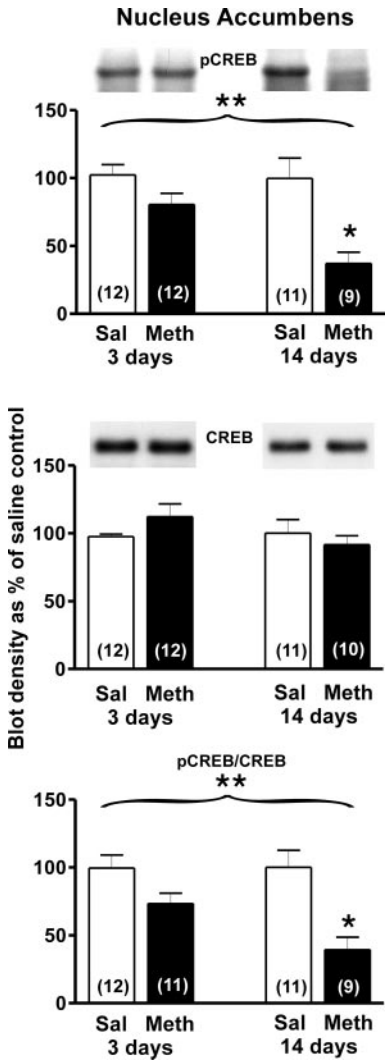

Fig. 7. pCREB and the ratio of pCREB to CREB is decreased in the nucleus accumbens of 14 day-withdrawn methamphetamine-sensitized rats. Immunoblots above graphs illustrate pCREB or CREB bands of accumbal tissue from the same treatment group/withdrawal time as each bar. Asterisks above graphs indicate mANOVA significance; treatment effects occurred for pCREB ($p = 0.004$) and pCREB/CREB ($p = 0.002$). Asterisks above individual bars indicate differences between pretreatment groups; post hoc Newman-Keuls test. *, $p < 0.05$; **, $p < 0.01$.

was not altered for either the VP or the NAc, and ΔFosB levels were not changed in the VP (Student's t test between saline- and methamphetamine-treated rats; $p > 0.05$; n = 8–13), in direct contrast to what was obtained for these regions 14 days after a sensitizing regimen of methamphetamine.

Discussion

The doses of methamphetamine employed in this study produced the expected inverted "U" dose-motor effect curve; the earlier postinjection times of the first exposure increased locomotion (when the drug is first entering the brain). These were replaced with stereotypies as the full dose was absorbed into the brain. Repeated administration shortened the onset and augmented the intensity of the stereotypies, reflecting an up-regulated system. After 3 or 14 days of withdrawal, an acute methamphetamine challenge produced stereotypies of greatest intensity and duration in those rats pretreated with methamphetamine. By demonstrating that enhanced motor effects could be elicited for up to 2 weeks after repeated methamphetamine treatments, this experiment showed that the brain was in a persistently altered state. We have recently revealed that the behavioral sensitization induced by the methamphetamine treatment regimen used here does not reflect permanent damage, in that the behaviors can be reversed with postsensitization treatments with serotonergic and dopaminergic ligands (McDaid et al., 2006). Taken as a whole, these new findings validate the utility of the treatment/withdrawal protocol employed here for subsequent studies of the molecular processes that are associated with the capacity of rats to express enhanced responding to an acute methamphetamine challenge long after the sensitizing regimen is terminated.

Activity state and/or expression level of three proteins that represent different levels of neuronal signal transduction

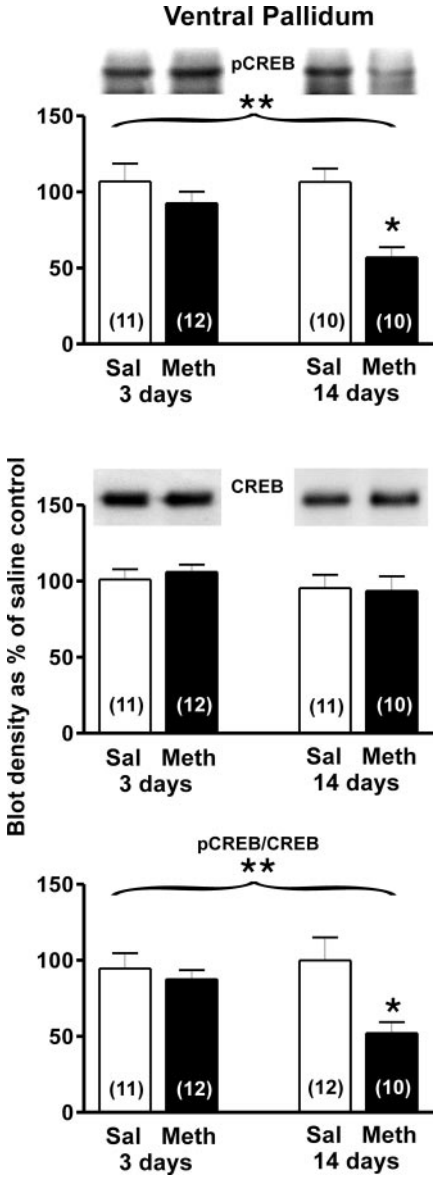

Fig. 8. pCREB and the ratio of pCREB to CREB is decreased in the ventral pallidum of 14 day-withdrawn methamphetamine-sensitized rats. Immunoblots above graphs illustrate pCREB or CREB bands of pallidal tissue from the same treatment group/withdrawal times as each bar. As illustrated by asterisks above the graphs, mANOVA revealed an effect of repeated treatment on pCREB levels (p = 0.001) and for the pCREB/CREB ratio (p = 0.002). Asterisks above individual bars indicate differences between pretreatment groups; post hoc Newman-Keuls test. *, $p < 0.05$; **, $p < 0.01$.

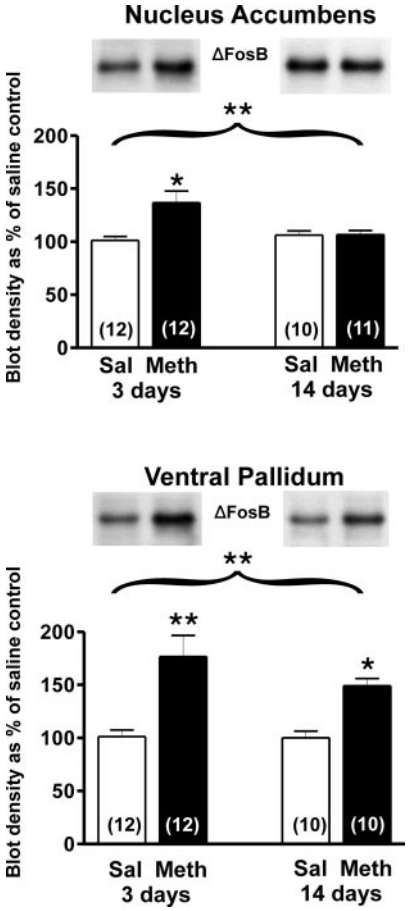

Fig. 9. ΔFosB is increased in the nucleus accumbens and ventral pallidum of 3 day-withdrawn methamphetamine-sensitized rats; this increase persists to 14 days of withdrawal in the ventral pallidum. Representative immunoblots from the different assays are illustrated above each bar. For the accumbens (top), a mANOVA revealed a repeated treatment effect (p = 0.009); for the ventral pallidum (bottom), there was a repeated treatment effect (p = 0.0003), identified by asterisks above graphs. Asterisks above individual bars indicate differences between pretreatment groups; post hoc Newman-Keuls test. *, $p < 0.05$; **, $p < 0.01$.

and gene transcription (i.e., ERK, CREB, and ΔFosB) were assessed in methamphetamine-sensitized rats. The results indicate that these signaling proteins were differentially regulated, demonstrating temporally related and limbic brain region-particular differences in activation or expression. The VTA is critically involved in development of sensitization to cocaine and amphetamine (White and Kalivas, 1998), and this is believed to involve glutamatergic inputs (Wolf, 1998). Several indices of excitatory glutamatergic transmission in the VTA are increased during the first few days after sensitizing treatments of cocaine and amphetamine, but these are transient and seem to be normalized by 5 to 14 days (Zhang et al., 1997; Giorgetti et al., 2001; Borgland et al., 2004). pERK, pCREB, and ΔFosB are implicated in stimulant-induced changes in glutamate transmission in several brain regions (White and Kalivas, 1998). In the VTA, CREB has been shown to regulate expression of the AMPA glutamatergic receptor subtype (Olson et al., 2005). However, we did not detect changes in VTA levels of any signaling proteins measured after either 3 or 14 days of withdrawal from repeated methamphetamine treatments. Our findings with the 14-day withdrawal period support the concept that the VTA is normalized by this post-treatment time. The observations at 3 days withdrawal extend the prior work to indicate that either 1) these signaling proteins do not underlie stimulant-induced changes in VTA glutamatergic function in general, or 2) methamphetamine deviates from the other stimulants in its ability to alter VTA transmission. Studies on VTA glutamate function in methamphetamine-sensitized rats are needed to make this determination. The FCtx is critical for sensitization development, and expression during short-term withdrawal from amphetamine (Wolf et al., 1995; Cador et al., 1999). Likewise, we observed changes in FCtx only at 3 days of withdrawal. The NAc is important in maintenance of motor sensitization to other psychostimulants (Wolf et al., 1993; Cador et al., 1995), and pCREB was decreased in this region at 14 days of withdrawal from methamphetamine. The ΔFosB elevations seen at 3 days, however, were normalized in the NAc by 14 days of withdrawal. In contrast, the VP, a target of the NAc, demonstrated changes in both pCREB and ΔFosB at 14 days of withdrawal. Further understanding of how these limbic regions contribute to expression of methamphetamine-induced sensitization is gained by considering putative roles of each of the molecular markers assayed in this study, and their inter-relationship in neuronal signal transduction/gene expression processes.

In vivo elevations in the ERK cascade caused by psychostimulants seem to best correlate with immediate exposure to a stimulus (Sgambato et al., 1998; Valjent et al., 2000; Lu et al., 2005). In the current study, the rats were killed without exposure to an acute challenge; thus, they lacked an immediate stimulus. The absence of changes in basal pERK/ERK in any of the brain areas assayed is in accord with a lack of changes in basal pERK in striatal regions after repeated methamphetamine treatment (Mizoguchi et al., 2004) and underscores the idea that pERK is involved in the consequences of acute stimuli, and its basal expression is not altered, per se, with long-term events of drug administration. Thus, ERK seems to be a critical determinant of the brain's capacity to process relevant stimuli, and this role is evident (and pERK levels are augmented) when an immediate stimulus is superimposed on a sensitized brain.

Levels of CREB were not altered by the methamphetamine treatment; therefore, changes observed in pCREB/CREB ratio are not reflecting changes in CREB expression levels, per se, but rather changes in activity or levels of kinases and/or phosphatases that target CREB. Because the basal expression of pERK was not changed, another kinase(s) probably provided this regulation in the current study. Cdk5 and protein kinase A (PKA) are probable candidates. For example, Cdk5 phosphorylates cAMP-regulated phosphoprotein (DARPP-32) at Thr75, which inhibits the activity of PKA (Bibb et al., 1999). PKA phosphorylates CREB at Ser133 (Montminy and Bilezikjian, 1987), and in a study where amphetamine is given once daily for 7 days to preweanling rats, striatal PKA activity is reduced after 72 days of withdrawal (tested at postnatal day 90) (Crawford et al., 2000). In addition, Cdk5 is up-regulated after 7 days withdrawal from repeated methamphetamine treatment in adult rats (Chen and Chen, 2005), and its overexpression in mice is accompanied by decreased basal pCREB without a change in pERK levels (Takahashi et al., 2005). Thus, pERK may influence short-term up-regulation of CREB, whereas other kinases, such as Cdk5-PKA cascades, may be more important in regulating phosphorylation of CREB at longer withdrawal times.

At 3 but not 14 days of withdrawal, FCtx levels of pCREB were increased. Activation of glutamate receptors increases CREB phosphorylation (Konradi et al., 1996) and increased levels of subunit 1 of the AMPA glutamatergic receptor channel occur in the medial prefrontal cortex after 3 days but not after 14 days of withdrawal from repeated amphetamine (Lu and Wolf, 1999). Similar events may underlie the increased excitability of cortical neurons seen 3 days after repeated cocaine treatmen, which, like pCREB levels observed in the present study, are normalized by 14 days (White et al., 1995).

pCREB levels were decreased in the NAc and VP at 14 days of withdrawal, a time when motor sensitization could be induced by methamphetamine. This parallels work showing that overexpression of pCREB in mouse NAc decreases cocaine-induced motor sensitization (Sakai et al., 2002) and that increased CREB in rat NAc decreases reward-motivated behaviors (Carlezon, Jr. et al., 1998). However, we observed a discord between pCREB levels and motor sensitization at 3 days of withdrawal; motor sensitization was demonstrated, but there were no changes in pCREB. These time-related differences suggest that either pCREB is not involved in motor sensitization or that a reduction of pCREB may be a long-term adaptation to repeated methamphetamine but is not necessary for behavioral sensitization during short-term withdrawal. It will interesting to ascertain whether these differences in basal levels of pCREB are reflected in responding to an acute challenge of methamphetamine. The pCREB reduction at 14 days of withdrawal in the NAc correlates with a dysregulation of neuronal excitability of neurons after 2 to 4 weeks withdrawal from repeated methamphetamine (Brady et al., 2005). The exact relationship between electrophysiological endpoints and pCREB-related signaling proteins remains to be determined, and additional electrophysiological studies (using, for example, the appropriate kinase inhibitors) would be of use in extrapolating causality from changes in pCREB levels to neuronal physiology.

ΔFosB was enhanced in the NAc and VP. These results concur with a report that mice overexpressing ΔFosB in the

NAc show an enhancement in motor responses to cocaine (Kelz et al., 1999). It is noteworthy that ΔFosB levels were at control values in the NAc by 14 days of withdrawal. This finding suggests that sustained accumbal elevation is not required for an acute methamphetamine challenge to induce sensitized motor responding.

The current comparisons between withdrawal times for several signaling proteins among different brain regions provide insight into the temporal and spatial dynamics of drug-induced sensitization. Prior work has revealed that acute psychostimulant administration increases pERK and pCREB (Choe et al., 2002), and although any accompanying change in ΔFosB is minimal, ΔFosB is resistant to metabolism and is thus relatively more persistent (Nestler, 2004). With repeated injections, pERK and pCREB normalize or potentially "overcompensate" by decreasing levels, whereas ΔFosB accumulates in brain tissue (Nestler, 2004). A similar response profile would lead to the observed results after withdrawal from repeated methamphetamine treatment in which accumbal and pallidal basal levels of pERK were unchanged, pCREB levels were decreased, and ΔFosB levels were elevated. The increased ΔFosB in the VP, but not in the NAc, at 14 days of withdrawal may reflect the greater pallidal increase at 3 days, which was sufficiently large as to remain elevated. Given the putative role for ΔFosB in the persistent effects of abused drugs, the discovery that ΔFosB is uniquely elevated in the VP at a time when motor sensitization can be evoked strongly suggests a role for the VP in the persistent behavioral consequences of repeated methamphetamine use.

In summary, these findings underscore the temporal (withdrawal time) and spatial (various brain regions) complexities of the persistent and/or tardive activation/expression of signaling proteins that underlie a drug-sensitized brain. Although the present findings expand our understanding of the neuroanatomical and molecular substrates that may play a role, this field is in its infancy. Much remains to be studied before a clear picture of the molecular, cellular, and circuit-related consequences to repeated drug exposure can be elucidated. Although daunting, it will be critical to integrate these multifaceted approaches to understand how abused drugs engage genetic processes to compose the behavioral phenotypes that hallmark addiction.

Acknowledgments

We thank Amanda Mickiewicz and Robin Voigt for their excellent technical assistance, Dr. Adriano Marchese for his valuable technical advice on immunoblotting procedures, and Dr. William A. Carlezon, Jr. for his helpful comments on an early version of the manuscript.

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