

REGULAR RESEARCH ARTICLE

Selective Activation of Striatal NGF-TrkA/p75NTR/ MAPK Intracellular Signaling in Rats That Show Suppression of Methamphetamine Intake 30 Days following Drug Abstinence

Oscar V. Torres, Subramaniam Jayanthi, Michael T. McCoy, Jean Lud Cadet

Molecular Neuropsychiatry Research Branch, DHHS/NIH/NIDA Intramural Research Program, Baltimore, Maryland.

O.V.T. and S.J. contributed equally to this work.

Correspondence: Jean Lud Cadet, MD, Chief, Molecular Neuropsychiatry Research Branch, DHHS/NIH/NIDA IRP, 251 Bayview Boulevard, Baltimore, MD 21224 (jcadet@intra.nida.nih.gov).

Abstract

Background: The continuing epidemic of methamphetamine addiction has prompted research aimed at understanding striatal dysfunctions potentially associated with long-term methamphetamine use.

Methods: Here, we investigated transcriptional and translational alterations in the expression of neurotrophic factors in the rat striatum at 30 days following methamphetamine self-administration and footshock punishment. Male Sprague-Dawley rats were trained to self-administer methamphetamine (0.1 mg/kg/injection, i.v.) or saline during twenty-two 9-hour sessions. Subsequently, rats were subjected to incremental footshocks for 13 additional methamphetamine self-administration sessions. This paradigm led to the identification of rats with shock-resistant and shock-sensitive phenotypes. Thirty days following the last footshock session, the dorsal striatum was dissected and processed for gene expression and protein analyses.

Results: PCR arrays revealed significant differences in neurotrophins and their receptors between the 2 phenotypes. Brain-derived neurotrophic factor and nerve growth factor protein levels were increased in the dorsal striatum of both shock-resistant and shock-sensitive rats. However, neurotrophic receptor tyrosine kinase 1 phosphorylation and nerve growth factor receptor protein expression were increased only in the shock-sensitive phenotype. Moreover, shock-sensitive rats showed increased abundance of several phosphorylated proteins known to participate in Ras/Raf/MEK/ERK signaling cascade including cRaf, ERK1/2, MSK1, and CREB.

Conclusions: These findings support the notion that animals with distinct phenotypes for methamphetamine intake in the presence of adverse consequences also display differential changes in an intracellular signaling cascade activated by nerve growth factor-TrkA/p75NTR interactions. Thus, the development of pharmacological agents that can activate nerve growth factor-dependent pathways may be a promising therapeutic approach to combat methamphetamine addiction.

Keywords: addiction, footshocks, neurotrophins, signal transduction

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Significance Statement

Methamphetamine addiction is characterized by continued drug use despite adverse life events associated with its use. To mimic the human conditions, we used a self-administration model of methamphetamine (METH) intake performed in conjunction with footshocks as adverse consequences. This paradigm helped to discriminate shock-sensitive (SS) from shock-resistant (SR) rats, with SR rats being considered an addicted phenotype. Our main findings indicated that there were specific differences in the striatal expression of genes coding for neurotrophic factors between the 2 METH self-administration (SA) phenotypes. Importantly, nerve growth factor (NGF) and its downstream signaling pathway (NGF-TrkA and p75NTR/MAPK signaling) were found to be selectively increased in the SS rats. These observations suggest that METH abstinence may be associated with an upregulated NGF-signaling cascade. Our data may have important therapeutic implications in the treatment of patients at different stages of METH addiction.

Introduction

Addiction to methamphetamine (METH) is considered a chronic neuropsychiatric disorder characterized by striatal dysfunctions, cognitive deficits, and loss of control over drug consumption despite adverse consequences associated with its use (Scott et al., 2007; Rusyniak, 2013). METH addiction is also associated with a high prevalence of relapse to drug-taking behaviors even after years of sobriety (Brecht and Herbeck, 2014; Gowin et al., 2015). These clinical observations have implicated long-lasting neuroplastic changes in brain regions involved with both reward mechanisms and compulsive diatheses (Belin et al., 2013). In fact, several groups of investigators have reported significant changes in gene expression within mesolimbic reward circuitry following noncontingent injections of METH in rodents [for a review see (Cadet and Krasnova, 2009)]. Using the self-administration (SA) model of METH intake in rats, we and others have also documented substantial transcriptional and translational changes within the rodent dorsal striatum (Krasnova et al., 2013; Cadet et al., 2015; Li et al., 2015; Caprioli et al., 2017). The dorsal striatum is indeed a key structure involved with the habitual manifestations of addiction (Koob and Volkow, 2010; Belin et al., 2013). Together, these findings support the notion that the path from casual drug use to habitual drug taking may depend on long-lasting neuroadaptations that include a shift in control from ventral reward circuits to more dorsal striatal habit circuits (Feil et al., 2010; Everitt and Robbins, 2013).

We recently developed a rat model in which we use footshocks as adverse consequences during METH SA. This model has helped to dichotomize rats into 2 distinct addiction-related phenotypes (Cadet et al., 2017). Specifically, these procedures were able to segregate rats that continued to take METH despite footshock punishment (shock-resistant [SR]) from rats that reduce their METH intake with increasing shock intensity (shock-sensitive [SS]). Interestingly, SR rats were found to exhibit higher incubation of METH-seeking behavior compared with SS rats (Krasnova et al., 2017; Torres et al., 2017).

In the present study, we examined tissue from animals used in a previous behavioral study (Torres et al., 2017) to test whether neurobiological alterations in the striatum might reflect molecular adaptations that may distinguish SR from SS even after a prolonged period of drug abstinence. We focused on the dorsal striatum, because neuroadaptations within this structure might underlie the transition from casual to compulsive drug-seeking behavior (Everitt and Robbins, 2013, 2016). The importance of striatum is also highlighted by substantial transcriptional and translational changes following models of METH SA (Krasnova et al., 2013; Cadet et al., 2015; Li et al., 2015; D'Arcy et al., 2016). Because neurotrophins are known to participate in the generation of long-lasting plastic changes in the brain (Alder et al., 2003; Bekinschtein et al., 2008), we tested the possibility

that alterations in their striatal expression might occur following prolonged abstinence from our METH SA procedures. Here, we provide evidence that the striatal NGF-TrkA/p75NTR/MAPK signaling pathway is selectively activated in SS rats at 30 days following METH SA and footshocks. Our results support the view that distinct signaling cascades may play important roles in mediating the long-lasting effects of METH taking depending on the level of control over drug consumption.

Methods

Animals and SA Procedures

Male Sprague-Dawley rats (Charles River) weighing 350 to 400 g were used in these experiments. Rats were anesthetized with a ketamine/xylazine mixture (50 and 5 mg/kg, i.p., respectively) and were inserted with catheters into the jugular vein. After recovery, rats were placed in Med Associates SA chambers and trained to self-administer METH (0.1 mg/kg/injection, i.v.) on an FR-1 schedule during three 3-hour sessions/d (9 h/d). Each 3-hour session was separated by a 30-minute time interval. Following SA training, rats were subjected to punishment where 50% of active lever-presses resulted in a mild foot-shock. Animals were classified as SS if they reduced their intake by 70%. Subgroups were divided into SR (n = 7), SS (n = 9), and saline controls (n = 5) as previously reported by Torres et al. (2017). Cue-induced drug-seeking behavior was assessed at 2 and 21 days post-shock essentially as described in Krasnova et al. (2014). Briefly, each test consisted of a 1-hour session without METH availability, during which time drug-associated lever presses resulted only in tone and light cues previously paired with METH infusions. All animal procedures were conducted following the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of the NIDA Intramural Research Program.

Tissue Collection and RNA Extraction

To measure potential differences in gene expression between the SR and SS phenotypes after a long period of abstinence, rats were killed 9 days following the second extinction test (30 days total after the last METH SA session). This time frame was based on our previous studies showing METH-induced changes in striatal expression of neurotrophins and genes involved in the CREB (cAMP responsive element binding protein) signaling pathway 1 month after METH SA (Krasnova et al., 2013, 2016). Following rapid decapitation, dorsal striatal tissue was dissected, immediately placed on dry ice, and stored at -80°C. Total RNA was then isolated using Qiagen RNeasy Mini kits and treated against genomic DNA contamination by using Qiagen RNase-free DNase kits (Qiagen) following the manufacturer's protocol. RNA levels

were then assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). RNA integrity numbers were also examined, as described by Schroeder et al. (2006), using an Agilent 2100 Bioanalyzer System in conjunction with the RNA LabChip assay (Agilent) with no sample showing signs of degradation.

RT² Profiler PCR Array

The RT² Profiler PCR Array (PARN-031Z, Qiagen) discovery platform was used to examine differences in striatal gene expression between SR and SS rats. Each array contained 84 gene-specific primers related to neurotrophic signaling, neuropeptides, and transcription factors. Total RNA (0.5 µg) was treated with 5x buffer GE, incubated at 42°C for 5 minutes, and placed on ice. Purified RNA was then reverse-transcribed to cDNA by using the RT² First Strand Kit (Qiagen) and RNase-free water following the manufacturer's instructions. Each 96-well RT² Profiler PCR Array was applied on a LightCycler 480 II PCR system (Roche Diagnostics) for each striatal sample. Each array plate contained a genomic DNA contamination control, 3 reverse transcription controls, 3 positive PCR controls, and 5 wells with different housekeeping genes (Beta-actin, Beta-2 microglobulin, Hypoxanthine phosphoribosyltransferase 1, Lactate dehydrogenase A, and Ribosomal Protein, Large, p1). Data analyses for threshold amplification cycle numbers (Tc) were performed using the complimentary web-based Qiagen software tools following the manufacturer's protocol and normalized to the 4 housekeeping genes. To confirm primer specificity of each gene product, amplicon melting curves were recorded and analyzed after every array experiment.

Independent qRT-PCR Analysis

RT² Profiler PCR Array data were verified by individual qRT-PCR comparisons. Briefly, unpooled total RNA (0.5 µg) was reverse-transcribed to cDNA using oligo dT primer from the Advantage RT for PCR kit (Clontech). Sequences for rat gene-specific primers corresponding to PCR targets were then designed using the LightCycler Probe Design software version 1 (Roche Diagnostics) and synthesized by the Synthesis and Sequencing Facility of Johns Hopkins University. qRT-PCR reactions were carried out in a final volume of 12.5 µL consisting of reverse transcribed cDNA, gene-specific primers, nucleic-acid free water, and iQ SYBR Green Supermix (Bio-Rad). All qRT-PCR experiments were conducted using a LightCycler 480 II instrument (Roche). qRT-PCR primers were designed for the specific amplification of rat *Bdnf* (brain-derived neurotrophic factor), *Ngf* (nerve growth factor), *TrkA* (neurotrophic receptor tyrosine kinase 1), *TrkB* (neurotrophic receptor tyrosine kinase 2), *Gfra2* (GDNF family receptor alpha 2), *Crh* (corticotrophin-releasing hormone), *Crhr1* (Crh receptor 1), *Crhr2* (Crh receptor 2), *Crhbp* (Crh binding protein), *Ucn2* (urocortin 2), *c-fos*, *fosb*, *Egr1* (early growth response 1), *Egr2* (early growth response 2), and *Egr3* (early growth response 3) (sequences are listed on supplementary Table 1). The purity of each amplicon was subsequently verified by melting curve analysis. Expression of mRNA levels for each gene was normalized to the reference gene beta-2-microglobulin. Results are reported as fold changes calculated by the ratios of normalized target gene products compared with the gene expression data of saline SA control group.

Western-Blot Analysis

Western-blot analyses were carried out essentially as previously described by our laboratory (Jayanthi et al., 2009). Briefly, striatal

tissue was homogenized in ice-cold lysis buffer A (10 mM HEPES, 1.5 mM MgCl₂, 10 mM KCl, and 1% igepal) containing protease and phosphatase inhibitor cocktail tablets (Roche Diagnostics). After homogenization, samples were centrifuged at 14 000 g for 5 minutes at 4°C. Supernatant was collected and used as cytosolic protein fraction. Nuclear fractions were then suspended in buffer B (20 mM HEPES, 840 mM NaCl, 1.5 mM MgCl₂, 4 mM EDTA, and 10% glycerol) containing protease and phosphatase inhibitor cocktail tablets (Roche Diagnostics). Individual protein concentrations were determined by utilizing the BCA assay kit (Thermo Fisher Scientific). Samples were then transferred on to PVDF membranes and incubated overnight at 4°C with a specific antibody raised against: Pro-BDNF (Millipore, #AB5613P), BDNF (Santa Cruz Biotechnology, #SC546), Pro-NGF and mature NGF (Santa Cruz, #SC365944), TrkB (Millipore, #07-225), pTrkB (Sigma-Aldrich, #SAB 4301317), TrkA (Cell Signaling, #2508), pTrkA (Cell Signaling, #9141), P75NTR (nerve growth factor receptor) (Cell Signaling, #2693), Sortilin (Thermo Fisher Scientific, #PA5-19481), p-cRaf (Cell Signaling, #9421), pMEK1/2 (mitogen activated protein kinase 1) (Cell Signaling, #9154), pErk1/2 (mitogen activated protein kinase 3) (Cell Signaling, #4370), pMSK1 (mitogen and stress activated protein kinase 1) (Cell Signaling, #9595), pCREB (Cell Signaling, #9198), H3ac (acetylated histone H3) (Active Motif, #39139), p-MeCP2 (methyl-CpG binding protein 2) (Active Motif, #39733), and p-mTOR (mammalian target of rapamycin) (Cell Signaling, #2971). To confirm equal protein loading, blots were reprobated with an antibody against α -Tubulin (Sigma) for 2 hours at room temperature. Optical densities were measured using the ChemiDoc MP Imaging system (Bio-Rad) and normalized using the signal intensity of α -tubulin. The results are reported as percentage of control changes calculated as the ratios of protein expression for each METH group compared with the saline SA control group.

Statistical Analysis

Profiler PCR Array data were analyzed using the Qiagen web-based software. qRT-PCR data were analyzed using 1-way ANOVAs followed by posthoc tests (Bonferroni) or planned comparisons (2-tailed Student tests) (SPSS 20). Western-blot data were also analyzed by 1-way ANOVAs followed by posthoc tests (Fisher's LSD) or planned comparisons (StatView, SAS). Individual planned comparisons were used in specific cases where the difference between SR and SS rats was based on an empirical framework. Correlations between RT² Profiler PCR Arrays and qRT-PCR data were assessed using linear regression (SPSS 20). All data are presented as means \pm SEM and considered statistically significant when $P \leq .05$.

Results

Transcriptional Alterations in the Dorsal Striatum 30 Days after METH SA and Footshock Punishment

To identify differentially expressed neuroplasticity-related genes that might distinguish SR from SS rats, we used the unbiased discovery platform Neurotrophins and Receptors RT² Profiler PCR Array system. This approach allowed us to potentially target a specific subset of genes that were found to be affected after cessation of METH SA experiments (Krasnova et al., 2013, 2016). Figure 1a illustrates METH-induced changes in the expression of neurotrophic and neuropeptide associated genes in the dorsal striatum. Specifically, mRNA expression for *Bdnf*, *Ngf*, *Vgf*, *Trkb*, *Ntf3* (neurotrophin 3), *Gfra1*, and *Gfra2* were

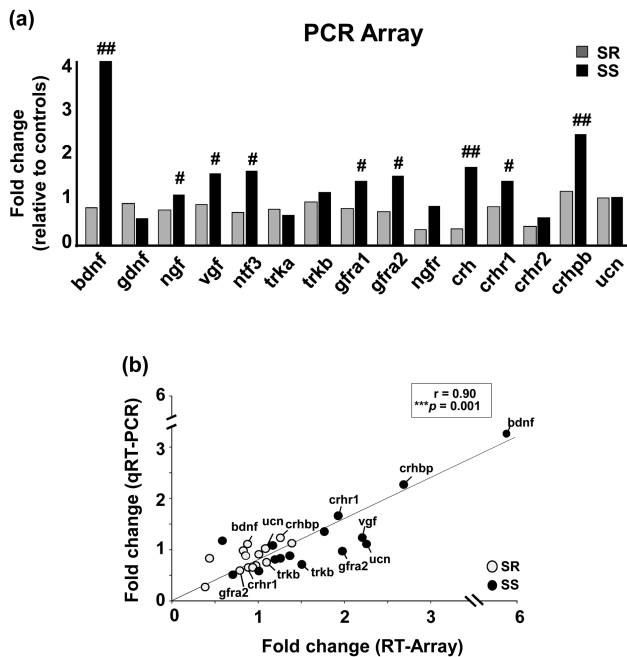


Figure 1. Transcriptional alterations 30 days after the punishment phase of methamphetamine (METH) self-administration (SA) in the dorsal striatum of shock-resistant (SR) and shock-sensitive (SS) rats. (a) The graph shows neurotrophic- and neuropeptide-associated genes that were differentially expressed between SR and SS rats as determined by RT² Profiler PCR Arrays. (b) The figure shows transcriptional responses obtained by Profiler Arrays (“x” axis) and individual qRT-PCRs (“y” axis) for upregulated genes in the dorsal striatum of SR rats (in grey circles) and SS rats (in black circles). Data are presented as fold-changes relative to saline control rats. A significant correlation was observed between the 2 analyses ($P = .001$). Key to statistics: * $P < .05$, ** $P < .01$ compared with SR rats.

upregulated in SS rats compared with SR rats at 30 days after METH SA and punishment. These findings may be related to the demonstration that METH exposure can increase synaptic spine density and structural plasticity in the dorsal striatum (Jedynak et al., 2007). We also found that *Crh*, *Crhr1*, and *Crhbp* mRNA levels were increased in SS rats relative to SR rats ($P < .05$). Array data were subsequently validated by running qRT-PCRs using the same RNA samples isolated from the dorsal striatum of these rats. We found a significant correlation ($r = 0.90$, $P < .01$) between the array and PCR data (Figure 1b).

Differential Expression of Neurotrophins, Neuropeptides, and Immediate Early Genes (IEGs) in SR and SS Phenotypes

qRT-PCR analysis showed significant alterations in *Bdnf* mRNA levels [$F_{(2,17)} = 7.86$, $P < .01$], with SS rats showing significant increases compared with saline controls and SR rats ($P < .01$) (Figure 2a). *Ngf* mRNA levels were not significantly higher in SS rats compared with SR rats [$F_{(2,17)} = 1.78$, $P = .21$]. *Gfra2* mRNA levels were also increased [$F_{(2,18)} = 7.97$, $P < .01$] in SS rats relative to SR and saline control rats. Interestingly, SS rats displayed a significant decrease in *Trka* [$F_{(2,18)} = 6.39$, $P < .01$] mRNA levels compared with SR rats. Our PCR analyses also confirmed the upregulation of some, but not all, neuropeptide-associated genes. *Crhr1* mRNA levels were increased in SS rats [$F_{(2,17)} = 5.14$, $P < .05$] (Figure 2b) but showed no changes in SR rats. Differential changes were also observed for *Crhbp* [$F_{(2,18)} = 3.7$, $P < .05$] and *Ucn2* [$F_{(2,18)} = 3.9$, $P < .05$] mRNA levels, with SS rats displaying

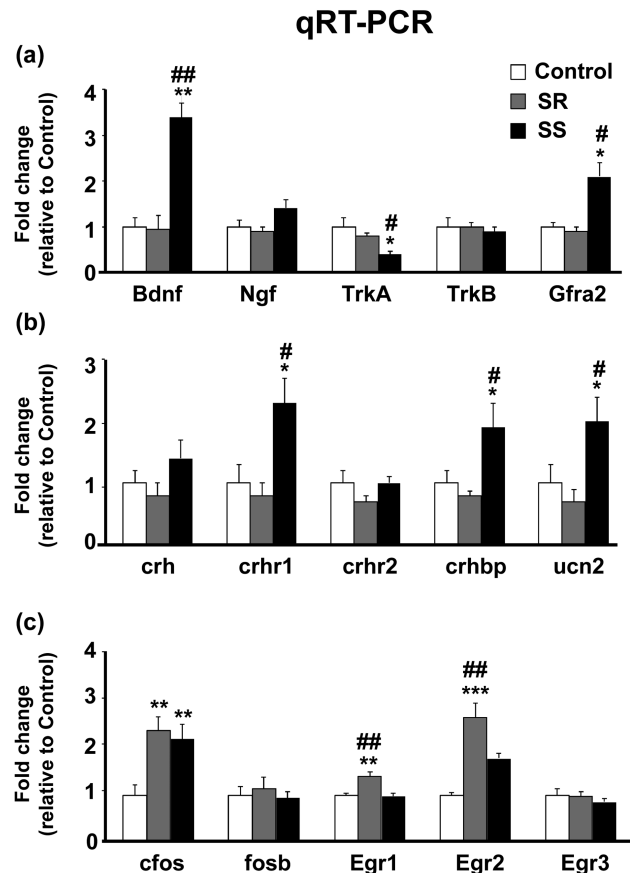


Figure 2. Distinct neurotrophin, neuropeptide, and immediate early genes (IEGs) expression in the dorsal striatum of shock-resistant (SR) and shock-sensitive (SS) rats. (a) Independent qRT-PCR analyses for neurotrophin mRNA levels in the dorsal striatum with increased expression of *Bdnf* in both SR and SS rats, decrease *TrkA* levels in SS rats, and increased *Gfra2* levels also in SS rats. (b) Differential expression neuropeptide-related genes and receptors in the dorsal striatum, with SS rats having increased mRNA levels for *crhr1*, *crhbp*, and *ucn2* compared with saline controls and SR rats. (c) Altered mRNA levels of IEGs in the dorsal striatum of SR and SS rats. Both SR and SS rats had increased expression of *c-fos* mRNA levels compared with saline controls, while only SR rats had increased *egr1* and *egr2* mRNA levels compared with saline and SS rats. Key to statistics: * $P < .05$, ** $P < .01$, *** $P < .001$ compared with saline controls; * $P < .05$, ** $P < .01$ compared with SR rats.

significant increases compared with saline control and SR rats ($P < .05$) (Figure 2b).

To test if other genes known to be regulated by trophic factors were also altered between the SR and SS phenotypes, we measured mRNA levels of some IEGs (Figure 2c). We found significant increases in *c-fos* mRNA levels [$F_{(2,17)} = 3.93$, $P < .05$] in both SR and SS rats compared with controls. No changes in *fosb* mRNA levels [$F_{(2,18)} = 0.30$, $P = .74$] were observed. There were also significant increases in *Egr1* [$F_{(2,17)} = 9.0$, $P < .01$] and *Egr2* [$F_{(2,18)} = 13.52$, $P < .01$] mRNA levels in SR rats compared with SS rats and controls (Figure 2c). There were no significant changes in *Egr3* mRNA levels [$F_{(2,18)} = 0.53$, $P < .59$].

Differential Expression of Neurotrophin-Related Proteins in the Dorsal Striatum of SR and SS Rats

To better elucidate the neuroplastic bases for the SR from SS phenotypes, we tested if changes in the striatal expression of neurotrophin mRNA levels were also associated with corresponding changes in protein products. There were significant decreases in

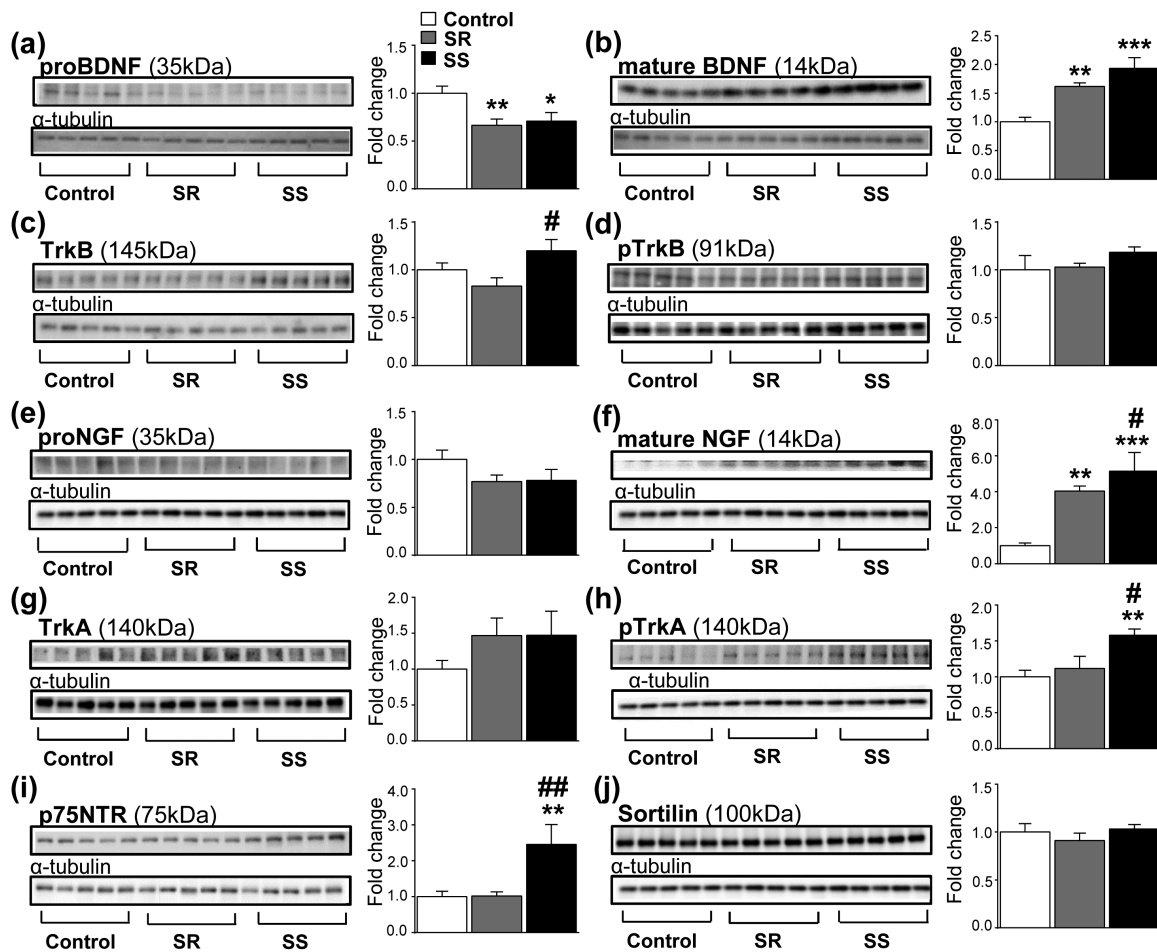


Figure 3. Expression of BDNF- and NGF-related proteins in the dorsal striatum following 30 days after methamphetamine (METH) self-administration (SA). (a) Significantly decreased pro-BDNF levels in shock-resistant (SR) rats compared with saline controls. (b) Increased mature BDNF in SR and shock-sensitive (SS) rats compared with saline controls. (c) TrkB levels are slightly increased in SS rats compared with SR rats. (d) Similar expression of pTrkB protein levels in SR and SS phenotypes. (e) No changes in pro-NGF levels in SR and SS rats. (f) Increased mature NGF protein levels in both SR and SS rats, with SS showing larger increases. (g) Comparable upregulated TrkA levels in SR and SS rats. (h) Increased pTrkA expression in SS rats compared with saline controls. (i) Increased of p75NTR expression in SS rats compared with saline controls and SR rats. (j) Similar levels of sortilin expression between SR and SS rats. Values represent means \pm SEM of fold changes relative to the controls. Key to statistics: * $P < .05$, ** $P < .01$, *** $P < .001$ compared with saline controls; # $P < .05$, ## $P < .01$ compared with SR rats.

striatal proBDNF levels [$F_{(2,15)} = 5.13, P < .05$] in SR rats compared with controls (Figure 3a). Figure 3b shows that there were also increases in mature BDNF levels [$F_{(2,16)} = 11.80, P < .001$] in both SR and SS rats. There were also significant changes in the BDNF receptor, TrkB, protein levels [$F_{(2,15)} = 3.63, P < .05$] (Figure 3c) but no significant changes in the phosphorylated form, pTrkB [$F_{(2,16)} = 1.58, P = .23$] (Figure 3d) between the SR and SS rats (Figure 3c).

We next focused our attention on proNGF, NGF, and TrkA protein levels. We detected no significant changes in proNGF levels [$F_{(2,15)} = 1.72, P = .21$] (Figure 3e) but found significant increases in mature NGF levels [$F_{(2,16)} = 42.45, P < .001$] in both SR and SS groups compared with the control group (Figure 3f). However, mature NGF protein levels were significantly higher in the SS rats compared with the SR rats ($P < .05$) (Figure 3f). There were no significant differences in TrkA protein levels between the groups (Figure 3g). However, there was increased abundance of phosphorylated TrkA protein [$F_{(2,15)} = 5.10, P < .05$] only in the SS phenotype (Figure 3h). Because NGF can also interact with the p75NTR receptor (Bucci et al., 2014), we tested the possibility that there might be differential protein expression of this receptor in the 2 phenotypes. Figure 3i shows that there were indeed significant changes in p75NTR protein levels [$F_{(2,15)} = 6.13, P < .05$], with the SS rats showing increased expression compared with SR and control

rats. Given that p75NTR can interact with sortilin to cause cellular damage (Nykjaer and Willnow, 2012) and because METH is also a known toxicant (Cadet and Krasnova, 2009), we measured striatal sortilin levels. Figure 3j shows that there were no significant changes in sortilin levels [$F_{(2,16)} = 0.81, P = .46$] in the 2 phenotypes (Figure 3j), supporting the idea that increased p75NTR may be related to enhanced neuroplasticity in the SS phenotype but not to the reported toxic effects of METH SA (Krasnova et al., 2010).

Activation of the Ras/Raf/MEK/ERK Pathway in the Dorsal Striatum of SS Rats

Neurotrophins exert their functions by activation of the Ras/Raf/MEK/ERK intracellular signaling cascade through phosphorylation of several proteins in that cascade (Reichardt, 2006; Cargnello and Roux, 2011). Importantly, it is known that p75NTR can interact with TrkA to potentiate activation of this cascade (Chao et al., 2006; Reichardt, 2006; Matusica et al., 2013), which is known to participate in the modulation of gene expression in response to neuronal activity (Flavell and Greenberg, 2008). We thus thought it likely that the SS phenotype might also show increased phosphorylation of proteins of the MAPK cascade. Indeed, Figure 4a showed a

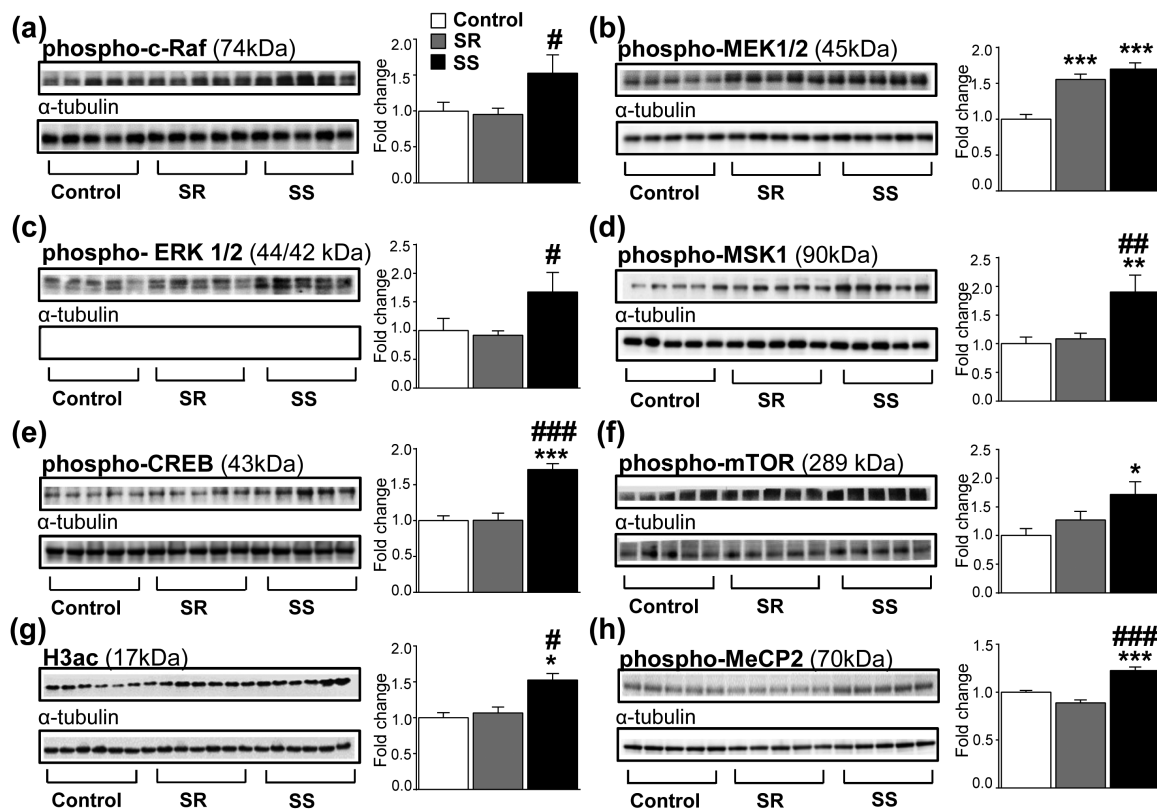


Figure 4. Activation of the MAPK/ERK intracellular signaling cascade in the dorsal striatum of shock-sensitive (SS) rats. (a) p-cRaf expression in SS rats compared with controls and SR rats. (b) Increased pMEK1/2 abundance in both SR and SS rats compared with saline controls. (c) Increased pERK 1/2 expression in SS rats compared with SR rats. (d) Increased pMSK1 expression in SS rats compared with saline controls and SR rats. (e) Increased pCREB expression in SS rats compared with saline controls and SR rats. (f) Increased p-mTOR levels in SS rats compared with control rats. (g) Increased H3ac levels in SS rats compared with saline controls and SR rats. (h) Increased p-MeCP2 expression in SS rats compared with saline controls and SR rats. Key to statistics: * $P < .05$, ** $P < .01$, *** $P < .001$ compared with saline controls; # $P < .05$, ## $P < .01$, ### $P < .001$ compared with SR rats.

trend [$F_{(2, 15)} = 3.51, P = .06$] of increased p-Raf abundance in SS rats, with planned comparisons revealing significant increases compared with SR rats ($P < .05$). Figure 4b also shows significant increases in pMEK1/2 abundance [$F_{(2, 16)} = 18.87, P < .001$] in both SR and SS rats relative to controls. Figure 4c illustrates a trend [$F_{(2, 15)} = 3.34, P = .06$] towards increased pERK abundance, with planned comparisons revealing significant increases in the SS rats compared with SR rats ($P < .05$). There were significant increases [$F_{(2, 15)} = 6.78, P < .01$] in pMSK1 abundance in SS rats compared with SR and control rats (Figure 4d). Similarly, CREB phosphorylation was significantly [$F_{(2, 16)} = 22.55, P < .01$] increased only in the SS phenotype (Figure 4e).

Neurotrophins are also known to increase activation of mTOR complexes via mTOR phosphorylation with secondary increase in translation processes (Laplante and Sabatini, 2012). Because such an increase in translation may provide a partial explanation for the increased p75NTR protein expression observed only in the sensitive phenotype, we measured mTOR phosphorylation and found significant increases in phosphorylated mTOR abundance [$F_{(2, 13)} = 4.22, P < .05$] in SS rats compared with controls (Figure 4f). We also measured the abundance of total histone 3 acetylation (H3ac) in the dorsal striatum and found significant increases in H3ac abundance [$F_{(2, 16)} = 4.67, P < .05$] in SS rats compared with SR and saline controls (Figure 4g). Phosphorylated MeCP2 protein levels were also increased in SS rats compared with SR and controls (Figure 4h).

Discussion

Recent work in our laboratory has indicated that, even in rats that had escalated their intake of METH during SA procedures, footshock punishment can lead to the segregation of distinct subgroups of rats that either continue to take the drug compulsively or suppress their responding for the drug (Cadet et al., 2017; Krasnova et al., 2017; Torres et al., 2017). Here, we report that SS rats showed increased activation of multiple proteins involved with the NGF-TrkA/p75NTR/MAPK signaling cascade in the dorsal striatum after 30 days of withdrawal from METH SA and punishment (Figure 5). SS rats also showed increases in the expression of neuropeptide and neurotrophic genes compared with SR rats. Specifically, neuropeptide-related genes that were increased in SS rats included *Crhr*, *Crhbp*, and *Ucn2*, whereas neurotrophic-associated genes that showed upregulation included *Bdnf* and *Gfra2*. These observations are interesting given that UCN and its receptors serve to integrate physiological stress responses that might have served as triggers for the appearance of the SS phenotype in reaction to negative stimuli.

Our behavioral studies are in line with the reports of other authors who have developed SA methods to identify rats with persistent drug-seeking behaviors despite adverse consequences (Deroche-Gamonet et al., 2004; Pelloux et al., 2007; Chen et al., 2013) or despite environmental signals of potential adversity (Vanderschuren and Everitt, 2004) to drug taking. Our studies have used this approach to identify

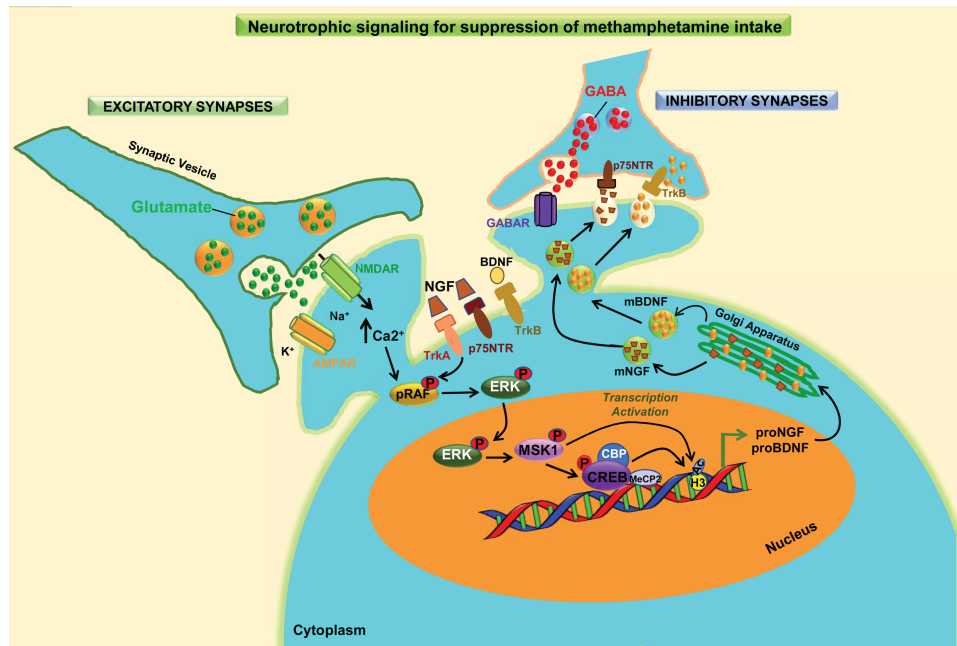


Figure 5. Preferential activation of the NGF signaling pathway in shock-sensitive methamphetamine (METH) self-administering (SA) rats. The figure indicates that METH SA is accompanied by increased signaling in excitatory synapses in all rats. Yet contingent footshocks led to the identification of 2 METH SA phenotypes: rats that continued to press an active lever to receive METH (shock-resistant, SR) and those that reduced their lever pressing, thereby reducing their METH intake (shock-sensitive, SS). Both sets of rats showed increased BDNF, whereas rats that reduce their lever pressing exhibited increased mature nerve growth factor (NGF) levels and greater abundance of phosphorylated TrkA, a receptor for NGF. Importantly, the levels of p75NTR, another NGF receptor, were also increased only in the genetically prone shock-sensitive rats. In addition, several phospho-proteins that participate in the NGF/RAF/ERK/MSK cascade were activated only in the shock-sensitive rats. Moreover, the shock-sensitive rats showed increased CREB phosphorylation in the dorsal striatum. Phosphorylated CREB is known to recruit the histone acetyl-transferase, CREB binding protein (CBP), an event that leads to increased H3 histone acetylation, as observed in the shock-sensitive rats. These series of molecular events could have led to increased GABAergic tone in the dorsal striatum and subsequent suppression of METH SA in the shock-sensitive animals. Therefore, the development of molecules that can specifically activate this striatal NGF-mediated phosphorylation cascade may be of therapeutic benefit to METH-addicted individuals.

distinct molecular profiles between persistent drug-seeking rats and those that reduce their METH intake. For example, we have previously demonstrated that SS rats are characterized by differential changes in DNA hydroxymethylation in the nucleus accumbens (Cadet et al., 2017). Within this same region, we also demonstrated that SS rats show increased mRNA levels of various histone deacetylases that participate in gene regulation (Cadet et al., 2016a). In a subsequent report, we also documented that SR rats exhibited increases in nucleus accumbens proenkephalin and prodynorphin mRNA levels (Cadet et al., 2016b). Herein, we expand on our previous work by demonstrating that, in the dorsal striatum, neurotrophins and activation of their downstream signaling cascade distinguishes SS from SR phenotypes following prolonged abstinence from METH intake.

Neurotrophins play important roles in regulating diverse neuronal processes including synaptic plasticity, cell survival, differentiation, and neuronal growth (Alder et al., 2003; Bekinschtein et al., 2008). Our observations of increased mature BDNF protein levels in SR and SS rats are consistent with previous reports showing increased BDNF expression in rats that self-administered METH chronically (Krasnova et al., 2013; Li et al., 2015). These observations suggest that increased BDNF expression may be a consequence of long-term METH exposure but do not necessarily reflect molecular adaptations that would help to distinguish compulsive from controlled drug-taking behaviors. This conclusion is supported by our findings that there were no differential changes in TrkB phosphorylation between the two phenotypes.

Because BDNF and TrkB protein levels did not account for neuronal differences between SS and SR rats, the possibility arose that NGF and its receptor, TrkA, might play a more prominent role in discriminating the 2 phenotypes. Indeed, we observed increases in NGF protein levels accompanied by selective increases in phosphorylated TrkA and p75NTR levels in the SS phenotype compared with the SR rats. Although NGF was originally described as a neurotrophic factor required for cell proliferation (Cohen et al., 1954), it is now clear that NGF can also participate in a number of neurobiological events (Conner et al., 2009; Manni et al., 2013). For example, within the striatum NGF is produced by GABAergic interneurons (Bizon et al., 1999) and has significant protective and plastic effects on striatal cholinergic neurons under normal and pathological conditions (Fischer et al., 1998; Gratacos et al., 2001). Additionally, NGF can cause hypertrophy of striatal cholinergic neurons, increased levels of choline acetyltransferase mRNA, and reduced spontaneous neuronal activity (Forander et al., 1996). Moreover, the biological effects of NGF occur through its binding to TrkA and p75NTR, with distinct functional and structural interactions between the 2 receptors (Matusica et al., 2013; Bucci et al., 2014; Covaceuszach et al., 2015). For instance, p75NTR can co-precipitate along with TrkA (Bibel et al., 1999), with TrkA affinity for NGF increasing in the presence of p75NTR (Esposito et al., 2001). p75NTR can also prolong cell-surface TrkA-dependent signaling (Makkerh et al., 2005), thereby enhancing TrkA signaling capacity (Verdi et al., 1994; Epa et al., 2004). Interestingly, knockdown of p75NTR expression significantly reduced excessive alcohol intake in rats

(Darcq et al., 2016), whereas our own findings suggest that increased p75NTR might be involved in suppressing METH-seeking behavior, since the SS rats also show less cue-induced drug seeking (Torres et al., 2017). This statement hints to the possibility that SS rats might have a better ability to learn and establish memory for adverse events through the activation of the NGF signaling cascade. In support of this hypothesis, a previous report suggested that enhancement of contextual fear memory in rodents is mediated by neurotrophic-dependent activation of the Erk1/2(MAPK) pathway (Revest et al., 2014). Similarly, increased neurotrophic factors in the dorsal striatum appear to be associated with enhanced performance on the lever-press escape/avoidance paradigm in rats (Albeck et al., 2005).

Given that stimulation of TrkA by NGF induces auto-phosphorylation of this receptor and promotes ERK1/2 activation (Diolaiti et al., 2007), we thought it likely that NGF/TrkA interactions might differentially activate downstream signaling between the 2 phenotypes. This supposition was confirmed by our findings that only striatal tissues from the SS rats showed increased phosphorylation of proteins associated with the MAPK/MEK/ERK intracellular cascade. NGF-TrkA interactions are known to activate multiple intracellular signaling pathways including the Ras/MAPK, PI3K, and PLC γ cascades (Marlin and Li, 2015). However, the Ras/MAPK cascade has received much attention due to the prominent role it plays in neuronal plasticity (Bruehl-Jungerman et al., 2007; Cargnello and Roux, 2011; Correa et al., 2012), memory formation (Adams and Sweatt, 2002), and transcriptional responses (Davis et al., 2000). In neuronal cells, the binding of mature NGF onto TrkA activates the adaptor GRB2-SOS protein complex, which increases the rate of GDP-GTP exchange on Ras, leading to Ras activation (Bucci et al., 2014; Marlin and Li, 2015). Activation of Ras induces a sequential activation of Raf, a MAPK kinase kinase. Activation of Raf subtypes induces phosphorylation of MEK that then promotes the phosphorylation of ERK1/2 (Plotnikov et al., 2011; Ciccarelli and Giustetto, 2014). Translocation of pERK1/2 into the cell nucleus results in the phosphorylation of MSK1 (Plotnikov et al., 2011). Phosphorylated-MSK1 directly causes the phosphorylation of CREB (Arthur and Cohen, 2000) that binds onto cAMP response elements. Phosphorylated-CREB then recruits histone acetyltransferases that promote histone acetylation and increase accessibility of the genome for molecular machinery to initiate transcription (Nestler, 2012). This discussion is compatible with our observations of increased phosphorylation of Raf, MEK, ERK, and MSK1 with subsequent increases in CREB phosphorylation in SS rats. In addition, our finding of increased H3ac in SS rats agrees with the above discussion and postulates a selective activation of long-lasting plasticity events that enhance memory formation in this group of animals. The finding that phospho-mTOR is increased in SS, but not SR, rats is also of interest, since NGF is known to activate this protein (Zhang and Ma, 2014; Wang et al., 2017) that can regulate translation rates, metabolic processes, and synaptic plasticity in neurons (Cho, 2011). Furthermore, the observations that pMeCP2 levels were selectively increased in SS rats support our notion of enhanced learning in this group of rats, since mice deficient in pMeCP2 show memory deficits (Moretti et al., 2006). Although MeCP2 is known as gene silencer, recent evidence suggests it also binds to methylated non-CG sites (Chen et al., 2015) and can function as a transcriptional activator by association with pCREB (Chahrour et al., 2008). However, it is important to note a recent study that reported decreased METH SA after virally mediated knockdown of MeCP2 in the nucleus accumbens (Lewis et al., 2016). These

findings implicate potentially distinct roles of this protein in various brain regions following METH SA. Taken together, our findings and those of others suggest that increased neurotrophic factors and activation of the Ras/Raf/MEK/ERK pathway may serve to prevent the development of plastic changes that promote compulsive METH intake.

In conclusion, we found that rats that reduce their METH intake in the presence of punishment show significant increases in striatal NGF levels, TrkA phosphorylation, p75NTR expression, and phosphorylation of the Ras/Raf/MEK/ERK signaling cascade. These molecular adaptations implicate neurotrophin-mediated signaling pathways during late stages of withdrawal from METH SA in a brain region associated with habitual and compulsive behaviors. Thus, our punishment-based model highlights the possibility of identifying specific molecular alterations that may help to distinguish rats with distinct profiles of drug intake after periods of drug withdrawal when a propensity to relapse might be pronounced. Therefore, the use of such models may be quite useful in the development of therapeutic modalities against drug addiction.

Supplementary Material

Supplementary data are available at *International Journal of Neuropsychopharmacology* online.

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Statement of Interest

None.

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