Published in final edited form as:

Neurosci Lett. 2012 October 24; 528(2): 210–214. doi:10.1016/j.neulet.2012.07.048.

Upregulation of Npas4 protein expression by chronic administration of amphetamine in rat nucleus accumbens *in vivo*

Ming-Lei Guo^{1,*}, Bing Xue¹, Dao-Zhong Jin¹, Zhen-Guo Liu², Eugene E. Fibuch³, Li-Min Mao¹, and John Q. Wang^{1,3,*}

¹Department of Basic Medical Science, School of Medicine, University of Missouri-Kansas City, Kansas City, MO 64108, USA

²Department of Neurology, Shanghai Jiao Tong University School of Medicine, Shanghai, China

³Department of Anesthesiology, School of Medicine, University of Missouri-Kansas City, Kansas City, MO 64108, USA

Abstract

The neuronal PAS domain protein 4 (Npas4) is a transcription factor that is almost exclusively expressed in the mammalian brain. As an activity-dependent transcription factor, Npas4 regulates the transcription of discrete genes and transcriptionally controls the experience-dependent learning and memory. In this study, we explored the impact of the psychostimulant amphetamine (AMPH) on Npas4 protein expression in the rat striatum. We found that acute systemic injection of AMPH had a minimal effect on protein levels of Npas4 in the caudate putamen (CPu) and nucleus accumbens (NAc), while AMPH readily increased protein products of the immediate early gene c-Fos in these regions. In contrast, repeated administration of AMPH (5 mg/kg, once daily for 5 days) triggered a significant increase in Npas4 expression in the NAc, although repeated AMPH did not alter Npas4 in the CPu. These data demonstrate that Npas4 is an AMPH-sensitive transcription factor. It is inducible selectively in the NAc in response to repeated AMPH administration.

Keywords

Immediate early gene; transcription factor; c-Fos; Le-PAS; NXF; PASD10; striatum; caudate

1. Introduction

The neuronal PAS domain protein 4 (Npas4, also known as NXF, Le-PAS and PASD10) is a brain-specific transcription factor and is expressed in various regions of adult mammalian brains, including the striatum, cortex, and hippocampus [23]. As a basic helix-loop-helix transcription factor, Npas4 contains a unique PAS domain, through which Npas4 interacts with other proteins to form functional transcription factor complexes [16]. These complexes regulate target gene expression in response to diverse stimuli. As such, Npas4 acts as an

*Corresponding authors: Dr. Ming-Lei Guo, Tel:1-816-235-6723, Fax: 1-816-235-5574; Department of Basic Medical Science, University of Missouri-Kansas City, School of Medicine, 2411 Holmes Street, Kansas City, Missouri 64108, USA. Dr. John Q. Wang, Tel: 1-826-235-1786; Fax: 1-816-235-5574. guomi@umkc.edu (M.L. Guo), wangjq@umkc.edu (J.Q. Wang).

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

important regulator in gene expression in relation to a variety of physiological and pathophysiological activities [5].

As an activity-dependent transcription factor, Npas4 expression was readily induced under various conditions, including cerebral ischemia [23], seizure [3], and chronic restraint stress [27]. Changes in its expression were also seen in one form of synaptic plasticity, i.e., long-term potentiation [1,20]. After induction, Npas4 is engaged in regulating gene transcription and thus controlling homeostatic neuronal activity [11]. Most recently, Npas4 in the hippocampus was induced by contextual fear experience in mice, which contributes to contextual memory formation and fear memory [9,21]. In the striatum, Npas4 seems to be sensitive to psychostimulant exposure. Acute injection of cocaine induced a rapid and transient increase in *Npas4* mRNA expression in the mouse striatum [18]. Thus, Npas4 is among drug-responsive genes and might be involved in the regulation of drug effects.

The psychostimulant amphetamine (AMPH) is known to exert a significant impact on expression of discrete sets of genes in the mesolimbic reward system. A well-known AMPH responsive gene family is immediate early genes, including c-Fos and other related members [10,15,22]. A number of early studies have demonstrated that acute systemic injection of AMPH produced a rapid and transient increase in c-Fos protein expression in the striatum [4,17,26]. As activity-dependent transcription factors, c-Fos and other immediate early genes play a critical role in constructing neuroadaptations critical for the enduring addictive properties of stimulants. Npas4 is also considered a neuronal activity-dependent immediate early gene. Its expression at the protein level in striatal neurons may be sensitive to stimulants, although the direct evidence for or against this is lacking at present.

In this study, we investigated the effect of AMPH on Npas4 protein expression in the rat striatum *in vivo*. We first tested the effect of acute systemic injection of AMPH at a behaviorally active dose (5 mg/kg) on Npas4 expression in the dorsal caudate putamen (CPu) and the ventral nucleus accumbens (NAc). We then examined the effect of AMPH after repeated injections (5 mg/kg, once daily for 5 days) on striatal Npas4 expression. In all experiments, c-Fos expression was monitored in parallel as a positive control.

2. Materials and methods

2.1. Animals

Adult male Wistar rats weighing 250–300 g (Charles River, New York, NY) were individually housed in a controlled environment at a constant temperature of 23°C and humidity of 50 ± 10% with food and water available *ad libitum*. The animal room was on a 12-h/12-h light/dark cycle. Rats were allowed 5–6 days of habituation to the animal colony. All animal use and procedures were in strict accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

2.2. Systemic drug injection

D-amphetamine sulfate (Sigma-Aldrich, St. Louis, MO) was freshly dissolved into 0.9% saline solution before each administration. In acute AMPH administration experiments, each rat received a single intraperitoneal (i.p.) injection at a dose of 5 mg/kg. Selection of this dose is because AMPH administered at this dose caused typical behavioral stimulation and immediate early gene expression in the striatum [24,25]. Rats were sacrificed 1 or 2 h after drug injection. Rats received a saline injection at the same volume served as controls. In chronic AMPH administration experiments, rats received the same dose of AMPH (5 mg/kg, i.p.) daily for five consecutive days. At the fifth day, rats were sacrificed 2 h after the final AMPH injection. Rats treated with five daily injections of saline served as controls.

2.3. Western blot analysis

Rats were anesthetized with equithesin (5 ml/kg, i.p.) and decapitated at time points indicated. Brains were quickly removed and cut into coronal sections. The CPu and NAc were dissected from sections and homogenized in the RIPA buffer (Thermo Scientific) with a protease inhibitor cocktail (Thermo Scientific) and a phosphatase inhibitor cocktail (Thermo Scientific). Protein concentrations were determined. Western blot was performed as described previously [6,12]. Briefly, the equal amount of proteins was separated on SDS NuPAGE Novex 4–12% gels (Invitrogen, Carlsbad, CA). Proteins were transferred to the polyvinylidene fluoride membrane (Millipore, Bedford, MA) and blocked in blocking buffer (5% nonfat dry milk in phosphate-buffered saline and 0.1% Tween 20) for 1 h. The blots were washed and incubated in the blocking buffer containing a primary rabbit antibody against Npas4 (1:2000, a generous gift from Dr. M.E. Greenberg) [11], c-Fos (1:1000, Santa Cruz; Cat. #: 7202) and β -actin (1:4000, Sigma; Cat. #: A2066). The incubation was carried out overnight at 4°C and followed by 1 h incubation in a horseradish peroxidase-linked secondary antibody against rabbit at 1:5000. Immunoblots were developed with the enhanced chemiluminescence reagents (ECL; Amersham Pharmacia Biotech, Piscataway, NJ). Kaleidoscope-prestained standards (Bio-Rad, Hercules, CA) and MagicMark XP Western protein standards (Invitrogen) were used for protein size determination. The density of immunoblots was measured using the Kodak 1D Image Analysis software.

2.4. Statistics

The results were presented as means $\pm$ S.E.M. and were evaluated using Student's t-test. Probability levels of < 0.05 were considered statistically significant.

3. Results

3.1. Effects of acute AMPH administration on Npas4 expression

We first investigated the effect of dopamine stimulation with an acute systemic injection of AMPH on expression of Npas4 in the rat striatum. To this end, we subjected rats to a single dose of AMPH (5 mg/kg, i.p.). We sacrificed rats 1 h after drug injection. Changes in Npas4 protein abundance in the two striatal structures (CPu and NAc) were analyzed using Western blotting with a pre-validated anti-Npas4 antibody [11]. We found that AMPH did not alter Npas4 expression in the CPu. The protein level of Npas4 in this region remained stable in AMPH-treated rats compared to saline-treated rats ($93.8 \pm 17.3\%$ of saline, $P > 0.05$; Fig. 1A). In contrast, AMPH induced a significant increase in c-Fos protein levels in the CPu. c-Fos is among immediate early genes that are known to be upregulated in striatal neurons following acute stimulant administration [5,10,31]. The response of c-Fos to AMPH therefore served as a positive control. Similar results were observed in the NAc. AMPH did not alter Npas4 protein levels ($86.5 \pm 24.3\%$ of saline, $P > 0.05$), while the drug elevated c-Fos expression in the region (Fig. 1B). These data demonstrate that acute AMPH administration was not able to affect Npas4 expression in striatal neurons.

To further verify the lack of effects of AMPH on Npas4 expression, we tested the effect of AMPH at a different time point. In this study, we sacrificed rats 2 h after an acute injection of AMPH (5 mg/kg, i.p.). We found that AMPH still caused no significant change in Npas4 protein levels in the CPu of AMPH-treated rats relative to saline-treated rats ($80.6 \pm 10.1\%$ of saline, $P > 0.05$; Fig. 2A). Whereas AMPH remained to produce a marked increase in c-Fos expression in the CPu (Fig. 2A). In the NAc, we did not observe a significant effect of AMPH on Npas4 expression ($124.4 \pm 14.2\%$ of saline, $P > 0.05$), while AMPH increased c-Fos expression (Fig. 2B). These data provide further evidence supporting that AMPH after acute injection has no ability to modify Npas4 expression in striatal neurons.

3.2. Effects of repeated AMPH administration on Npas4 expression

Given the lack of effects of AMPH after acute injection on striatal Npas4 expression, we next explored whether repeated AMPH administration affects Npas4 expression in the striatum. In this study, rats were treated with five daily injections of AMPH (5 mg/kg, i.p.; once daily for 5 days) and were sacrificed 2 h after the final injection. Immunoblots were performed to detect alterations in Npas4 and c-Fos expression in the CPu and NAc. The protein level of Npas4 in the CPu was no different between AMPH and saline groups ($97.3 \pm 9.8\%$ of saline, $P > 0.05$; Fig. 3A). The level of c-Fos was however significantly higher in AMPH-treated rats than saline-treated rats. Interestingly, Npas4 in the NAc showed a significant response to repeated AMPH administration. The NAc Npas4 protein level in AMPH-treated rats was elevated to $154.8 \pm 15.7\%$ of saline ($P < 0.05$; Fig. 3B). The c-Fos level was also increased in the NAc. These results indicated that repeated AMPH exposure led to an increase in Npas4 expression in a confined region of striatum (NAc).

4. Discussion

This study investigated the effect of dopamine stimulation with AMPH on Npas4 expression in the rat striatum. We found that acute administration of AMPH did not alter Npas4 protein expression in both the CPu and NAc. In contrast to acute AMPH injection, repeated administration of the drug induced a significant increase in Npas4 expression in the NAc, although repeated AMPH did not alter Npas4 expression in the CPu. As expected, both acute and repeated administration of AMPH led to an increase in c-Fos expression in the CPu and NAc. These data demonstrate that Npas4 is a protein sensitive to psychostimulant exposure. Repeated exposure to AMPH is seemingly required to upregulate Npas4 expression in the specific mesolimbic region.

Npas4 is considered to be a brain specific transcription factor [23]. It responds to cellular excitation and was upregulated in its protein abundance under many physiological and pathophysiological conditions in an activity-dependent manner [3,11,27]. A significant role of Npas4 has been recently demonstrated in experience-dependent synaptic plasticity and learning and memory. In Pavlovian fear conditioning, an amygdala-dependent form of learning and memory, Npas4 mRNAs and proteins were elevated in the lateral nucleus of the rat amygdala [19]. Npas4 was also induced in the mouse hippocampus after contextual learning [21]. Thus, Npas4 was suggested to be a master regulator of learning and memory and controls learning- and activity-regulated gene expression critical for memory formation [21]. Drug addiction is a neural process involving experience-dependent learning and memory. Npas4 is therefore likely to play a central role in this process. In fact, Npas4 was sensitive to stimulant exposure in this study. Repeated AMPH administration caused an increase in Npas4 expression in NAc neurons, indicating a potential role of Npas4 in processing effects of AMPH.

Acute cocaine or methamphetamine injection induced a rapid and transient increase in *Npas4* mRNA expression in the mouse striatum [13,18]. However, in this study, acute AMPH injection did not alter Npas4 protein expression in the rat striatum. Differences in drugs (cocaine versus AMPH), animals (rats versus mice), and gene products (mRNAs versus proteins) may explain the discrepancy. In contrast to acute drug injection, repeated injections of AMPH induced a moderate but significant increase in Npas4 expression. Apparently, repeated exposure to AMPH became sufficient to produce a significant effect. This nature seems to indicate that certain neuroadaptations occur during repeated drug exposure, which triggers Npas4 to be more sensitive to subsequent drug administration. Of note, the repeated injection regimen used in this study caused a well-known phenomenon of behavioral sensitization [24,25], which models the intensification of drug craving in stimulant addiction. The region-specific induction of Npas4 is another characteristic as

Npas4 expression was increased in the NAc but not CPu. This means that Npas4 may exert its role in regulating drug action primarily via NAc neurons at the time point surveyed.

Npas4 is a member of immediate early gene transcription factors. It is then worthy comparing Npas4 to other immediate early genes in their responses to stimulants. Npas4 did not respond to acute AMPH injection, while Fos family proteins (such as c-Fos and FosB) were elevated [this study,5,27]. In response to repeated AMPH or cocaine administration, striatal c-Fos induction was partially desensitized [7,17]. In contrast, Npas4 in the NAc was enhanced. This enhancement in accumbal Npas4 is noticeably similar to accumulation of Δ FosB, a splice product of *fosB*, in the striatum following repeated stimulant exposure [2,9,14]. A number of studies have demonstrated the role of Δ FosB in the addictive property of stimulants [2,8,9]. It will be intriguing to define whether Npas4 plays a similar role in drug-regulated learning and memory in the future.

Acknowledgments

We thank Dr. M.E. Greenberg for providing the anti-Npas4 antibody and Dr. Y. Lin's technical support. This work was supported by NIH grants DA010355 (J.Q.W.) and MH061469 (J.Q.W.) and by a grant from Saint Luke's Hospital Foundation.

References

1. Coba MP, Valor LM, Kopanitsa MV, Afinowi NO, Grant SG. Kinase networks integrate profiles of N-methyl-D-aspartate receptor-mediated gene expression in hippocampus. *J Biol Chem*. 2008; 283:34101–34107. [PubMed: 18815127]
2. Colby CR, Whisler K, Steffen C, Nestler EJ, Self DW. Striatal cell type-specific overexpression of DeltaFosB enhances incentive for cocaine. *J Neurosci*. 2003; 23:2488–2493. [PubMed: 12657709]
3. Flood WD, Moyer RW, Tsykin A, Sutherland GR, Koblar SA. Nxf and Fbxo33: novel seizure-responsive genes in mice. *Eur J Neurosci*. 2004; 20:1819–1826. [PubMed: 15380003]
4. Graybiel AM, Moraralla R, Robertson HA. Amphetamine and cocaine induce drug-specific activation of the c-fos gene in striosome-matrix compartments and limbic subdivision of the striatum. *Proc Natl Acad Sci USA*. 1990; 87:6912–6916. [PubMed: 2118661]
5. Gu YZ, Hogenesch JB, Bradfield CA. The PAS superfamily: sensors of environmental and developmental signals. *Annu Rev Pharmacol Toxicol*. 2000; 40:519–561. [PubMed: 10836146]
6. Guo ML, Fibuch EE, Liu XY, Choe ES, Buch S, Mao LM, Wang JQ. CaMKII α interacts with M4 muscarinic receptors to control receptor and psychomotor function. *EMBO J*. 2010; 29:2070–2081. [PubMed: 20461055]
7. Hope A, Kosofsky B, Hyman SE, Nestler EJ. Regulation of immediate early gene expression and AP-1 binding in the rat nucleus accumbens by chronic cocaine. *Proc Natl Acad Sci U S A*. 1992; 89:5764–5768. [PubMed: 1631058]
8. Hyman SE, Malenka RC, Nestler EJ. Neural mechanisms of addiction: the role of reward-related learning and memory. *Annu Rev Neurosci*. 2006; 29:565–598. [PubMed: 16776597]
9. Kelz MB, Chen J, Carlezon WA Jr, Whisler K, Gilden L, Beckmann AM, Steffen C, Zhang YJ, Marotti L, Self DW, Tkatch T, Baranaukas G, Surmeier DJ, Neve RL, Duman RS, Picciotto MR, Nestler EJ. Expression of the transcription factor deltaFosB in the brain controls sensitivity to cocaine. *Nature*. 1999; 401:272–276. [PubMed: 10499584]
10. Lillrank SM, Lipska BK, Bachus SE, Wood GK, Weinberger DR. Amphetamine-induced c-fos mRNA expression is altered in rats with neonatal ventral hippocampal damage. *Synapse*. 1996; 23:292–301. [PubMed: 8855514]
11. Lin Y, Bloodgood BL, Hauser JL, Lapan AD, Koon AC, Kim TK, Hu LS, Malik AN, Greenberg ME. Activity-dependent regulation of inhibitory synapse development by Npas4. *Nature*. 2008; 455:1198–1204. [PubMed: 18815592]

12. Liu XY, Mao LM, Zhang GC, Papasian CJ, Fibuch EE, Lan HX, Zhou HF, Xu M, Wang JQ. Activity-dependent modulation of limbic dopamine D3 receptors by CaMKII. *Neuron*. 2009; 61:425–438. [PubMed: 19217379]
13. Martin TA, Jayanthi S, McCoy MT, Brannock C, Ladenheim B, Garrett T, Lehrmann E, Becker KG, Cadet JL. Methamphetamine causes differential alterations in gene expression and patterns of histone acetylation/hypoacetylation in the rat nucleus accumbens. *PLoS ONE*. 2012; 7:e34236. [PubMed: 22470541]
14. McClung CA, Ulerly PG, Perrotti LI, Zachariou V, Berton O, Nestler EJ. DeltaFosB: a molecular switch for long-term adaptation in the brain. *Brain Res Mol Brain Res*. 2004; 132:146–154. [PubMed: 15582154]
15. Murphy CA, Russig H, Pezze MA, Ferger B, Feldon J. Amphetamine withdrawal modulates FosB expression in mesolimbic dopaminergic target nuclei: effects of different schedules of administration. *Neuropharmacology*. 2003; 44:926–939. [PubMed: 12726824]
16. Ooe N, Satio K, Mikami N, Nakatuka I, Kaneko H. Identification of a novel basic helix-loop-helix-PAS factor, NXF, reveals a Sim2 competitive, positive regulatory role in dendritic-cytoskeleton modulator drebrin gene expression. *Mol Cell Biol*. 2004; 24:608–616. [PubMed: 14701734]
17. Persico AM, Schindler CW, O'Hara BF, Brannock MT, Uhl GR. Brian transcription factor expression: effects of acute and chronic amphetamine and injection stress. *Mol Brain Res*. 1993; 20:91–100. [PubMed: 8255186]
18. Piechota M, Korostynski M, Solecki W, Gierzyk A, Slezak M, Bilecki W, Ziolkowska B, Kostrzewa E, Cymerman I, Swiech L, Jaworski J, Przewlocki R. The dissection of transcriptional modules regulated by various drugs of abuse in the mouse striatum. *Genome Biol*. 2010; 11:R48. [PubMed: 20459597]
19. Ploski JE, Monsey MS, Nguyen T, DiLeone RJ, Schafe GE. The neuronal PAS domain protein 4 (Npas4) is required for new and reactivated fear memories. *PLoS ONE*. 2011; 6:e23760. [PubMed: 21887312]
20. Ploski JE, Park KW, Ping J, Monsey MS, Schafe GE. Identification of plasticity-associated genes regulated by Pavlovian fear conditioning in the lateral amygdala. *J Neurochem*. 2010; 112:636–650. [PubMed: 19912470]
21. Ramamoorthi K, Fropf R, Belfort GM, Fitzmaurice HL, McKinney RM, Neve RL, Otto T, Lin Y. Npas4 regulates a transcriptional program in CA3 required for contextual memory formation. *Science*. 2011; 334:1669–1675. [PubMed: 22194569]
22. Renthall W, Carle TL, Maze I, Covington HE 3rd, Truong HT, Alibhai I, Kumar A, Montgomery RL, Olson EN, Nestle EJ. Delta FosB mediates epigenetic desensitization of the c-fos gene after chronic amphetamine exposure. *J Neurosci*. 2008; 28:7344–7349. [PubMed: 18632938]
23. Shamloo M, Soriano L, von Schack D, Rickhag M, Chin DJ, Gonzalez-Zulueta M, Gido G, Urfer R, Wieloch T, Nikolich K. Npas4, a novel helix-loop-helix PAS domain protein, is regulated in response to cerebral ischemia. *Eur J Neurosci*. 2006; 24:2705–2720. [PubMed: 17156197]
24. Wang JQ, McGinty JF. Dose-dependent alteration in *zif/268* and preprodynorphin mRNA expression induced by amphetamine and methamphetamine in rat forebrain. *J Pharmacol Exp Ther*. 1995; 273:909–917. [PubMed: 7752096]
25. Wang JQ, McGinty JF. Alterations in striatal *zif/268*, preprodynorphin and preproenkephalin mRNA expression induced by repeated amphetamine administration in rats. *Brain Res*. 1995; 673:262–274. [PubMed: 7606440]
26. Young ST, Porrino LJ, Iadarola MJ. Cocaine induces striatal c-Fos-immunoreactive proteins via dopaminergic D1 receptors. *Proc Natl Acad Sci USA*. 1991; 88:1291–1295. [PubMed: 1825356]
27. Yun J, Koike H, Ibi D, Toth E, Mizoguchi H, Nitta A, Yoneyama M, Ogita K, Yoneda Y, Nabeshima T, Nagai T, Yamada K. Chronic restraint stress impairs neurogenesis and hippocampus-dependent fear memory in mice: possible involvement of a brain-specific transcription factor Npas4. *J Neurochem*. 2010; 114:1840–1851. [PubMed: 20626564]

Highlights

- Acute AMPH injection had no effect on Npas4 protein level both in CPu and NAc *in vivo*.
- Chronic AMPH exposure did not alter the Npas4 protein levels in CPu.
- Chronic AMPH exposure readily increased Npas4 protein levels in NAc

Fig. 1.

Effects of acute AMPH administration on Npas4 and c-Fos protein expression in the CPu and NAc at 1 h after AMPH injection. (A) Effects of AMPH on Npas4 and c-Fos expression in the CPu. (B) Effects of AMPH on Npas4 and c-Fos expression in the NAc. Note that AMPH caused no change in Npas4 protein levels while it increased c-Fos expression in the two areas. Representative immunoblots are shown left to quantification of immunoblot results. Rats were treated with a single dose of AMPH (5 mg/kg, i.p.) and were sacrificed 1 h after drug injection. The quantified data are expressed as means $\pm$ S.E.M. (n = 5 per group). * $p < 0.05$ versus saline.

Fig. 2.

Effects of acute AMPH administration on Npas4 and c-Fos protein expression in the CPu and NAc at 2 h after AMPH injection. (A) Effects of AMPH on Npas4 and c-Fos expression in the CPu. (B) Effects of AMPH on Npas4 and c-Fos expression in the NAc. Representative immunoblots are shown left to quantification of immunoblot results. Rats were treated with a single dose of AMPH (5 mg/kg, i.p.) and were sacrificed 2 h after drug injection. The quantified data are expressed as means $\pm$ S.E.M. (n = 5 per group). * $p < 0.05$ versus saline.

Fig. 3.

Effects of repeated AMPH administration on Npas4 and c-Fos protein expression in the CPu and NAc. (A) Effects of repeated AMPH administration on Npas4 and c-Fos expression in the CPu. (B) Effects of repeated AMPH administration on Npas4 and c-Fos expression in the NAc. Note that AMPH induced a significant increase in Npas4 expression in the NAc. Representative immunoblots are shown left to quantification of immunoblot results. Rats were treated with AMPH (5 mg/kg, i.p.; once daily for 5 days) and were sacrificed 2 h after the final AMPH injection. The quantified data are expressed as means $\pm$ S.E.M. ($n = 5$ per group). * $p < 0.05$ versus saline.