Maintenance of amphetamine-induced place preference does not correlate with astrocytosis

Yasmina B. Martín[#], Esther Gramage[#], Gonzalo Herradón*

Pharmacology lab, Department of Pharmaceutical and Food Sciences. Facultad de Farmacia. Universidad CEU San Pablo, Madrid, Spain.

# Both authors contributed equally to this work

*Corresponding author:

Gonzalo Herradon, PhD

Lab. Pharmacology

Faculty of Pharmacy

Universidad CEU San Pablo

Urb. Montepríncipe

28668 Boadilla del Monte, Madrid, Spain.

Tel: 34-91-3724700 (Ext. 4840)

Fax: 34-91-3510475

e-mail: herradon@ceu.es

ABSTRACT

Astrocytosis, a process in which astrocytes undergo proliferation and enhancement of glial fibrillary acidic protein (GFAP) expression, has been suggested to play important roles in the maintenance of dependence to amphetamine and its derivatives. It was previously shown that mice with genetic deletion of pleiotrophin (PTN), a neurotrophic factor upregulated in different brain areas after administration of amphetamine, show a longer lasting amphetamine-induced conditioned place preference (CPP) when compared to wild type mice. In this work, we aimed to pursue the possibility of a different astrocytic response induced by amphetamine in PTN-/- and PTN+/+ mice, which could underlie the higher vulnerability of PTN-/- mice to maintain amphetamine CPP. In confirmation of previous studies, we found that PTN-/- mice significantly maintained amphetamine (3 mg/kg)-induced CPP 5 days after the last drug administration compared to PTN+/+ mice. Interestingly, the number of astrocytes in nucleus accumbens (NAcc), cingulate cortex (CG) and caudate putamen (CPu) did not differ between mice that maintained and did not maintain amphetamine-induced CPP independently of the genotype considered. However, we found that PTN-/- mice showed significantly decreased numbers of astrocytes in CG and CPu compared to PTN+/+ mice independently of whether they maintained amphetamine-induced CPP 5 days after the last drug administration or not. The data demonstrate that maintenance of amphetamine-induced CPP depends on the endogenous expression of PTN. The data tend to discard a correlation between activated astrocytes and maintenance of amphetamine conditioning effects and suggest PTN as a potential modulator of activation of astrocytes after amphetamine treatment.

Keywords: Pleiotrophin, addiction, neurotrophic factor, astrocyte, conditioned place preference, striatum.

1. INTRODUCTION

Alterations in the number, morphology and physiology of glial cells in critical areas for development of addictive behaviours such as nucleus accumbens (NAcc), caudate putamen (CPu) and cingulate cortex (CG) after exposure to drugs of abuse, may contribute to the vulnerability in maintaining drug seeking behaviours (Miguel-Hidalgo, 2009). These alterations include astrocytosis (Raivich et al., 1999), a process in which astrocytes undergo proliferation, morphological changes and enhancement of glial fibrillary acidic protein (GFAP) expression, which has been suggested as playing an important role in the development of drug dependence (Narita et al., 2008). Administration of amphetamine and its derivatives and most psychostimulants induces astrocytosis (Itzhak and Achat-Mendes, 2004; Fattore et al., 2002; Hebert and O´Callaghan, 2000). For instance, the levels of GFAP in the mouse NAcc and CG were found to be increased after chronic treatment with methamphetamine (Narita et al., 2005). Interestingly, the neurotrophic activity of these astrocytes seems to be important for the development and maintenance of drug addictive behaviours (Miguel-Hidalgo, 2009). For instance, it has been demonstrated that increased expression of Glial-Derived Neurotrophic Factor (GDNF) efficiently blocks methamphetamine-induced conditioned place preference (Niwa et al., 2007).

Another neurotrophin known to be expressed in astrocytes, pleiotrophin (PTN), has been recently confirmed as an important regulator of drug of abuse-induced neurotoxic and rewarding effects (see reviews by Herradon et al., 2009; Gramage and Herradon, 2011). Endogenous expression of PTN has been recently found to be key in the modulation of amphetamine-induced cognitive, addictive and neurotoxic effects (Herradon and Ezquerra, 2009; Gramage et al., 2010a,b, in press). In those reports, PTN

knockout (PTN-/-) mice exhibited longer lasting amphetamine-induced conditioning effects (Gramage et al., 2010a) and exacerbated neurotoxic effects induced by amphetamine in the striatum and substantia nigra (Gramage et al, 2010b). In these studies, the astrocytosis observed four days after a neurotoxic regime of amphetamine injections (10 mg/kg, 4 administrations, one every two hours) was significantly increased in the CPu and substantia nigra of PTN-/- mice compared to wild type (PTN+/+) mice. Whether or not this differential astrocytic response generated by PTN-/- mice in response to the neurotoxic regime of amphetamine injections could be observed after administration of amphetamine rewarding regimes remains to be studied. In the present study, we have pursued the possibility of a different astrocytic response induced by amphetamine in both mouse genotypes, PTN-/- and PTN+/+, which could underlie the higher vulnerability of mice lacking endogenous PTN to maintain amphetamine (3 mg/kg)-induced place preference 5 days after the last administration of the drug (Gramage et al., 2010a). We have tested the numbers of GFAP+ cells in NAcc, CG and CPu of mice from both genotypes that differ in the maintenance of amphetamine-induced place preference. These brain areas were chosen because a correlation was previously shown between development of astrocytosis in NAcc, CPu and CG and the rewarding effects of amphetamine (Narita et al., 2005, 2008).

2. MATERIALS AND METHODS

2.1. Pleiotrophin genetically deficient (PTN-/-) mice

PTN-/- mice, generated as previously described (Amet et al., 2001; Herradon et al., 2004; Gramage et al., 2012), were kindly provided by Dr. Thomas F. Deuel (The Scripps Research Institute, La Jolla, CA). The PTN gene consists of five exons encoding an 18-kDa protein with a 32 amino acid signal peptide. The replacement targeting vector generated a PTN null allele (PTN $^{2\text{-}4neo}$) by deleting exons 2 to 4. In both behavioural and immunohistochemical studies, we used male PTN-/- and wild type (PTN+/+) animals of 9-10 weeks (20-25 g). It has to be noted that the lack of gross alterations of basic behavior was previously described in PTN-/- mice (Pavlov et al., 2002; Gramage et al., in press).

All the animals used in this study were maintained in accordance with European Union Laboratory Animal Care Rules (86/609/ECC directive) and the protocols were approved by the Animal Research Committee of USP-CEU. Mice were housed at 22°C ± 1°C with a 12 hr light/12 hr dark cycle (lights on at 7am) with standard mouse chow (Harlan, Barcelona, Spain) and water provided ad libitum.

2.2. Conditioned Place Preference (CPP)

2.2.1. Apparatus

The apparatus used consisted of two Plexiglas square compartments of the same size (20 cm long x 14 cm high x 27 cm wide). One compartment had black plexiglas floor and walls and the other had black plexiglas floor and white walls. During the

amphetamine- and saline-paired sessions, the compartments were closed by a removable black guillotine door.

2.2.2. CPP procedure

The procedure selected for this study was based on a modification of the method previously used in our laboratory (Morales et al., 2007). The procedure to evaluate amphetamine-induced conditioning consisted of a 5-day schedule with three phases: preconditioning (day 1), conditioning (days 2-4) and testing (day 5). During preconditioning, PTN+/+ mice and PTN-/- mice were free to explore the two compartments for a 30-min period; their behaviour was monitored by a videotracking system (San Diego, California, USA) to calculate the time spent in each compartment. Placement was counterbalanced within each treatment group such that half the animals started in one chamber and half started in the other. As expected, and previously shown (Gramage et al., 2010a, 2011), the compartment with white walls was the less-preferred compartment by both mouse genotypes (~30 % stay of total time in the preconditioning phase).

In experiments to evaluate amphetamine-induced CPP, the conditioning phase consisted of a 3-day schedule of double conditioning sessions. The first one involved a morning session starting at 8 am, in which animals received a single injection of saline i.p. (10 ml/kg) and were immediately confined to the initially preferred compartment for 30 min. In the evening session starting at 3 pm, the animals were injected (i.p.) with 3 mg/kg amphetamine (PTN+/+, n = 12; PTN-/-, n = 18) and confined to the initially less-preferred chamber for 30 min. During the following two days, the procedure used was the same but the order of the treatments (morning/evening) was changed to avoid the influence of circadian variability. In control experiments, PTN+/+ and PTN-/- mice

received injections of saline i.p. (10 ml/kg) at both times (morning/evening) during the three days of the conditioning phase.

The testing phase was carried out on day 5 of the schedule. In this phase the animals freely moved throughout the apparatus for 30 min, exactly as in the preconditioning phase (day 1). The time spent in each compartment was also registered. The percentage of time-spent (stay) in the less-preferred compartment was then calculated in all cases and the difference between the time spent in the drug-paired (or saline-paired in the case of controls) compartment in this phase and the time spent in the same compartment in the preconditioning was considered as indicative of the degree of conditioning induced by the drug.

In previous studies we already showed that amphetamine (3 mg/kg) exerts a similar CPP in PTN-/- and PTN+/+ mice as assessed in the testing phase (day 5) of this procedure (Gramage et al, 2010a). In those studies, it was also shown that amphetamine-induced CPP was only significantly maintained in PTN-/- mice 5 days after the last administration of the drug. In the present studies we aimed to test the possibility of a different astrocytic response in both genotypes correlating with the genotypic differences found in the maintenance of amphetamine CPP. For this purpose, after the testing phase (day 5), animals were returned to their cages and were neither injected nor re-exposed to the CPP apparatus for a 4-day period. After that period of time (5 days after last drug administration; day 9 of the procedure), place conditioning was re-examined by conducting a new 30-min free choice test.

2.3. Immunohistochemistry studies

As indicated above, the purpose of the immunohistochemical analysis was to study the astrocytic response in different brain areas of mice from both genotypes that maintained and did not maintain amphetamine-induced CPP on day 9 of the procedure. The selection criteria of mice for immunohistochemical analysis according to maintenance of amphetamine-induced CPP was as follows. Mice that increased their stay in the drug-paired compartment by 15% or more in the testing phase (day 5) compared to the preconditioning phase were considered to be efficiently conditioned by the drug. After the test on day 9 of the CPP procedure, these mice were separated in two different groups per genotype: the ones that did not maintain (NM) drug-induced CPP, whose percentages of stay in the drug-paired compartment were reduced by more than 10% compared to the testing phase (day 5), and the ones that maintained (M) drug-induced CPP, whose percentages of stay in the amphetamine-paired compartment were similar or even greater to those in the testing phase (day 5).

Mice from both M and NM groups in every genotype were euthanized and brains rapidly removed and conserved in p-formaldehyde for 7 days and transferred to a solution of 0.1 M phosphate buffer containing 0.02% sodium azide for storage at 4 ºC. Following protocols previously described by our group (Alonso et al., 2007; Gramage et al., 2011), the brains were cut at a thickness of 30 μm using a vibratome (Leica, Wetzlar, Germany) and freefloating sections of NAcc, CG and CPu, were processed as follows: After endogenous peroxidase blocking by 0.3% H_2O_2, the sections were washed with PBS and placed in a blocking solution containing 5% normal horse serum and 0.3% Triton X-100 in PBS (STPBS) for 60 min. Sections were then incubated overnight at 4 ºC with anti-GFAP (1:1000 in STPBS) antibodies (Millipore, Madrid, Spain). The sections were then rinsed in PBS three times for 10 min and incubated for 30 minutes with the biotinylated secondary antibodies in PBS at room temperature. The

sections were rinsed in PBS three times for 5 minutes and then the avidin–biotin reaction was performed using Vectastain Elite ABC peroxidase kit following the protocol suggested by the manufacturer. The immunoreactivity was visualized using 0.06% diaminobenzidine and 0.03% H_2O_2 diluted in PBS. The sections were rinsed 5 minutes with phosphate buffer and mounted on gelatin/chrome alume-coated slides, air-dried overnight, dehydrated through graded ethanols, cleared in xylene, and coverslipped with DPX medium. Photomicrographs were captured with a digital camera coupled to an optical microscope (DMLS, Leica, Solms, Germany). The counting of GFAP-positive cells was accomplished with the help of the software Scion Image 4.02 (Scion Corporation, Frederick, MD, USA). As previously performed (Gramage et al, 2010a), cell counts were made in standardized areas (0.14 mm^2) obtaining the mean from 6 sections per animal and brain area.

2.4. Statistics

In the CPP studies, the percentage of time spent in the drug-paired compartment (less-preferred compartment in day 1) within each genotype was compared by one-way analysis of varianze (ANOVA) with repeated measures followed by Tukey's post-hoc tests, considering experimental phase (days 1, 5 and 9) as factor. Significance was considered at the 0.05 level.

Data obtained from image analysis of immunostaining were analysed using two-way ANOVA. Relevant differences were analyzed pair-wise by post-hoc comparisons with Bonferroni's post-hoc tests, considering genotype (PTN-/-, PTN+/+) and maintenance rate (mice that did not maintain (NM) and mice that maintained (M) drug-induced CPP on day 9 of the procedure) as variables. In addition, the linear correlation coefficient r

was calculated to measure the strength of a linear relationship between the CPP score of animals on day 9 (increase in the percentage of stay in drug-paired compartment on day 9 vs. day 1) and GFAP+ cell counts in every brain area of these mice. $P < 0.05$ was considered as statistically significant. All statistical analyses were performed using Graphpad prism 5 program.

3. RESULTS

3.1. Place Conditioning

In PTN+/+ mice, ANOVA revealed a significant effect of experimental phase in place conditioning studies with 3 mg/kg amphetamine ($F = 5.596$; $P < 0.0108$) (Fig. 1A). Post-hoc comparisons uncovered that amphetamine induced a significant place conditioning in PTN+/+ mice since they exhibited a higher increase in the time spent in the amphetamine-paired compartment on the testing phase (day 5) compared to preconditioning values (day 1). In confirmation of previous studies (Gramage et al, 2010a), five days after the last injection of amphetamine (day 9), PTN+/+ mice did not exhibit drug-induced CPP since the time-spent in the amphetamine-paired compartment was not found to be statistically different from preconditioning (day 1) values (Fig. 1A).
In PTN-/- mice, ANOVA revealed a significant effect of experimental phase in place conditioning studies with amphetamine ($F = 11.33$; $P < 0.0002$) (Fig. 1B). Post-hoc comparisons uncovered that amphetamine induced a significant place conditioning in PTN-/- mice as it occurred in PTN+/+ mice. However, in contrast to the results obtained in PTN+/+ mice, PTN-/- mice showed a robust amphetamine-induced CPP 5 days after the last injection of amphetamine, being the time spent in amphetamine-paired compartment effectively similar to that in the testing phase (day 5) (Fig. 1B).
As previously shown (Gramage et al., 2010a), saline conditioning in both compartments did not alter the percentage of stay in the compartments in control mice from both genotypes (data not shown).

3.2. Astrocytosis in brain areas of mice that maintain and that do not maintain amphetamine-induced place preference five days after last administration of amphetamine

We aimed to test a possible correlation between the development of astrocytosis in NAcc, CG and CPu and the maintenance of amphetamine-induced place preference 5 days after the last administration of the drug in mice from both genotypes. According to the criterion summarized in the methods section, we observed that 50% of PTN+/+ mice maintained amphetamine-induced CPP by day 9 of the procedure, whereas 73% of PTN-/- mice maintained drug-induced CPP at this time point. Accordingly, brains from mice from every genotype that maintained and that did not maintain amphetamine CPP by day 9 were processed for immunostaining procedures.

First, it has to be noted that we did not observe any significant differences in the generation of astrocytosis in the NAcc between mice from genotypes or between mice that did maintain and mice that did not maintain amphetamine-induced place preference on day 9 (results not shown). However, ANOVA revealed a significant effect of the genotype in the numbers of GFAP+ cells observed in CPu ($F(1,11) = 37.76$, $P < 0.0001$) and CG ($F(1,11) = 12.02$, $P = 0.0053$) of mice conditioned with amphetamine. The number of GFAP+ cells was found to be significantly increased in the CPu (Fig. 2) and in the CG (Fig. 3) of PTN+/+ mice compared to PTN-/- mice conditioned with amphetamine independently of whether they maintained amphetamine-induced CPP on day 9 or not. No significant correlations between CPP scores of animals on day 9 of the procedure and GFAP+ cell counts in CPu (WT+/+: $r = 0.15$, $p = 0.91$; PTN-/-: $r = 0.06$, $p = 0.93$) or in CG (WT+/+: $r = -0.42$, $p = 0.72$; PTN-/-: $r = 0.05$, $p = 0.94$) were found. In control mice, as expected in brain areas not characterized by the presence of

astrocytes in basal conditions, and shown in previous reports (Gramage et al., 2010a), very few GFAP-positive astrocytes were observed in the CPu and in the CG of saline-treated (conditioned) mice (data not shown).

4. DISCUSSION

Recent evidence suggests that drugs of abuse-induced glial changes significantly contribute to the vulnerability to and the persistence in drug addiction (Miguel-Hidalgo, 2009). Also, recent evidences clearly demonstrate that neurotrophins such as Brain-derived neurotrophic factor (BDNF) and GDNF play important roles in drug addiction (Boger et al, 2007; Niwa et al, 2007; Whitfield et al, 2011; McGinty et al, 2010). In the present report, we have performed a series of experiments designed to clarify the roles of a novel neurotrophic factor, PTN, in the rewarding effects of amphetamine, and the possible correlation between the development of astrocytosis in relevant brain areas to drug addiction and the maintenance of amphetamine-induced place preference. Here, we show that an intermediate dose of amphetamine (3 mg/kg) in the drug's dose range known to induce CPP (Tzschenke, 2007) caused similar CPP in PTN-/- and PTN+/+ mice. Importantly, in confirmation of previous studies (Gramage et al., 2010a), PTN-/- mice significantly maintained amphetamine-induced CPP 5 days after last administration of the drug compared to PTN+/+ mice. It is important to note that performance of PTN-/- mice of the same age as those used in the present study was found to be similar (Gramage et al., in press) or slightly decreased (Pavlov et al., 2002) compared to PTN+/+ mice in environmental cues-based learning and memory behavioural models. Therefore, it is likely that the sustained amphetamine-induced CPP found in PTN-/- mice is not due to a greater ability in learning tasks and, thus, is caused by enhanced amphetamine rewarding effects in these mice, suggesting PTN belongs to the group of neurotrophic factors with important functions in drug rewarding effects. For instance, it has been shown that infusion of BDNF into the NAcc results in persistent increases of cocaine self-administration and relapse whereas blocking BDNF

function decreases cocaine self-administration and relapse (Horger et al, 1999; Hall et al, 2003; Graham et al, 2007). In contrast, induction of GDNF and Tumor Necrosis Factor (TNF)-alpha expression has been shown to block the acquisition of methamphetamine-induced place preference (Niwa et al, 2007). In a similar manner, our data demonstrate that endogenous PTN serves to limit the maintenance of amphetamine-induced place preference.

Interestingly, all neurotrophic factors mentioned here, which are relevant to drug addiction, are also expressed in astrocytes (Appel et al, 1997; Lee et al, 2002; Miguel-Hidalgo, 2009). Astrocytes have been suggested to regulate the actions of some drugs of abuse by influencing synaptic transmission (Haydon et al, 2009) through their ability to release soluble factors such as neurotrophins (Narita et al, 2005). It has to be noted that intra-NAcc and intra-CG administration of astrocyte-conditioned medium aggravates methamphetamine-induced CPP (Narita et al, 2008). Since it has also been demonstrated that chronic administration of methamphetamine results in increased GFAP levels in NAcc and CG (Narita et al, 2005), it appeared reasonable to link the ability of a drug to generate astrocytosis with its rewarding effects. However, we have not found a correlation between the numbers of GFAP+ cells in CG, NAcc and CPu and the maintenance of amphetamine-induced place preference. The numbers of astrocytes in those brain areas were found to be effectively similar in mice that maintained and mice that did not maintain amphetamine-induced CPP 5 days after last drug administration independently of the genotype considered. However, very significant differences were identified between genotypes. In this manner, normal PTN+/+ mice conditioned with amphetamine showed significantly increased numbers of astrocytes in CG and CPu compared to PTN-/- mice. These results suggest that endogenous PTN potentiates the generation of astrocytosis in mice conditioned with a rewarding dose of

amphetamine independently of the degree of maintenance of amphetamine-induced CPP. Since intra-CG and intra-NAcc administrations of astrocyte-conditioned medium potentiate amphetamine-induced astrogliogenesis in CPP procedures (Narita et al, 2008) and PTN is known to be a rapidly secreted growth factor highly expressed in astrocytes (Martin et al., 2011), it seems reasonable to hypothesize that PTN is one of the soluble factors secreted by astrocytes contributing to astrogliogenesis in mice conditioned with amphetamine. This hypothesis is supported by the fact that PTN-/- mice conditioned with amphetamine exhibit a significantly decreased astrocytosis in CG and CPu compared to PTN+/+ mice.

Surprisingly, in our present model, differences in astrocytosis do not seem relevant in the genotypic differences found in the maintenance of amphetamine-induced place preference. It has to be noted that using other amphetamine regimes of administration, we previously demonstrated that amphetamine-induced astrocytosis is significantly enhanced in striatum and substancia nigra of PTN-/- mice compared to PTN+/+ (Gramage et al, 2010a; Gramage et al, 2010b). However, these apparent discrepancies can be reasonably attributed to the remarkable differences in the protocol of amphetamine administrations. Whereas in the latter studies, we used 4 amphetamine administrations (10 mg/kg, one every 2 hours) known to induce severe neurotoxic effects in the striatum (Gramage et al, 2010a; Gramage et al, 2010b), including an exacerbated astrocytosis and loss of dopaminergic terminals, in the present studies mice from both genotypes just received one administration of amphetamine (3 mg/kg) per day during the 3 days of the conditioning phase. It is very important to note that PTN is a confirmed survival factor for dopaminergic neurons in the nigrostriatal pathway (Hida et al., 2007; Gombash et al., 2012). Thus, it is reasonable to hypothesize that the relevance of the absence of endogenous PTN in PTN-/- mice may be high and

significantly contributes to the enhancement of the harmful effects induced by this neurotoxic regime of amphetamine administration, including astrocytosis, effects that are not observed after one administration of amphetamine (3 mg/kg) per day during the 3 days in the CPP procedure. In this manner, it has to be taken into account that the neurotoxic regime of administration of amphetamine causes a 6-fold increase in the number of striatal GFAP+ cells induced by the drug (Gramage et al., 2010a) compared to the present data after the CPP regime of administration of amphetamine.

Conclusion

The data presented here demonstrate that maintenance of amphetamine-induced conditioning effects depend on the endogenous expression of PTN. However, the data tend to discard a correlation between activated astrocytes and maintenance of amphetamine conditioning effects and suggest PTN as a potential modulator of activation of astrocytes after amphetamine treatment.

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FIGURE LEGENDS

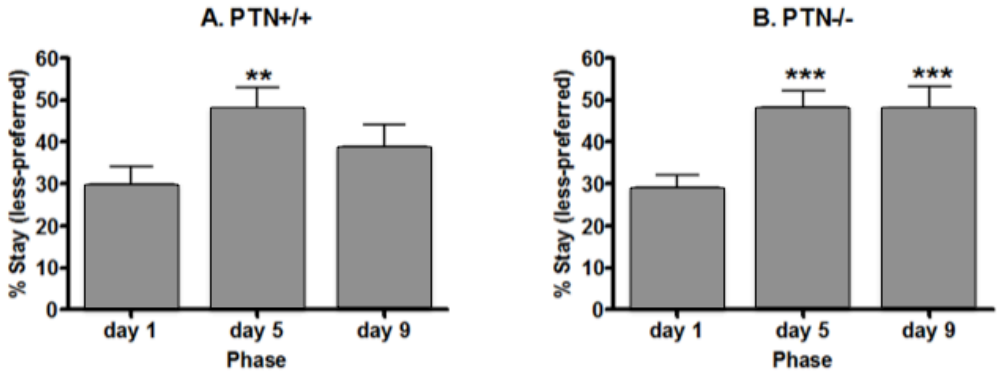

Fig. 1. Maintenance of amphetamine (3 mg/kg)-induced place preference. Results are presented as the mean ± S.E.M. of the percentage of the time spent by PTN+/+ (A) and PTN-/- (B) mice in the amphetamine (3 mg/kg)-paired (less-preferred) compartment during preconditioning (day 1) and testing phase (day 5), and 5 days after the last administration of the drug (day 9). ** $P < 0.01$, *** $P < 0.001$ vs. preconditioning (day 1).

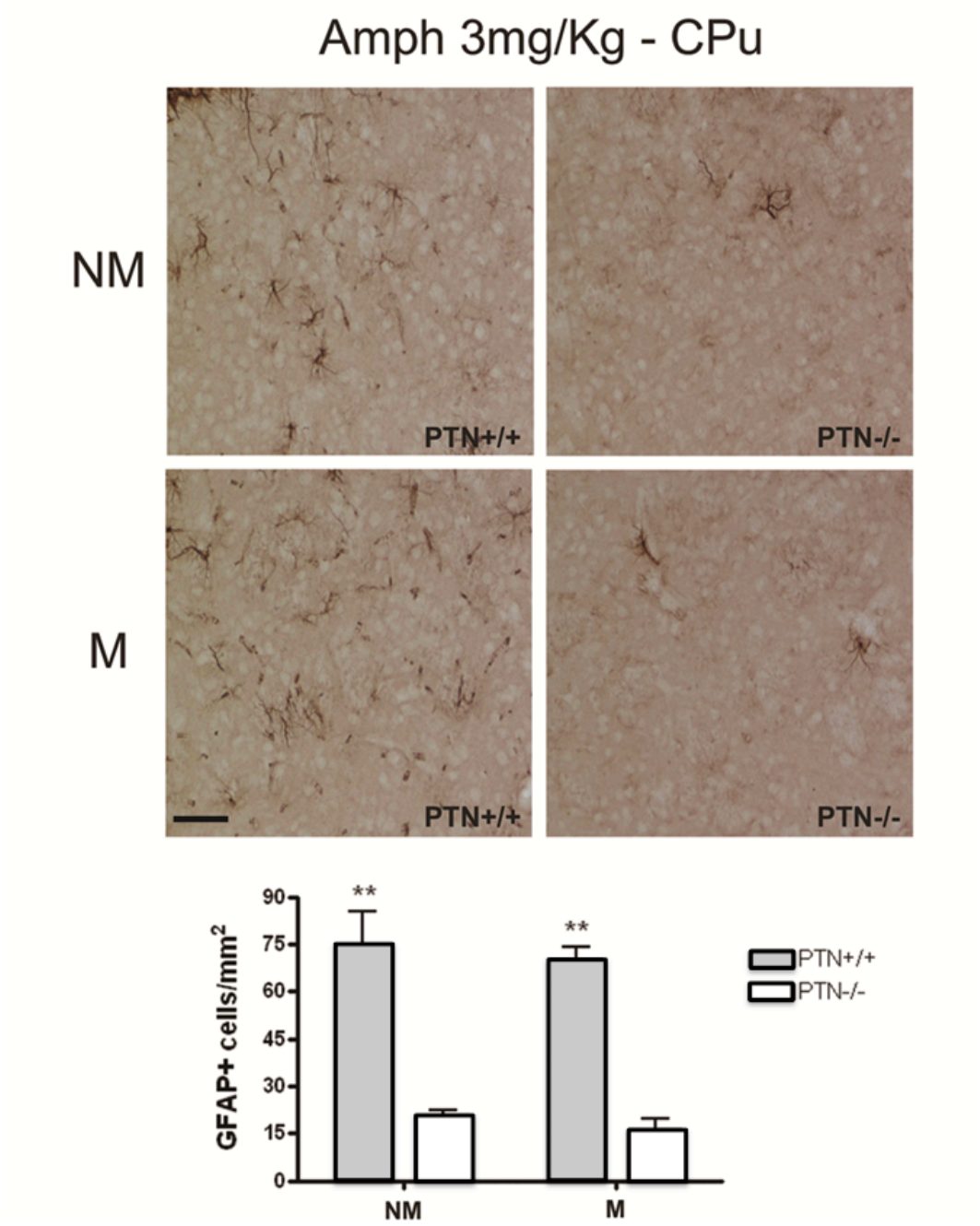

Fig. 2. Number of astrocytes in the caudate putamen (CPu) of PTN+/+ and PTN-/- mice conditioned with 3 mg/kg amphetamine. Photomicrographs are from GFAP-immunostained striatal sections of PTN-/- and PTN+/+ mice conditioned with 3 mg/kg amphetamine (Amph) that maintain (M) and that do not maintain (NM) amphetamine-

induced CPP 5 days after last drug administration. The graph represent quantification of data (mean ± S.E.M) obtained from the counts of GFAP-positive cells in the CPu. Scale bar = 50 µm. ** P < 0.01 vs. PTN-/-.

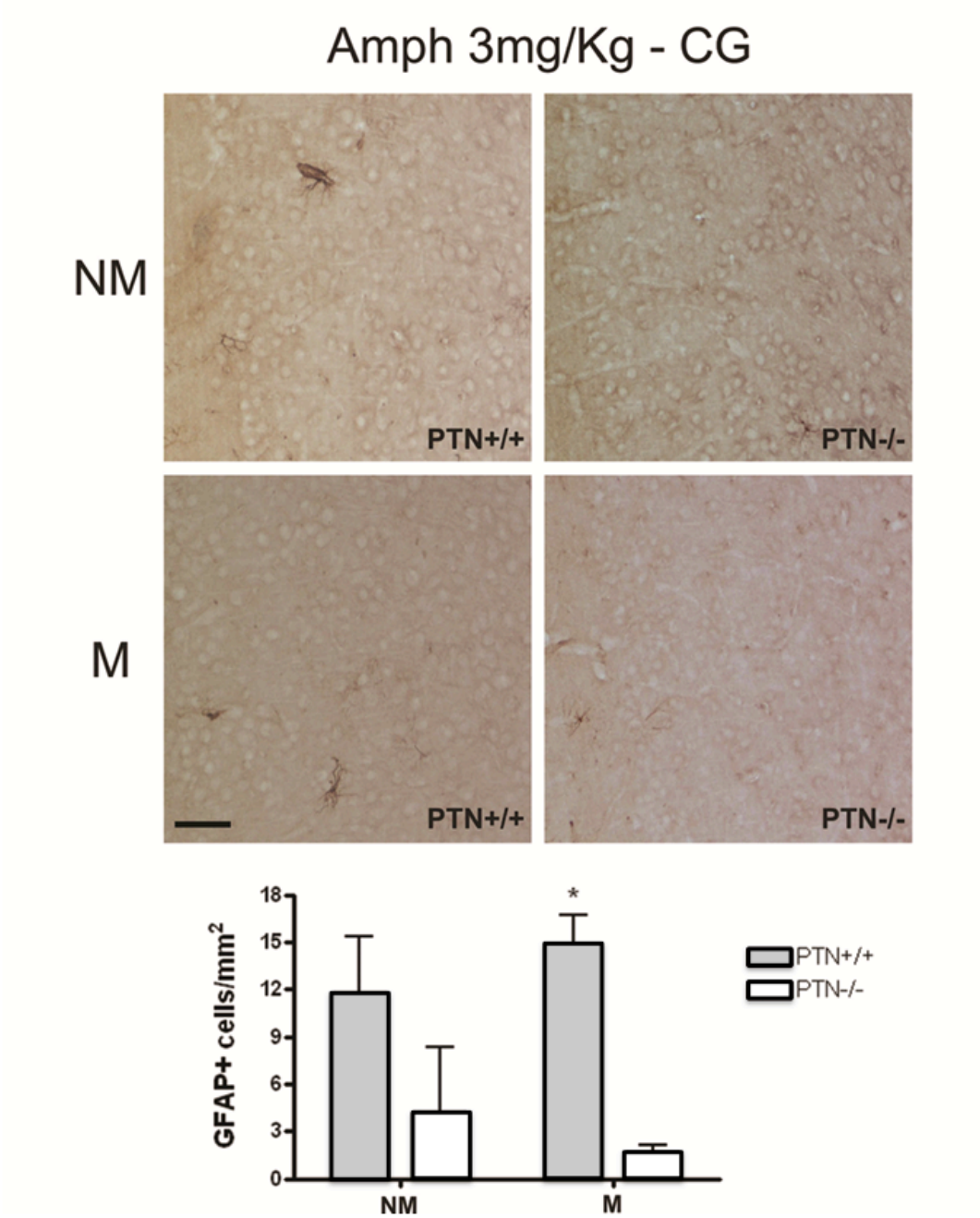

Fig. 3. Number of astrocytes in the cingulated cortex (CG) of PTN+/+ and PTN-/- mice conditioned with 3 mg/kg amphetamine. Photomicrographs are from GFAP-immunostained CG sections of PTN-/- and PTN+/+ mice conditioned with 3 mg/kg amphetamine (Amph) that maintain (M) and that do not maintain (NM) amphetamine-induced CPP 5 days after last drug administration. The graph represent quantification of data (mean ± S.E.M) obtained from the counts of GFAP-positive cells in the CG. Scale bar = 50 μm. * $P < 0.05$ vs. PTN-/-.