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The Role of Tissue-Type Plasminogen Activator System in Amphetamine-Induced Conditional Place Preference Extinction and Reinstatement

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Extracellular serine proteases of the plasminogen activator family (tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) may modulate synaptic adhesion and associate with learning behavior. Psychostimulants strongly induce their expression in the mesolimbic dopaminergic pathway, but cocaine preferentially induces uPA, whereas morphine and amphetamine preferentially induce tPA. tPA-expressing animals displayed enhanced conditional place preference (CPP) for amphetamine compared with uPA-overexpressing animals. Thus, modulation of the plasminogen system in the brain might be a potential target against drugs of abuse. In the present study, we aim to identify whether tPA is involved in the acquisition/learning phase or in the expression/retrieval phase of conditioned drug preference. For this purpose, animals were injected with lentiviruses expressing or silencing tPA in the NAc and place preference was assessed. We found that tPA expression is associated with acquisition of place preference and animals overexpressing tPA spend >87% of the time in the drug-associated compartment, compared with 60% for control animals. When ectopic expression of tPA has been inhibited by doxycycline during acquisition, animals do no more associate the environment with the drug. Suppression of endogenous tPA expression in animals treated with LV-siRNA fully suppresses place preference, and these animals appear to avoid the drug-associated box. tPA overexpression delays extinction, but priming with low doses of amphetamine reinstates place preference even after full extinction. Together, these data clearly indicate that tPA plays an important role in acquisition of amphetamine-induced CPP, but its role in CPP expression does not seem important.

Keywords: RNA interference; plasminogen activator; cocaine; drugs of abuse; lentivirus; *in vivo* gene transfer

INTRODUCTION

Activity-dependent synaptic plasticity and remodeling of the mesolimbic dopaminergic system play a crucial role in the development of drug dependence (Nestler, 2001). Previous studies have shown that psychostimulants strongly induce urokinase-type plasminogen activator (uPA) expression in the mesolimbic dopaminergic pathway, which has a major role in drug-mediated behavior and plasticity changes (Bahi *et al*, 2004a, 2006; Bahi and Dreyer, 2007). Extracellular serine proteases of the plasminogen activator family may modulate synaptic adhesion and associate with long-term potentiation and learning behavior. Furthermore, overexpression of uPA in the nucleus accumbens (NAc) shell strongly affects cocaine-induced conditional place preference (CPP) and delays extinction (Bahi and Dreyer, 2007). The NAc plays a central role in reward and is also an important structure regarding learning and memory (Dong *et al*, 2007; Gerdjikov *et al*, 2007; Ferretti *et al*, 2007). On the other hand, tissue-type plasminogen activator (tPA) is abundantly expressed in the central nervous system and contributes to the regulation of numerous aspects of synaptic plasticity and remodeling (Qian *et al*, 1993; Bu *et al*, 1994; Seeds *et al*, 1995; Hayden and Seeds, 1996; Davies and Silver, 1998). tPA participates in neurite outgrowth and neuronal development by cleaving proteins of the extracellular matrix and potentially forming a path for extending processes (Wu *et al*, 2000; Jacovina *et al*, 2001). It may serve as a potential mediator of L-LTP and may be involved in activity-related formation of perforated synapses in the hippocampus (Neuhoff *et al*, 1999).

A role for tPA in drug addiction has been suggested, since it enhances amphetamine and morphine behavioral sensitization (Nagai *et al*, 2004, 2005). Furthermore, tPA-knockout mice are not sensitized when tPA is absent and, if recombinant tPA is given back, they display a CPP score almost identical to that of wild-type mice; in addition, at low doses of cocaine, tPA-knockout mice showed enhanced

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locomotor activity when compared with their wild-type littermates, and displayed greater behavioral sensitization than the wild types (Ripley *et al*, 1999). Additionally, the reinforcing effects of morphine were reduced in tPA-knockout mice. Therefore, it has been suggested that modulation of the tPA system in the brain might be a potential target against drugs of abuse (Yan *et al*, 2007).

In previous studies, we presented further evidence for a significant function of extracellular proteases, tPA and uPA, in addiction and drug-related synaptic plasticity (Bahi and Dreyer, 2007). Our data suggested a differential role of plasminogen activators in this context. Psychostimulants induce both tPA and uPA in acute and chronic drug delivery, but cocaine preferentially induces uPA, whereas morphine and amphetamine preferentially induce tPA (Bahi and Dreyer, 2007). We showed that plasminogen activators tPA and uPA induce distinct behaviors. uPA-expressing animals displayed enhanced CPP for cocaine compared with tPA-overexpressing animals. By contrast, tPA-expressing animals displayed greater place preference compared with uPA-overexpressing animals when administered amphetamine or morphine (Bahi and Dreyer, 2007). These results could be interpreted according to differential pattern of activation and downstream targets. Also, when uPA was inhibited during the acquisition phase, animals no longer associated the environment with the drug and cocaine-induced place preference and reinstatement was largely dependent on active extracellular uPA. As we had proposed (Bahi and Dreyer, 2007), these observations suggest a putative role for inhibitors of specific plasminogen activators as useful tools for the treatment of addiction.

In the present study, we further investigated the role of the plasmin system in the expression of drug sensitization through emphasis on the role of tPA in amphetamine related behavior. The aim was to identify whether tPA is involved in the acquisition/learning phase or in the expression/retrieval phase of conditioned drug preference. For this purpose, animals were injected with lentiviruses expressing or silencing tPA in the NAc and place preference was assessed. The major finding was that tPA expression enhanced acquisition, but not expression, of amphetamine-induced CPP.

MATERIALS AND METHODS

Animals

Animals used in this experiment were male Wistar rats weighing 220–250 g that were obtained from Janvier (Genest-St-Isle, France). All animal experiments were carried out in accordance with the guidelines and regulations for Animal Experimentation, BAG, Bern, Switzerland. Three animals were housed per clear plastic cage with wire grid lids. Access to food and water was *ad libitum*, with illumination set on a 12-h light : 12-h dark cycle (light off at 0700 hours).

Lentivirus Construction of tPA-Expressing Lentivirus (LV-tPA), LV-GFP and LV-siRNAs

Using total RNA from cocaine-treated rat brain, the tPA cDNA was amplified and 6His-tagged by reverse transcription using M-MLV-RT (Invitrogen, Switzerland) following the manufacturer's instructions. We performed PCR amplification using the following set of rat tPA-specific primers: forward primer (with *Bam*HI) 5′-CGCG**GGATCC**ATGAAGGG AGAGCTGTTGTGC-3′ and reverse primer (with *Xho*I): 5′-GC CG**CTCGAG**TTAATGATGATGATGATGATGTTGCTTCATG TTGTCTTGAAT-3′. The cDNA was then digested with *Bam*HI and *Xho*I and cloned into similar sites in the lentiviral system transfer vector pTK431. A control vector construct, in which green fluorescent protein (GFP) expression is regulated by a tetracycline-inducible promoter, was generated by cloning a *Bam*HI/*Bgl*II DNA fragment containing the GFP gene into a *Bam*HI site in pTK431 (Bahi *et al*, 2004a, b, 2005a, b, 2006; Bahi and Dreyer, 2007).

To silence tPA expression *in vivo*, three targets were designed according to the rat tPA mRNA sequence (GenBank accession number M23697). The following targets within the tPA sequence were selected: first target, 2–25; second target, 1650–1673; third target, 733–756. an *Xho*I restriction site was 3′ added to each oligo. Using pSilencer 1.0-U6 (Ambion, UK) as a template and a U6 promoter-specific forward primer containing *Bam*HI (in bold) restriction site, GC**GGATCC**CGCTCTAGAACTAGTGC, each small interfering RNA (siRNA) target was added to the mouse U6 promoter by PCR (each target contains a 3′-specific, U6 promoter-specific primer). The PCR conditions were highly stringent to avoid mutations within the targets. The following PCR program was performed: 120 s at 94°C (initial denaturation) followed by 94°C for 45 s, 64°C for 45 s, and 72°C for 45 s, repeated over 35 cycles. The PCR reaction contains 4% dimethylsulfoxide (Sigma, Switzerland). The PCR product was digested with *Bam*HI and *Xho*I, cloned into similar sites in pTK431, and sequenced to verify the integrity of each construct.

After cloning and sequencing, all plasmids were $CsCl_2$-purified. Vesicular stomatitis virus G-pseudotyped lentiviruses were produced by the transient calcium phosphate cotransfection of human embryonic kidney 293T (HEK293T) cells with pTKs vectors together with pMDG-VSV-G and pΔNRF as described previously (Naldini *et al*, 1996; Bahi *et al*, 2004a, b, 2005a, b, 2006; Bahi and Dreyer, 2007). Lentiviral vector quantifications were performed according to the p24 ELISA kit (KPL, USA) in accordance with the manufacturer's instructions.

Surgery

All surgical procedures were performed as previously described (Bahi *et al*, 2004a, b, 2005a, b, 2006; Bahi and Dreyer, 2007). Briefly, rats were anesthetized with a mix of ketamine/xylazine (100 mg/kg/10 mg/kg, i.p.), and then, using a stereotactically guided 5-μl Hamilton syringe, 2 μl per site of concentrated lentiviral solution (approximately 200 000 ng of p24 antigen per milliliter) was bilaterally injected into the NAc at the following coordinates: anterior, +1.4; lateral, ±1.6; ventral, −6.8; Paxinos and Watson (1998). The rate of infusion was 1 μl/min, and the needle was left in place for an additional 5 min prior to withdrawal. After surgery, animals were injected subcutaneously with 5 ml of pre-warmed saline to avoid animal dehydration, and were allowed 7 day to recover prior to experimentation.

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CPP Test

CPP was performed as described previously (Mueller and Stewart, 2000). Briefly, in the pre-conditioning period, rats ($n = 9$) were allowed to move freely between two interconnected compartments (consisting of either wire grid or mesh floor) daily for 20 min for 3 days (days 1–3). On day 3, the amount of time spent in each chamber was monitored and used to assess unconditioned preference. Prior to conditioning, the rats were divided into two groups ($n = 18$ per group). The first group of animals received water supplemented with 5% sucrose, and the second group of animals received water supplemented with 5% sucrose and 0.02% doxycycline only during conditioning 'acquisition' (days 3–9). During the conditioning phase, the rats were injected on days 4, 6, and 8 with amphetamine (5 mg/kg, i.p.; $n = 9$ per group) and immediately confined in the floor-mesh box for 20 min. On days 5, 7, and 9, the rats were injected with 0.9% saline (1 ml/kg) and placed in the wire grid chamber for 20 min. During the conditioning phase, passage between chambers was blocked by a guillotine door. On day 10, the post-CPP test was performed without drug treatment and without doxycycline. Animals were placed in the interconnecting passage between the two chambers, with the guillotine door removed, and were allowed free access to the entire setup. The time spent in each chamber was measured for 20 min. Drug-induced place preference was expressed by Post *vs* Pre, which was calculated as follows: [(Post value)−(Pre value)], where Post and Pre values indicate the difference in time spent at the drug-conditioning site during post-conditioning and pre-conditioning periods, respectively.

Extinction by repeated testing. After conditioning and following the initial CPP test, rats ($n = 9$) underwent 20-min tests daily for 10 days. No injections were performed during this extinction period.

Reinstatement by amphetamine priming after extinction. After conditioning and following the initial CPP test, rats ($n = 9$) underwent 20-min tests daily for 10 days. No injections were performed during this extinction period. The animals did not receive amphetamine during this period. Following this period of extinction, all animals ($n = 9$) received a priming injection of 0.9% saline (1 ml/kg) tested for CPP, or were injected with amphetamine (2 mg/kg, i.p.) immediately before the final test for CPP.

Maintenance of the CPP. After CPP test, rats ($n = 9$) underwent a 5-week period of non-exposure to the test apparatus to determine whether retention of CPP would be maintained. During this time, animals were not injected and not tested, but were kept in their home cages. After this period, rats were placed in the apparatus and provided free access to both the compartments over a 20-min period.

Immunohistochemistry

Brain section staining was performed according to previously published procedures (Bahi *et al*, 2004a; Bahi and Dreyer, 2007). Briefly, rat brains were rapidly removed and frozen in isopentane at −30°C for 3 min and kept at −25°C. Coronal sections were cut at 14 μm with a cryostat (Leitz), placed on gelatinized glass slides, and air-dried at room temperature for 20 min. Antigens were localized using the avidin–biotin–peroxidase technique. Slices were fixed in 4% PFA for 15 min, and washed three times with 1 × PBS. Endogenous peroxidase activity was quenched with 2% hydrogen peroxide in water (H_2O_2) for 40 min at room temperature. Nonspecific binding was blocked for 30 min at RT in 1 × PBS containing 1% bovine serum albumin, 1% Triton X-100, and 3% normal goat serum. Sections were then rinsed and incubated overnight with mouse anti-histidine antibody (MCA1396, 1:12 000; Serotec) diluted in 1 × PBS containing 1% Triton X-100 and 1% normal goat serum. Sections were then washed three times with 1 × PBS and incubated with the biotinylated secondary antibody (goat anti-mouse immunoglobulin G, 1:500; Vector Laboratories, Burlingame, CA) for 45 min at room temperature. Sections were rinsed three times for 5 min in 1 × PBS at room temperature, followed by incubation in avidin–biotin complex (Vector Laboratories) in 1 × PBS solution. After three rinses in 1 × PBS, all sections were developed in 0.025% 3–3′ diaminobenzidine tetrahydrochloride plus 0.02% H_2O_2 for 10–15 min. Sections were then dehydrated, mounted in permanent medium (Eukitt), and examined with a Zeiss light microscope.

Statistical Analysis

All data were expressed as means ± SEM and analyzed using SPSS v11 software. In the analysis of CPP, statistical analysis was performed using an analysis of variance. First, an overall F-test analysis was performed to identify significant differences among any of the group means. In case of statistically significant F-ratio scores ($p < 0.05$), a second analysis using Tukey's test has been carried out, where sets of two groups at a time were compared to specifically determine significance differences. Acceptable significance level was set at $p < 0.05$.

RESULTS

LV-tPA Enhances Amphetamine-Induced Place Preference

Twelve groups of animals were prepared. Four groups consisted of animals injected with LV-GFP into the NAc. Another set of four groups of animals was injected with LV-tPA into the Nac, and a third set of four groups was infected with a lentivirus expressing siRNAs silencing locally endogenous tPA expression in the NAc. Two groups of each set were fed doxycycline, and the other groups of each set were fed 5% sucrose without doxycycline. Animals were then trained for CPP: rats were habituated for 3 days and tested for pre-conditioning, then trained for 6 days for CPP, and then tested for post-conditioning. Some groups were trained with saline injections, while others were treated with amphetamine injections, during the conditioning phase of CPP training. Thereafter, place preference was evaluated.

The data from this experiment are summarized in Figure 1. Before conditioning (at day 3), animals displayed no place preference for the two compartments (Figure 1a and b). Under all conditions, CPP was unbiased, since

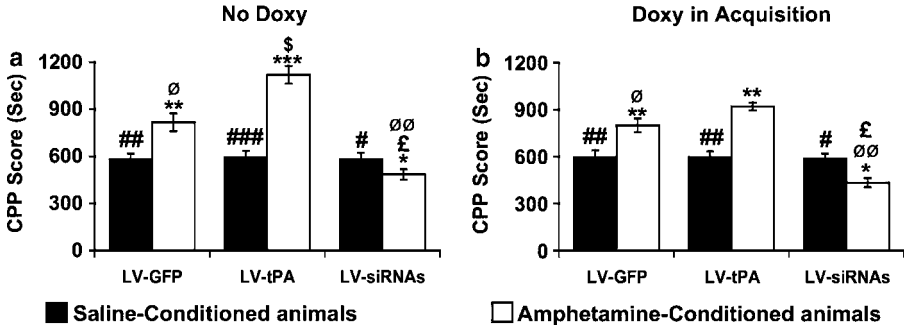

Figure 1 LV-tPA enhances amphetamine-induced place preference. Animals were injected with either LV-GFP, LV-tPA, or LV-siRNAs. Rats were habituated to the CPP cages for 3 days and tested for pre-conditioning; then they were trained for CPP using amphetamine (5 mg/kg) or 0.9% saline (1 ml/kg) for 6 days and tested for post-conditioning (a and b). Six groups of animals received 5% sucrose in the drinking water during conditioning (CPP acquisition) ('No Doxy', a), six groups received water supplemented with 5% sucrose and 0.02% doxycycline during conditioning ('Doxy in Acquisition', b), and doxycycline was removed 24 h prior to post-conditioning. Values indicate means ± SEM. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.005$ compared with pre-conditioning (expression); $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.005$ compared with amphetamine-conditioned animals; $^{\varnothing}p<0.05$, $^{\varnothing\varnothing}p<0.01$ compared with LV-tPA injected animals; $^{£}p<0.05$ compared with LV-tPA injected animals; $^{\$}p<0.05$ compared with animals fed doxycycline during conditioning (acquisition).

during this preconditioning phase, rats spent the same amount of time in both the chambers (data not shown; $F_{(11,60)}=0.853$; $p>0.589$). After conditioning (at day 10), no place preference was observed in all six groups injected with saline during conditioning (Figure 1a, $F_{(5,30)}=0.529$; $p>0.735$). Upon amphetamine conditioning, GFP-treated animals displayed a slight preference for the drug-paired box and spent 25% more time in this compartment (Figure 1a, $F_{(5,30)}=134.986$; $p<0.0001$). Further, animals stereotactically injected with LV-tPA, overexpressing tPA in the NAc, displayed much stronger preference for the drug-associated compartment, where they spent >87% of the time (Figure 1a, $F_{(5,30)}=134.986$; $p<0.0001$). In contrast, when tPA expression was suppressed in the NAc, in animals injected with LV-siRNAs, place preference was reduced even below the levels of the GFP control animals (Figure 1a, $F_{(5,30)}=134.986$; $p<0.0001$).

Other groups of animals were infected with the same lentiviruses into the NAc, but were fed doxycycline during acquisition. Thereafter, at the end of the training period (day 10), doxycycline was removed from the drinking water and animals were given doxycyline-free water. Under these conditions, control animals, treated with LV-GFP, displayed no significant CPP score difference compared with control animals fed given the normaldoxycycline-free regimen throughout (compare LV-GFP in Figure 1a and b, $F_{(5,30)}=134.986$; $p>0.98$). By contrast, in animals treated with LV-tPA the CPP score upon amphetamine conditioning was strongly reduced in comparison with animals given the normal regimen ($F_{(5,30)}=134.986$; $p<0.0001$), and, under these conditions, LV-tPA animals still displayed difference in CPP compared with LV-GFP-treated animals ($F_{(5,30)}=86.763$; $p<0.004$; Figure 1b). Furthermore, doxycycline feeding had no effect on LV-siRNA-treated animals (Figure 1b, $F_{(5,30)}=9.963$; $p>0.663$). Therefore, avoiding overexpression of tPA during the acquisition phase of place preference suppresses amphetamine-induced place preference.

LV-tPA-Mediated Delayed Extinction of Amphetamine-Induced Place Preference

Animals injected with either LV-GFP, LV-tPA, or LV-siRNAs were trained with daily injections of amphetamine or saline as described under Materials and Methods. Finally, after CPP monitoring on day 10, they were placed daily into the setup for 20 min/day for 12 days, but drug was not injected during this extinction period. As shown in Figure 2, saline-treated animals displayed no place preference and a stable CPP score throughout the four time points considered for the statistical analysis, even if animals have been fed doxycycline during the acquisition period (Figure 2a and b, day 1, $F_{(5,30)}=1.19$; $p>0.337$, or Figure 2a and b, day 7, $F_{(5,30)}=0.66$; $p>0.657$, and Figure 2a and b, day 12,: $F_{(5,30)}=0.329$; $p>0.892$). No difference was observed between the beginning and the end of the CPP extinction period (Figure 2a and b, day 1 *vs* day 12, $F_{(5,30)}=0.349$; $p>0.849$).

By contrast, in groups injected with amphetamine during the acquisition period, strong place preference and CPP score were induced among tPA-treated animals (Figure 1b, $F_{(5,30)}=134.986$; $p<0.0001$). During extinction, tPA-injected animals still display higher CPP score at day 1 (Figure 2a′, $F_{(5,30)}=260.475$; $p<0.0001$) or day 7 (Figure 2a′, $F_{(5,30)}=56.695$; $p<0.0001$), and large differences between days 1 and 12 (Figure 2a′, $F_{(5,30)}=110.339$; $p<0.0001$). However, no difference was observed at day 12 between all the groups. LV-GFP-treated animals, as well as LV-siRNA-treated animals, also displayed extinction upon drug withdrawal ($F_{(11,60)}=74.445$; $p<0.0001$). At the end of the withdrawal period, all groups of animals displayed no preference anymore for the drug-associated compartment (Figure 2a′, day 12, $F_{(5,30)}=1.531$; $p>0.210$). Clearly, GFP-treated animals, as well as siRNA-treated animals, were unable to distinguish between the two compartments already 7 days after the CPP test ($F_{(3,20)}=0.247$; $p>0.862$, and $F_{(3,20)}=1.602$; $p>0.22$, respectively), whereas tPA-treated animals displayed a longer extinction latency ($F_{(3,20)}=95.544$; $p<0.0001$), which lasted for approximately 12 days, in part due to the fact that place preference was much higher initially in this group. The same observations could be made with animals fed doxycycline during acquisition (Figure 2b′), although under these conditions tPA-treated animals displayed lower CPP score at the end of the training period (as already shown in Figure 1b, $F_{(11,60)}=35.185$; $p<0.0001$).

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Priming with Low Dose of Amphetamine, but not Saline, Reinstates Amphetamine-Induced Place Preference by LV-tPA After Extinction

At the end of the extinction period, all animals were injected with saline and tested for CPP, but no place preference was observed in all the groups of animals (data not shown; $F_{(11,60)} = 0.715$; $p > 0.719$). Thereafter, animals were given one single injection of amphetamine (2 mg/kg, i.p.) and tested for CPP during 20 min (Figure 3a and b). This priming injection stimulated place preference and all groups of animals immediately recapitulated the CPP score observed at the end of the training sessions (compare day 10; Figure 1, $F_{(11,60)} = 79.857$; $p < 0.0001$). Animals that had been trained with saline during the conditioning phase, displayed no place preference in all the groups ($F_{(5,30)} = 1.047$; $p > 0.409$), but for animals trained with amphetamine during conditioning, despite drug withdrawal for 12 days the CPP scores observed at the post-conditioning session were immediately recovered upon this single priming drug injection, whatever the feeding regimen (Figure 3, $F_{(5,30)} = 140.54$; $p < 0.0001$).

LV-tPA Maintained Amphetamine-Induced Place Preference

Animals were injected with LV-GFP, LV-tPA, or LV-siRNAs and trained for CPP as described above. After CPP monitoring on day 10, rats were placed back in their home

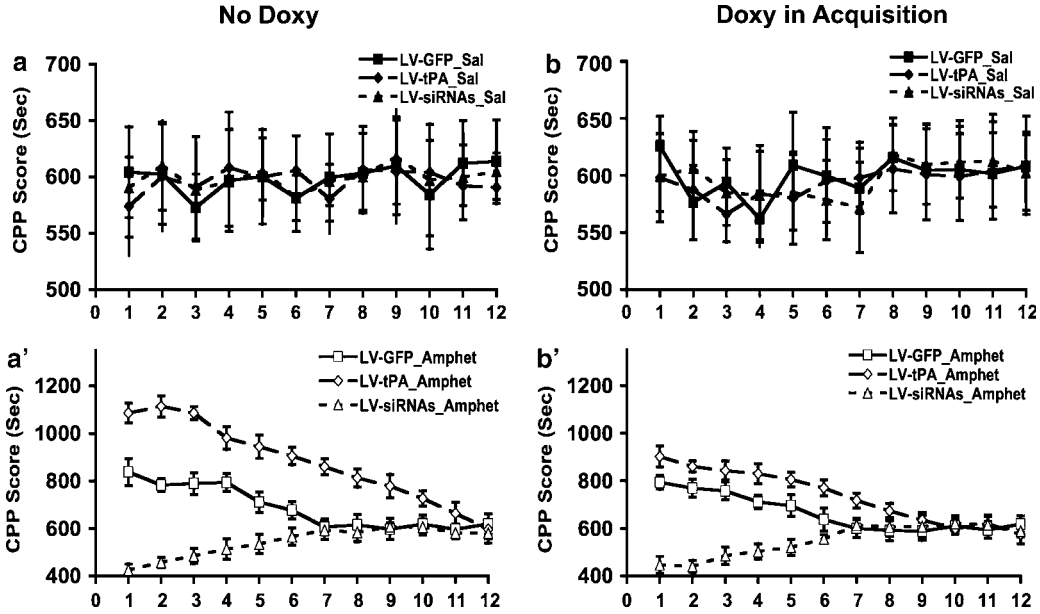

Figure 2 LV-tPA-mediated delayed extinction of Amphetamine-induced place preference. Animals were injected with either LV-GFP, LV-tPA, or LV-siRNAs and trained with daily injections *of.* saline or amphetamine (see Materials and Methods). After CPP monitoring on day 10, they were placed daily into the setup for 20 min/day for 12 days, but drug or saline were not injected during this extinction period. (a) Saline-injected animals fed 5% sucrose regimen during acquisition; (b) saline-injected animals fed doxycycline regimen during acquisition; (c) amphetamine-injected animals fed 5% sucrose regimen during acquisition; and (d) amphetamine-injected animals fed doxycycline regimen during acquisition. Values indicate means ± SEM.

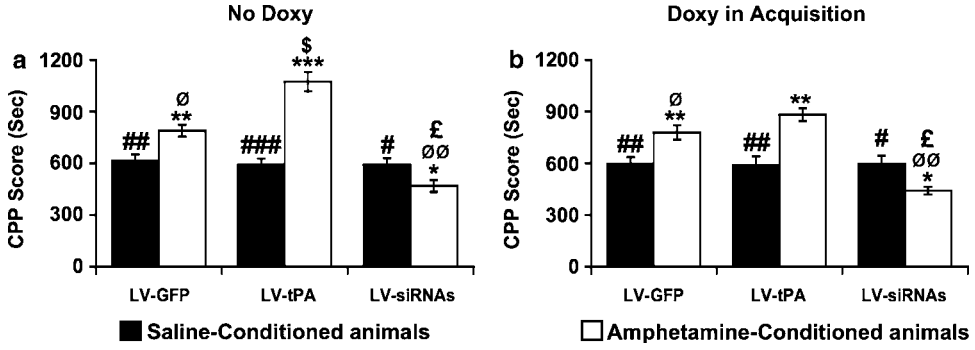

Figure 3 Low dose of amphetamine but not saline priming reinstates amphetamine-induced place preference by LV-tPA after extinction. Animals were injected with LV-GFP, LV-tPA, or LV-siRNAs and trained for CPP as in Figure 1. After CPP monitoring on day 10, rats were placed daily into the setup for 20 min during 12 days. Animals were then given one single injection of 0.9% saline (1 ml/kg, i.p.) or amphetamine (0.5 mg/kg, i.p.) and tested for CPP during 20 min. Values indicate means ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$ compared with saline-primed animals. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.005$ compared with amphetamine-conditioned animals; $^{\varnothing}p < 0.05$, $^{\varnothing\varnothing}p < 0.01$ compared with LV-tPA-injected animals; $^{£}p < 0.05$ compared with LV-tPA-injected animals; $^{\$}p < 0.05$ compared with animals fed doxycycline during conditioning (acquisition).

cages without testing or injection for 5 weeks. After this withdrawal period, animals were placed into the CPP setup and submitted to a final test for CPP during 20 min, with no injection of either saline or amphetamine. Placed into this environment, animals immediately displayed CPP scores almost identical to those achieved at the end of the training period ($F_{(11,60)} = 74.177$; $p < 0.0001$). No significant difference was observed for each respective group between CPP scores observed in Figures 1, 4a and b, respectively (within group comparison, $p > 0.134$). Under these conditions, tPA-overexpressing animals that had been conditioned with amphetamine again spent >85% of the time in the drug-associated compartment ($F_{(3,20)} = 115.18$; $p < 0.0001$), whereas if tPA expression had been downregulated during conditioning (either with doxycycline, that is, downregulating ectopic expression, or with siRNAs, that is, suppressing endogenous expression), the time spent in the drug-associated compartment was considerably reduced ($F_{(3,20)} = 24.65$; $p < 0.01$). In all cases, scores were identical to those obtained immediately after conditioning.

Immunocytochemistry

Animals treated with LV-GFP, LV-tPA, or LV-siRNAs were decapitated and brains were removed for immunocytochemistry. As shown in Figure 5, lentivirus treatment led to strong overexpression of ectopic tPA in brains from animals given normal regimen, as detected using 6His monoclonal antibody, whereas after a doxycycline regimen, tPA in the NAc was reduced to levels similar to those found in control animals (ie, treated with LV-GFP) (Figure 5). On the other hand, animals treated with LV-siRNA display no tPA expression in the NAc, indicating almost full suppression of the gene (Figure 5). These data were further confirmed by zymography, which showed that the active enzyme was fully absent in the NAc from animals treated with LV-siRNAs, but was present in the NAc of LV-tPA-treated animals (data not shown). tPA activity was enhanced in the absence of doxycycline, and upon cocaine priming, but was absent when animals were injected LV-GFP or LV-siRNAs, or when animals were given doxycycline in the drinking water during acquisition, and also when animals received saline injection for priming. This correlates with behavioral observations and indicates that the proteolytic activity is probably necessary for the acquisition of CPP.

DISCUSSION

The data in this study suggest that overexpression of tPA in the NAc strongly affects CPP, and may also delay CPP extinction. Ectopic overexpression of tPA in the NAc was achieved by administration of LV-tPA, which was active during the acquisition phase of place preference conditioning using amphetamine as an unconditioned stimulus. The

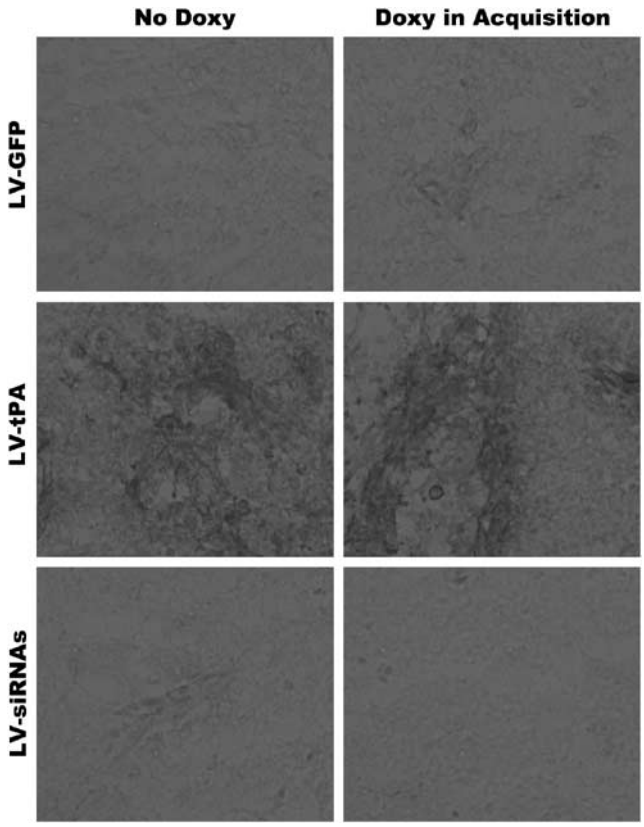

Figure 5 tPA immunohistochemistry at the site of stereotactic injection of LV-GFP, uPA expressing lentivirus (LV-uPA), or LV-siRNAs in the NAc. Animals were killed at the end of the experiment, and brains were dissected out and processed for immunohistochemistry (see section Materials and Methods for details). Rats were injected with LV-GFP (upper panels), LV-uPA (middle panels), or LV-siRNAs (lower panels). Rats were treated with normal doxycycline-free regimen (left panels) or with 0.02% doxycycline during acquisition (right panels). Magnification: × 60.

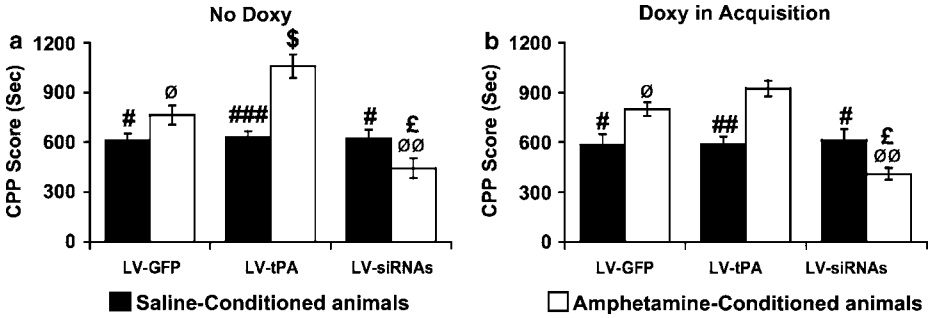

Figure 4 LV-tPA maintained amphetamine-induced place preference. Animals were injected with LV-GFP, LV-tPA, or LV-siRNAs and trained for CPP as in Figure 1. After CPP monitoring on day 10, rats were placed back in their home cages without testing or injection during 5 weeks. After this period, animals were submitted to a final test for CPP during 20 min. Values indicate means ± SEM. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with amphetamine-conditioned animals; $^{\varnothing}p < 0.05$, $^{\varnothing\varnothing}p < 0.01$ compared with LV-tPA-injected animals; $^{£}p < 0.05$ compared with LV-tPA injected animals; $^{\$}p < 0.01$ compared with animals fed doxycycline during conditioning (acquisition).

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results showed that tPA-overexpressing animals spent more than 87% of the time in the amphetamine-associated compartment, compared with 60% preference displayed by conditioned animals given the control infusion, LV-GFP. When ectopic expression of tPA was inhibited by doxycycline throughout the acquisition period, LV-tPA-treated animals failed to show augmented CPP. These latter results confirmed that the LV-tPA needed to be active, and producing tPA, in order to promote the development of greater preference to an amphetamine-associated context.

While the foregoing results involved the supplementation of endogenous tPA with virally delivered tPA, an additional group of animals provided the converse condition of lowering endogenous tPA levels using LV-siRNA treatment that targets tPA mRNA. This treatment was also localized to the NAc and revealed effects opposite of those obtained with LV-tPA treatment. That is, amphetamine-conditioned animals given LV-siRNA during the acquisition phase developed significant reduction in preference to the drug-associated context. In spending significantly less time in the drug-associated chamber than LV-GFP controls (and LV-tPA animals), these data suggest that reducing endogenous levels of tPA promotes the development of a conditioned aversion. Moreover, this is a learned response, given that extinction trials resulted in a reduction of context avoidance in LV-siRNA animals previously conditioned with amphetamine.

Whether the latter observations are confined to stimulant drugs, remains to be determined. Conceptually, it is relevant that low or impaired production of tPA in mice was shown to enhance emotional reactivity to psychogenic stressors and impair learning (Ammassari-Teule *et al*, 2001; Madani *et al*, 2003; Pawlak *et al*, 2005). Moreover, stimulants, such as amphetamine, are sympathomimetics that produce significant elevations in various central and peripheral parameters of physiological arousal (eg, activation of the noradrenergic system). Therefore, it could be hypothesized that reductions in tPA reduce the threshold for arousal in response to CNS stimulants, reducing their reinforcing efficacy for drug-seeking behavior. This is conceivable in light of studies of tPA-deficient mice showing greater locomotor responses to low doses of cocaine, when compared with similarly treated wild-type mice (Ripley *et al*, 1999). In addition, using a self-administration paradigm, tPA-knockout mice showed less responding for morphine, as compared with wild-type controls (Yan *et al*, 2007), suggesting that a deficiency in tPA renders drugs of abuse less rewarding and/or reinforcing. The present studies are not inconsistent with this possibility, although further studies are required to clarify precisely what motivational and cognitive mechanisms are being affected by tPA modulation using the lentivirus system.

The present study also tested extinction over a 12-day period subsequent to the development of CPP in LV-tPA animals. The results suggested that extinction was delayed in amphetamine-conditioned LV-tPA rats, although it should be emphasized that the starting preference for these animals was substantially higher than for the amphetamine-treated LV-GFP and LV-siRNA groups. Nevertheless, it is notable that after 1 week of un-reinforced trials, the previously conditioned LV-tPA animals are still showing a high preference for the conditioned context, which by this time was well above the equivalent preference for both chambers shown by all other groups. This may demonstrate that the original CS-UCS association between context and amphetamine treatment is quite strong (as evident on day 1; Figure 2, bottom panel), and sufficiently stable to prevent a rapid return to preconditioning levels of preference. Moreover, upon priming with a small dose of amphetamine, the LV-tPA group reverted to the greater preference to the drug-associated context, when compared with the previously conditioned LV-GFP group receiving a similar priming dose of amphetamine. This indicates that elevations in tPA during acquisition of CPP create very strong learning associations that can re-enlist response biases to pre-extinction levels. This observation is of obvious clinical relevance given that relapse to drug-seeking behavior is a recurring and intractable problem.

It is also important to note that tPA appears to play an important role more in acquisition of amphetamine-induced CPP, rather than in its expression. Indeed, even though the active enzyme is overexpressed after acquisition, eventual extinction is observed. In addition, we have shown that tPA is induced upon chronic drug administration (Bahi and Dreyer, 2007), in agreement with findings from other groups (Nagai *et al*, 2005, 2006). Nicotine increased tPA protein levels and promoted the release of tPA into the extracellular space in the NAc. Furthermore, our previous studies have shown similar effects for uPA upon cocaine injection, whereas inhibition of uPA impairs reconsolidation of the cocaine-induced place preference even after extinction and while animals are under the influence of cocaine during reinstatement testing, indicating that uPA may mediate cocaine-associated contextual memory (Bahi and Dreyer, 2007).

The present findings are consistent with the view that tPA activator plays a key role in neuroplasticity and is a neuromodulator in the CNS (Yamada *et al*, 2005). Moreover, as previously mentioned, the tPA–plasmin system is involved in the rewarding effects of drugs of abuse (eg, morphine), possibly through regulation of dopamine release in the NAc (Nagai *et al*, 2004, 2005, 2006; Ito *et al*, 2006, 2007; Yan *et al*, 2007). Indeed, similar to the present findings involving the use of LV-siRNA, tPA-knockout mice showed a reduction in morphine-, methamphetamine-, and nicotine-induced CPP and locomotor sensitization (Nagai *et al*, 2004, 2005, 2006; Ito *et al*, 2006, 2007; Yan *et al*, 2007). Furthermore, exogenous recombinant human tPA or plasmin restored the defect of morphine-induced dopamine release in the NAc and hyperactivity in tPA-knockout mice (Nagai *et al*, 2004). No significant difference in food-reinforced operant behavior was observed between tPA-knockout and wild-type control mice, but tPA-knockout mice showed an increase in morphine self-administration, suggesting that tPA is critically involved in the reinforcing properties of morphine in mice (Yan *et al*, 2007). The present results are consistent with these previous findings and the conclusion that the tPA system may represent a potential target in the therapeutic treatment of drug addiction.

Drug addiction exploits learning and memory systems (O'Brien *et al*, 1992; Bonci and Malenka, 1999; Thomas *et al*, 2000; Wise, 2000; Hyman, 2005). Memories for events, places, foods, behaviors, and emotions play an important

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role in addiction. Persistent drug-seeking/taking behavior involves the consolidation and retention of information associated with the psychopharmacologic properties of drugs (Wright and Harding, 2004). Reconsolidation serves to maintain, strengthen, or modify memories. Specifically, retrieval of previously consolidated memory traces may induce an additional activity-dependent labile period during which the memory can be modified. Furthermore, chronic drug exposure results in neuronal plasticity, as exemplified by morphological changes in neurons (Robinson and Kolb, 1999; Robinson *et al*, 2001). Consequently, molecules such as tPA that are involved in plasticity may influence acquisition and/or reconsolidation of drug-related information via these mechanisms.

Understanding the molecular mechanisms of reconsolidation may provide crucial insights into the behaviors of normal individuals and into psychiatric disorders such as addiction that are characterized by exceptionally strong and salient emotional memories. Anxiolytic and amnestic agents have been proposed as useful tools in eliminating memories after retrieval; for example, benzodiazepines have recently been shown to disrupt reconsolidation of contextual fear in rats (Tronson and Taylor, 2007). As an alternative to these strategies, our studies demonstrate the potential clinical relevance of focusing attention on the plasminogen system. Using the lentivirus system, we showed that tPA may be involved in learning and amphetamine-associated contextual memory. Although we observed a conditioned aversion when silencing endogenous tPA with LV-siRNA, our studies support the notion that memory and/or retrieval cues for drug-associated contexts may be diminished by pharmacological lowering of tPA. This may lead to development of new treatment strategies for human addiction.

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