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Involvement of tissue plasminogen activator "tPA" in ethanol-induced locomotor sensitization and conditioned-place preference

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Ethanol is one of the most abused drugs in the western societies. It is well established that mesolimbic dopaminergic neurons mediate the rewarding properties of ethanol. In our previous studies we have shown that the serine protease tissue plasminogen activator (tPA) is involved in the rewarding properties of morphine and amphetamine. In the current study, we investigated the role of tPA in ethanol-induced behavioral sensitization and conditioned-place preference (CPP). Ethanol treatment dose-dependently induced tPA enzymatic activity in the nucleus accumbens (NAc). In addition, ethanol-induced increase in tPA activity was completely inhibited by pre-treatment with the dopamine D1 and D2 receptor antagonists SCH23390 and raclopride respectively. Furthermore, ethanol-induced locomotor stimulation, behavioral sensitization and conditioned-place preference were enhanced following tPA over-expression in the NAc using a lentiviral vector. In contrast, tPA knock down in the NAc with specific shRNA blocked the rewarding properties of ethanol. The defect of locomotor stimulation in shRNA-injected mice was reversed by microinjections of exogenous recombinant tPA into the nucleus accumbens. Collectively, these results indicate, for the first time, that activation of tPA is effective in enhancing the rewarding effects of ethanol. Targeting the tissue plasminogen activator system would provide new therapeutic approaches to the treatment of alcoholism.

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1. Introduction

Alcohol dependence can be characterized by a pattern of compulsive ethanol drinking or loss of control of intake by an individual in spite of the adverse devastating negative consequences of its abuse [1]. It is well established that alcohol intake increases the release of dopamine and subsequently increases gene expression in mesencephalic brain areas related to reinforcement and reward, such as the nucleus accumbens (NAc) or the ventral part of striatum [2–6]. In these regions, alcohol-induced opioid release stimulates dopamine neurons by acting directly on the NAc and by disinhibiting GABA neurons projecting into the ventral tegmental area (VTA) [7–13].

Several recent reports have suggested a close interaction between the plasminogen system and drug of abuse. Particularly, many studies have addressed the role of tissue plasminogen activator (tPA) in amphetamine, nicotine and morphine-induced behavioral changes and reward [14–22]. Extracellular proteases are found in a large amount of human tissues and function to maintain the integrity of the extracellular matrix, to modulate the interaction of the cells during development and to contribute to tissue remodeling [23–25]. Regulation of the extracellular matrix by proteases and protease inhibitors is a fundamental biological process for normal growth, development and repair in the central nervous system (CNS) [26,27]. tPA is a serine protease that catalyzes the conversion of plasminogen to plasmin and plays a role in fibrinolysis [28]. In addition, tPA is expressed by many types of neurons in the developing and adult brain [for review see [15,29]]. tPA is highly expressed in the adult rodent brain in regions involved in learning and memory (hippocampus) [30,31], fear and anxiety (amygdala) [32–34], motor learning (cerebellum) [35,36], and addiction [14,33,37–40]. The understanding of physiological functions of tPA in the CNS has expanded together with its roles in pathological situations including neuronal degeneration due to excitotoxicity [41–43], Alzheimer's disease [44–47] and amyotrophic lateral sclerosis (ALS) [48]. These findings suggest that tPA is involved in the regulation of numerous aspects of neuronal remodeling and particularly drug-induced synaptic plasticity.

In 2004, Nagai and co-workers showed that repeated methamphetamine injections dose-dependently induced tPA mRNA

Abbreviations: CMV, cytomagalovirus; CPP, conditioned-place preference; GABA, gamma-aminobutyric acid; GFP, green fluorescent protein; HEK293T, human embryonic kidney 293T; KO, knock-out; NAc, nucleus accumbens; shRNA, short herpin RNA; tPA, tissue-plasminogen activator; VTA, ventral tegmental area.

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expression in multiple brain regions including the NAc. In addition, methamphetamine-induced behavioral sensitization and conditioned place preference (CPP) were significantly reduced in tPA-KO mice compared to their wild-type littermates [21]. The same group has also shown that the tPA–plasmin system plays a crucial role in nicotine-induced reward and dopamine release [39] as well as morphine-induced dopamine release and behaviors but not in the anti-nociceptive effects of morphine [17–19]. Using lentiviral-mediated gene transfer approach our previous research has demonstrated that tPA-overexpressing rats had greater acute locomotor stimulating response, behavioral sensitization and conditioned place preference upon morphine and amphetamine treatments and that, these effects were inhibited using specific-shRNA-expressing viral vectors [14]. In an independent study we have reported that lentiviral tPA over expression in the NAc was involved in the acquisition, extinction and reinstatement but not in the expression, of amphetamine-induced place preference. [16]. Together, these data clearly indicate that tPA plays an important role in morphine and amphetamine-induced behavioral changes.

To extend our previous findings, we performed a series of experiments to examine the effects of ethanol on tPA enzymatic activity. Using lentiviral-mediated gene transfer and shRNA expression, we investigated how tPA manipulation in the NAc may influence ethanol-induced locomotor sensitization and conditioned place preference.

2. Materials and methods

2.1. Animals

Male C57BL/6 mice weighing 25–30 g were group housed in standard plexiglas cages 1 week before the experimental procedure started. All mice were kept under standard laboratory conditions (12/12 h light–dark cycle, lights off at 7 a.m., 22 °C, 55% relative humidity) with free access to tap water and standard mouse chow diet. All animal care and use were in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. All experimental procedures were approved by the local Research Ethics Committee.

2.2. Ethanol solution and drugs

Ethanol was diluted in 0.9% isotonic saline (10%, v/v). The D1 receptor antagonist SCH23390 and the D2 receptor antagonist raclopride were purchased from Sigma–Aldrich Chemie GmbH (Buchs, Switzerland) and dissolved in saline and injected into the intraperitoneal cavity. Based on previous published studies, SCH23390 and raclopride were used at 0.05 and 0.1 mg/kg respectively [49–51]. SCH23390 or raclopride were administered intraperitoneally 30 min before the ethanol treatment. Control animals were given the same volume of vehicle.

2.3. Construction and production of lentiviral vectors LV-GFP, LV-tPA and LV-shRNA

These vectors were prepared as described previously [14,16]. Briefly, for LV-tPA, tPA was amplified from total brain cDNA using specific primers. The amplicon was then digested with *Bam HI* and *Xho I* and ligated into pTK431 previously digested with the same restriction enzymes. LV-GFP was made from a control vectors construct, in which green fluorescent protein (GFP) is expressed by a CMV promoter. For LV-shRNA, using PCR amplification shRNA oligos were added to the mouse U6 promoter using pSilencer 1.0-U6 (Ambion, UK) as a template and the PCR product was digested with *Bam HI* and *Xho I* and cloned into similar sites in pTK431. 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 [14,16,52–59].

2.4. Microinjection of LV-GFP, LV-tPA, LV-shRNA and recombinant tPA into the NAc

Microinjection of lentiviral vectors or recombinant tPA into the NAc was performed according to the previous study [60]. First, mice were tested for their baseline preference and conditioned with either saline or ethanol for 10 days (see Table 1). Viral vectors were injected after conditioning. Mice were anesthetized with isoflurane and placed in a stereotaxic apparatus. A pair of metal guide cannulas was stereotaxically implanted bilaterally into the NAc using following coordinates: +1.6 mm antero-posterior, ±0.8 mm lateral from the bregma, and 4 mm ventral from the skull (dorsal striatum coordinates: AP +1.6 mm, lateral ±1.2 mm and DV 2.5 mm)

Table 1
Time-lines of the experimental procedure.

Days	0	1–10	11–12	12–20	21
	Baseline	Conditioning	Viral injection	Recovery	CPP test

[61]. Following recovery from the operation, mice were used for EtOH–CPP expression. For rescuing experiments using recombinant tPA, an infusion cannula was inserted through the guide cannula until they protruded 1 mm beyond the inner end. Vehicle or tPA (10, 30 or 100 ng) were microinjected bilaterally into the NAc through the infusion cannulas at a rate of 0.2 μl/min for 5 min (final volume 1 μl/site). Saline or ethanol (1 g/kg; i.p.) was administered 10 min after the microinjections, and then locomotor activity was measured for 60 min as described below. Determination of the location of the infusion cannula placements was assessed at the completion of the experiments.

2.5. Measurement of locomotor activity

Mice were placed individually in a transparent acrylic cage with a black frosting Plexiglas floor, and locomotor activity was measured every 5 min for 60 min using digital counters with infrared sensor (Activity Monitor, Med Associates, VA, USA). All mice were habituated to the test environment for 30 min before the measurement of locomotor activity. Mice were then injected with isotonic saline (0.9%; i.p.) or ethanol (0.5, 1 or 2 g/kg; i.p.), and the locomotor activity was measured in daily sessions for 10 days.

2.6. Conditioned place preference testing apparatus

Eight conditioning Plexiglas boxes were enclosed in light and sound-attenuating chambers. The place preference boxes were composed of two distinct tactile conditioning environments (consisting of either wire grid or mesh floor). All experimental procedures were conducted without lights in these chambers. Photodetectors and infrared light sources were mounted 2 cm above the floor of the conditioning box at 2.5-cm intervals along the sides of the box. A computer recorded activity and position of mouse within the conditioning box (Med Associates, VA, USA).

2.7. General behavioral procedures for conditioned place preference experiments

2.7.1. Pre-conditioning (habituation)

A 1-day habituation procedure preceded conditioning in all experiments. After a saline injection (10 ml/kg; i.p.), the mouse was placed in the center with free access to both conditioning chambers and the time the mouse spent in each of the two chambers during a 15-min test period was recorded. This pre-test determined baseline preferences (whether the mouse spent significantly more time in one test chamber than in the other, regarded as equipment bias). Mice that spent more than 60% of the time in either one of the compartments during the pre-conditioning baseline (habituation) session were excluded from the study. This allowed us to use an unbiased design in which both compartments were equally preferred before the conditioning session and to randomly assign the compartment paired with ethanol.

2.7.2. Conditioning

The place conditioning phase started 1 day after the pre-conditioning phase. This phase consisted of ten, 30-min sessions (five saline and five ethanol pairings). These sessions were conducted once each day (from day 1 to day 10). On each of these days, separate groups of animals received one conditioning session with ethanol (1 g/kg, 10%, w/v; i.p.) and one with isotonic saline (10 ml/kg; i.p.). During these sessions in alternative days, the animals were confined to one compartment by closing the removable wall. Animals of each group were injected with ethanol and were immediately confined to one compartment of the apparatus for 30 min. Following administration of saline, the animals were confined to the other compartment for 30 min. Locomotor activity was recorded during each of these five saline and five ethanol conditioning sessions to determine the effects of LV-GFP, LV-tPA and LV-shRNA on ethanol-induced hyperactivity. In the saline control group, mice received saline injection on all 10 conditioning days. Treatment compartment and order of presentation of ethanol and saline were counterbalanced for each group.

2.7.3. Post-conditioning (CPP-test)

On day 11 and after a saline injection (10 ml/kg; i.p.) the animals were allowed free access to both compartments. No ethanol injection was given on the test day. Animals were placed in the center of the chamber with free access to both test chambers for 15 min and the time the mouse spent in each chamber was automatically recorded and used as a test for CPP.

2.8. Measurement of tPA activity

Immediately after the completion of behavioral testing, brains were removed after rapid decapitation. The NAc was punched from 2-mm sections with an 18-gauge syringe and placed immediately in lysis buffer. Fifty micrograms of protein from each sample were incubated with a specific tPA substrate from AMC tPA

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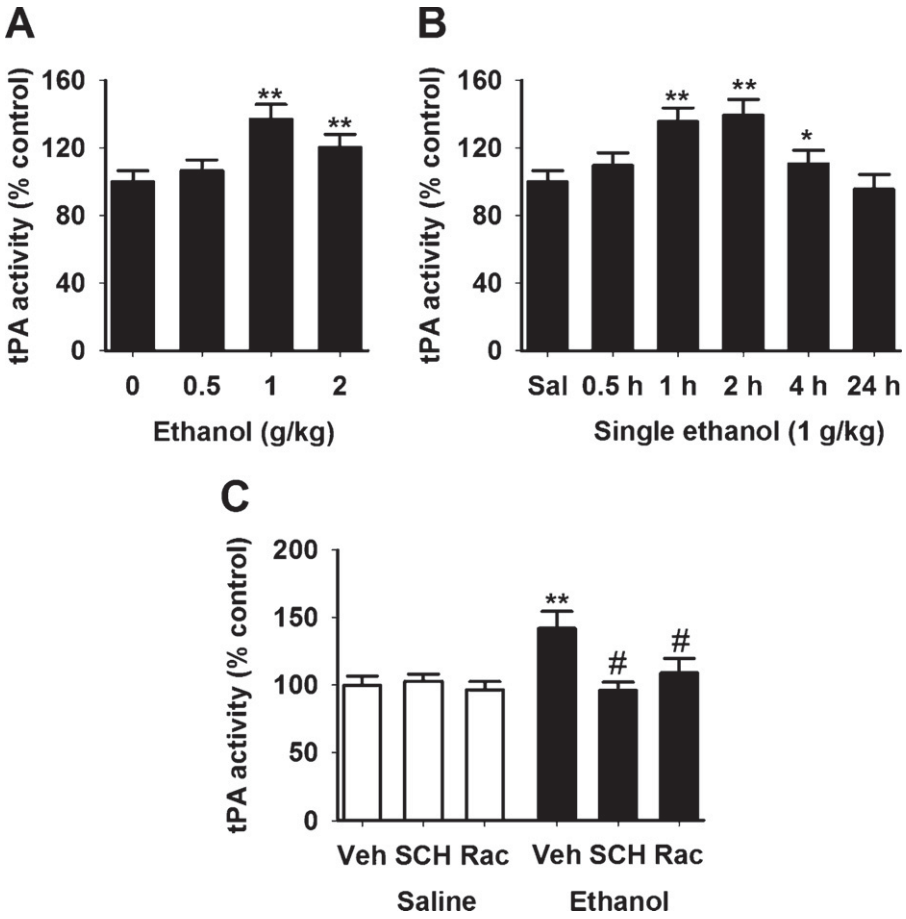

Fig. 1. Changes in tPA enzymatic activity in the NAc after ethanol treatment. (A) Ethanol dose dependently increases tPA activity in the NAc. Mice were treated with ethanol i.p. at doses of 0, 0.5, 1 or 2 g/kg. Mice were then killed and tPA activity was measured in the NAc extracts. Values indicate means ± SEM ($n = 6$ for all doses). $^{**}P < 0.01$ compared to saline-treated group. (B) Ethanol-induced tPA activity kinetics revealed a maximum effect after 2 h post injection. Mice were i.p. injected with 1 g/kg ethanol and killed as described previously after 0.5, 1, 2, 4 or 24 h post injection. Values indicate means ± SEM [saline ($n = 4$); 0.5-h ($n = 6$); 1-h ($n = 6$), 2-h ($n = 5$), 4-h ($n = 6$), 24-h ($n = 8$)]. $^{**}P < 0.01$ and $^{*}P < 0.05$ compared to saline-treated group. (C) Ethanol-induced tPA activity in the NAc was antagonized by dopamine D1 and D2 receptors antagonists. Mice were injected with SCH23390 (0.05 mg/kg) or raclopride (0.1 mg/kg) i.p. 30 min before ethanol (1 g/kg, i.p.) treatment. Mice were then killed and tPA activity was measured as described previously. Values indicate means ± SEM. [Vehicle ($n = 10$); SCH23390 ($n = 12$); raclopride ($n = 9$).] $^{**}P < 0.01$ compared to saline-treated group. $^{\#}P < 0.05$ compared to vehicle-treated group.

Activity Assay Kit (Fremont CA, USA) according to the manufacturer's protocol and published studies [62–64]. Reactions were stopped and colorimetric intensity was measured at 442 nm in a microplate spectrophotometer. Data represent values obtained from three independent experiments performed in triplicate.

2.9. Statistical analysis

For statistical comparisons, the software package SPSS (version 17.0) was used. All data were expressed as means ± SEM. In the analysis of the effect of time course for the behavioral sensitization, an ANOVA with repeated measures was used and followed by the Bonferooni's test when F values were significant. In the analysis of tPA activity, acute locomotion and conditioned place preference, an ANOVA was used. In cases of a significant main effect, post-hoc comparisons were performed with Bonferroni's test. The criterion for statistical significance was $P < 0.05$.

3. Results

3.1. Ethanol increases tPA activity in the nucleus accumbens

It has been shown that tPA is stored in synaptic vesicles and released after cell depolarization [62]. We hypothesize that if ethanol-induced dopamine release is affecting the tPA system, ethanol should increase tPA activity in the NAc. We examined tPA activity in the NAc protein extracts after ethanol administration using an enzymatic assay. In this experiment, four different groups of mice were injected with increasing doses of ethanol (0, 0.5, 1 and 2 g/kg; i.p.) and killed 60 min later. As shown in Fig. 1A, ethanol treatment increased tPA activity in the NAc. In addition the effect of ethanol was dose dependent [$F_{(3,20)} = 9.512$; $P < 0.05$; $n = 6$ for all groups] (Fig. 1A). Post-hoc analysis revealed that, compared to saline injected mice, 0.5 g/kg ethanol treatment did not affect tPA enzymatic activity ($P > 0.05$). In addition, single ethanol injection (1 g/kg) significantly increased tPA activity in the NAc 1 h (135%), 2 h (139%) and 4 h (110%) after the treatment [$F_{(5,29)} = 6.588$; $P < 0.05$; saline ($n = 4$); 0.5 h ($n = 6$); 1 h ($n = 6$), 2 h ($n = 5$), 4 h ($n = 6$), 24 h ($n = 8$)] (Fig. 1B). These results suggest that acute ethanol administration induces an up-regulation of tPA activity in the NAc. Furthermore, ethanol-induced increase in tPA activity in the NAc was inhibited by pretreatment with dopamine D1 receptor antagonist SCH23390 ($P < 0.05$) and with the D2 receptor antagonist raclopride ($P < 0.05$) (Fig. 1C). In between subject analysis has revealed a main effect of ethanol treatment [$F_{(1,28)} = 12.278$; $P < 0.05$]. The within subject evaluation has shown an ethanol × drug interaction [$F_{(2,28)} = 8.375$; $P < 0.05$; vehicle ($n = 10$); SCH23390 ($n = 12$); raclopride ($n = 9$)]. These results suggest that ethanol stimulates release of tPA through both dopamine D1 and D2, receptors.

3.2. Ethanol-induced locomotor stimulation in LV-GFP, LV-tPA and LV-shRNA mice

In order to clarify the physiological significance of the regulation of tPA activity in the NAc, we compared the locomotor

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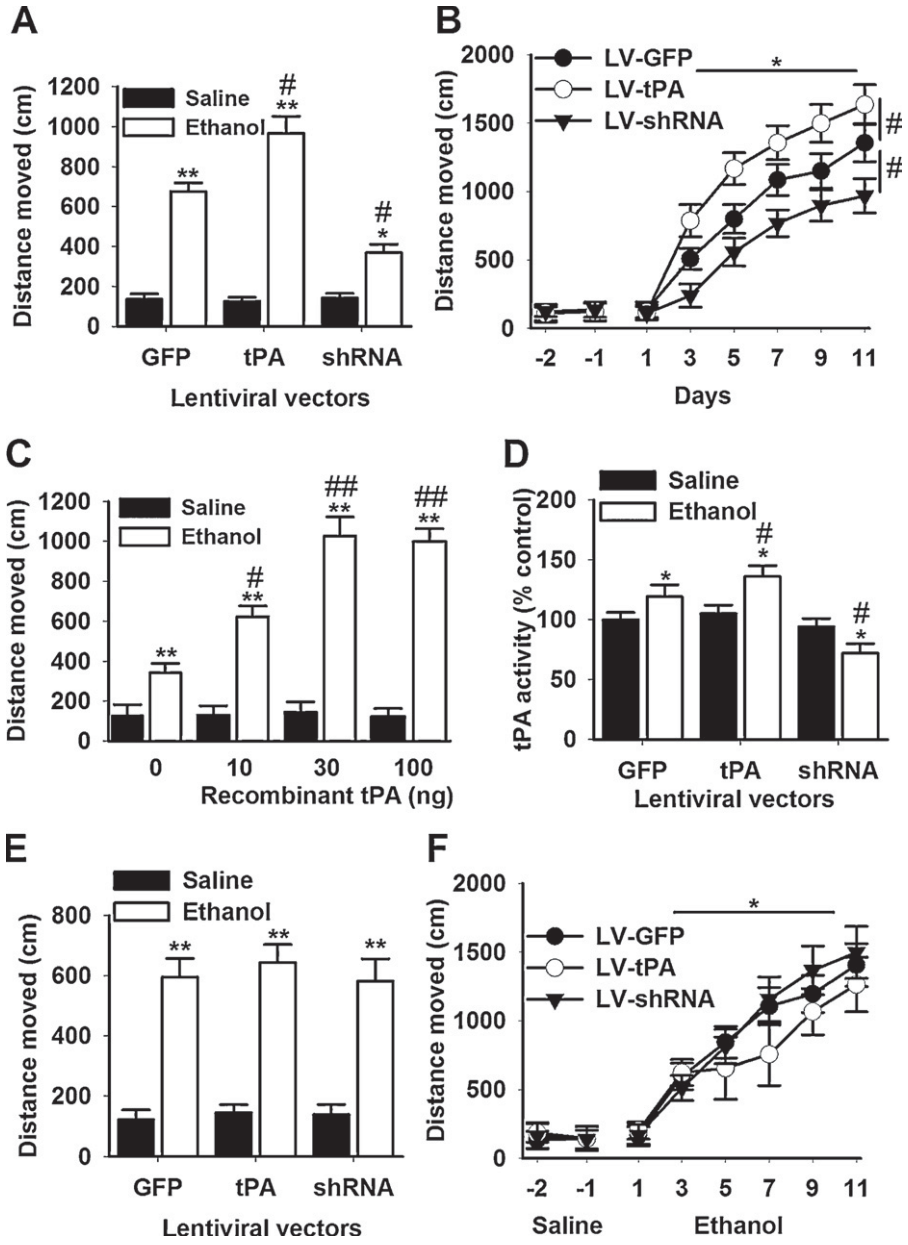

Fig. 2. Ethanol-induced locomotor stimulation in mice expressing GFP, tPA or shRNA. (A) Mice were stereotaxically injected in the NAc with lentiviral vectors expressing either GFP, tPA or shRNA. After recovery, mice were acutely injected with either saline or ethanol (1 g/kg, i.p.) and locomotor activity was measured 15 min later. Values indicate means ± SEM. [LV-GFP ($n = 11$); LV-tPA ($n = 13$); LV-shRNA ($n = 10$)]. $^{**}P < 0.01$ and $^{*}P < 0.05$ compared to saline-treated group. $^{\#}P < 0.05$ compared to LV-GFP-injected group. (B) The same animals as previously were injected daily with saline (2 days) for baseline locomotor activity and then daily with ethanol (0.5 g/kg, i.p.) for 11 days and locomotor activity monitored to assess behavioral sensitization. $^{*}P < 0.05$ compared to day 1. $^{\#}P < 0.05$ compared to LV-GFP-injected group. (C) Mice were stereotaxically injected in the NAc with LV-shRNA. After recovery mice were infused with 0, 10, 30 or 100 ng of recombinant tPA. 10 min later mice were injected with either saline or ethanol (1 g/kg, i.p.) and locomotor activity was measured. $^{**}P < 0.01$ and $^{*}P < 0.05$ compared to saline-treated group. $^{\#\#}P < 0.01$ and $^{\#}P < 0.05$ compared to 0 ng tPA-infused group. [saline ($n = 13$); ethanol ($n = 16$)]. (D) After repeated ethanol treatment (panel "B" above), mice were killed at day 11 and tPA enzymatic activity was measured in the NAc. [LV-GFP (EtOH, $n = 8$/saline, $n = 3$); LV-tPA (EtOH, $n = 9$/saline, $n = 4$); LV-shRNA (EtOH, $n = 7$/saline, $n = 3$).] $^{*}P < 0.05$ compared to saline-treated group. $^{\#}P < 0.05$ compared to LV-GFP-injected group. (E) Mice were sterotaxically injected in the dorsal striatum with lentiviral vectors expressing either GFP, tPA or shRNA. After recovery, mice were acutely injected with either saline or ethanol (1 g/kg, i.p.) and locomotor activity was measured. Values indicate means ± SEM. [LV-GFP ($n = 11$); LV-tPA ($n = 12$); LV-shRNA ($n = 11$)]. $^{**}P < 0.05$ compared to saline-treated group. (F) The same animals as previously were injected with saline (2 days) for baseline locomotor activity and then daily with ethanol (0.5 g/kg, i.p.) for 11 days and locomotor activity was monitored to assess behavioral sensitization. $^{*}P < 0.05$ compared to day 1.

stimulating effects of ethanol in LV-GFP, LV-tPA and LV-shRNA injected mice. Results of ethanol-induced locomotor activity were evaluated using one-way ANOVAs repeated measure, with the virus (LV-GFP, LV-tPA and LV-shRNA; 3 levels) defined as a between-subject factor and treatment (saline and ethanol; 2 levels) as within-subject factor. As shown in Fig. 2A, the locomotor response to 1 g/kg; i.p. ethanol was significantly enhanced [$F_{(1,31)} = 10.537$; $P < 0.05$; LV-GFP ($n = 11$); LV-tPA ($n = 13$); LV-shRNA ($n = 10$)] in mice that were treated with ethanol as compared to saline. In addition, analysis has revealed an ethanol × virus interaction [$F_{(2,31)} = 7.649$; $P < 0.05$]. Post-hoc evaluation has shown that, compared to control animals injected with LV-GFP, tPA over-expression enhances locomotor activity. In contrast, knocking down tPA expression by mean of LV-shRNA in the NAc reduces ethanol-induced locomotor stimulation. When the time course of ethanol (0.5 g/kg)-induced locomotor sensitization in LV-tPA and LV-shRNA mice was compared with that in LV-GFP mice, the sensitization was found to be significantly high in

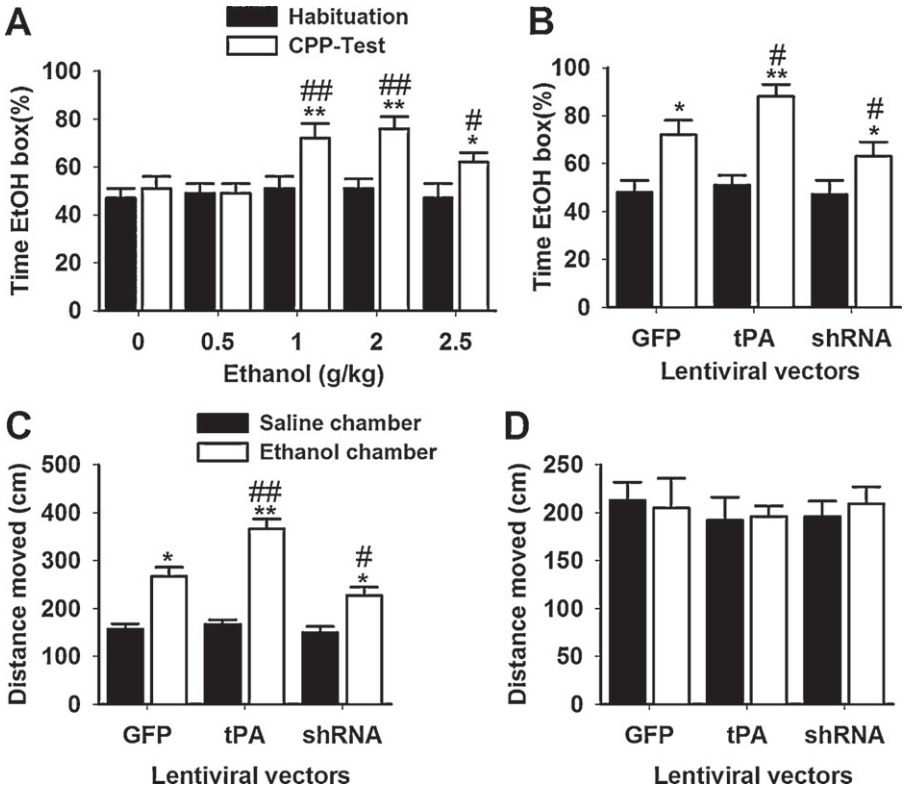

Fig. 3. Ethanol-induced place preference in mice expressing GFP, tPA or shRNA in the NAc. (A) Optimization of ethanol doses for place preference (CPP). Ethanol-CPP is dose-dependent. After an initial 15-min determination of place preference baseline, mice were place conditioned with ethanol (0, 0.5, 1, 2 or 2.5 g/kg; i.p.) or in the opposite chamber. Final CPP was determined on day 11. All mice demonstrated similar preconditioning place preferences (habituation). Mice place conditioned with 0 and 0.5 g/kg ethanol did not demonstrate a change from the initial place preference response. In contrast, place conditioning with 1, 2 or 2.5 g/kg ethanol produced a significant preference for the ethanol-paired environment. Values indicate means ± SEM. [0-g/kg (n = 7); 0.5-g/kg (n = 9); 1-g/kg (n = 8); 2-g/kg (n = 9); 2.5-g/kg (n = 8)] $^{**}P < 0.01$ and $^{*}P < 0.05$ compared to habituation; $^{\#\#}P < 0.01$ and $^{\#}P < 0.05$ compared to 0-g/kg. (B) Mice were tested for their baseline and stereotaxically injected with either LV-GFP, LV-tPA or LV-shRNA. Mice were then conditioned with ethanol (1 g/kg, i.p.) and tested for CPP. Values indicate means ± SEM. [LV-GFP (n = 10); LV-tPA (n = 11); LV-shRNA (n = 9).] $^{**}P < 0.01$ and $^{*}P < 0.05$ compared to habituation. $^{\#}P < 0.05$ compared to LV-GFP-injected group. (C) In the same mice, ethanol significantly increased locomotor activity in LV-tPA, but had no effect on LV-shRNA, injected mice compared to LV-GFP during conditioning. $^{**}P < 0.01$ and $^{*}P < 0.05$ compared to habituation. $^{\#\#}P < 0.01$ and $^{\#}P < 0.05$ compared to LV-GFP-injected group. (D) In absence of ethanol LV-GFP, LV-tPA and LV-shRNA did not affect locomotor activity during place preference test.

tPA-over-expressing mice [$F_{(7,217)} = 6.942$; $P < 0.05$; LV-GFP (n = 11); LV-tPA (n = 13); LV-shRNA (n = 10)]. In contrast, tPA knock down in the NAc impaired ethanol-induced behavioral sensitization (Fig. 2B). We then examined whether exogenous recombinant tPA can reverse LV-shRNA-induced decrease in the ethanol locomotor sensitization response. The attenuation of locomotor stimulation in ethanol-treated LV-shRNA mice was dose-dependently significantly reversed by microinjections of exogenous recombinant tPA [$F_{(3,81)} = 8.622$; $P < 0.05$; saline (n = 13); ethanol (n = 16)] into the nucleus accumbens, although tPA microinjection itself had no effect on locomotor activity in saline-treated LV-shRNA mice (Fig. 2C). To determine whether observed locomotor stimulation changes are related to the tPA activity, the enzymatic activity of tPA in repeated ethanol treatment groups was assessed. LV-tPA over-expression resulted into enhanced extracellular enzymatic activity of this protease in the NAc [$F_{(2,21)} = 13.106$; $P < 0.05$; LV-GFP (n = 8); LV-tPA (n = 9); LV-shRNA (n = 7)] (Fig. 2D). A within-subject analysis revealed a main effect of ethanol treatment [$F_{(1,21)} = 12.677$; $P < 0.05$]. However, when gene expression of tPA was inhibited with specific shRNA-expressing lentiviral vectors, a reduced enzymatic activity was observed.

Because it is possible that tPA activity may also be modulated by the ethanol in the dorsal striatum, we examined the ethanol-induced tPA activity in this brain region. As shown in Fig. 2E, the locomotor response to 1 g/kg; i.p. ethanol was significantly enhanced [$F_{(1,31)} = 12.967$; $P < 0.05$; LV-GFP (n = 11); LV-tPA (n = 12); LV-shRNA (n = 11)] in mice that were treated with ethanol as compared to saline. In contrast to the NAc, within-subject analysis has revealed no ethanol × virus interaction [$F_{(2,31)} = 0.678$; $P > 0.05$]. Post-hoc evaluation has shown that, compared to control animals injected with LV-GFP, tPA over-expression or knock down using LV-siRNA did not affect locomotor activity. Similarly, when the time course of ethanol (0.5 g/kg)-induced locomotor sensitization in LV-tPA and LV-shRNA mice was compared with that in LV-GFP mice, the sensitization was not found to be affected by tPA expression using viral vectors [$F_{(7,217)} = 0.975$; $P > 0.05$; LV-GFP (n = 11); LV-tPA (n = 12); LV-shRNA (n = 11)] (Fig. 2F).

3.3. Ethanol-induced place preference in LV-GFP, LV-tPA and LV-shRNA-injected NAc

Because we observed robust changes in tPA enzymatic activity in the nucleus accumbens, the areas of the brain important for the rewarding effects of drugs of abuse [65–68], we focused on the role of tPA in the rewarding effects of ethanol, which can be assessed using the conditioned place preference test [69–73].

Optimization of ethanol place conditioning was conducted with ethanol doses of 0, 0.5, 1, 2 or 2.5 g/kg once daily for 5 days. As shown in Fig. 3A, the one-way ANOVA analysis indicated a significant main effect of ethanol dose [$F_{(4,36)} = 6.974$; $P < 0.05$; n = 7 for 0 mg/kg, n = 9 for 0.5 mg/kg, n = 8 for 1 mg/kg, n = 9 for 2 mg/kg and n = 8 for 2.5 mg/kg]. Mice place conditioned with 0 or 0.5 g/kg ethanol displayed no change in preference from baseline (P = 0.394 and P = 0.487 respectively). However, subsequent post-hoc evaluation for each dose revealed that mice place conditioned 30 min daily with 1, 2 or 2.5 g/kg doses of ethanol spent significantly more time

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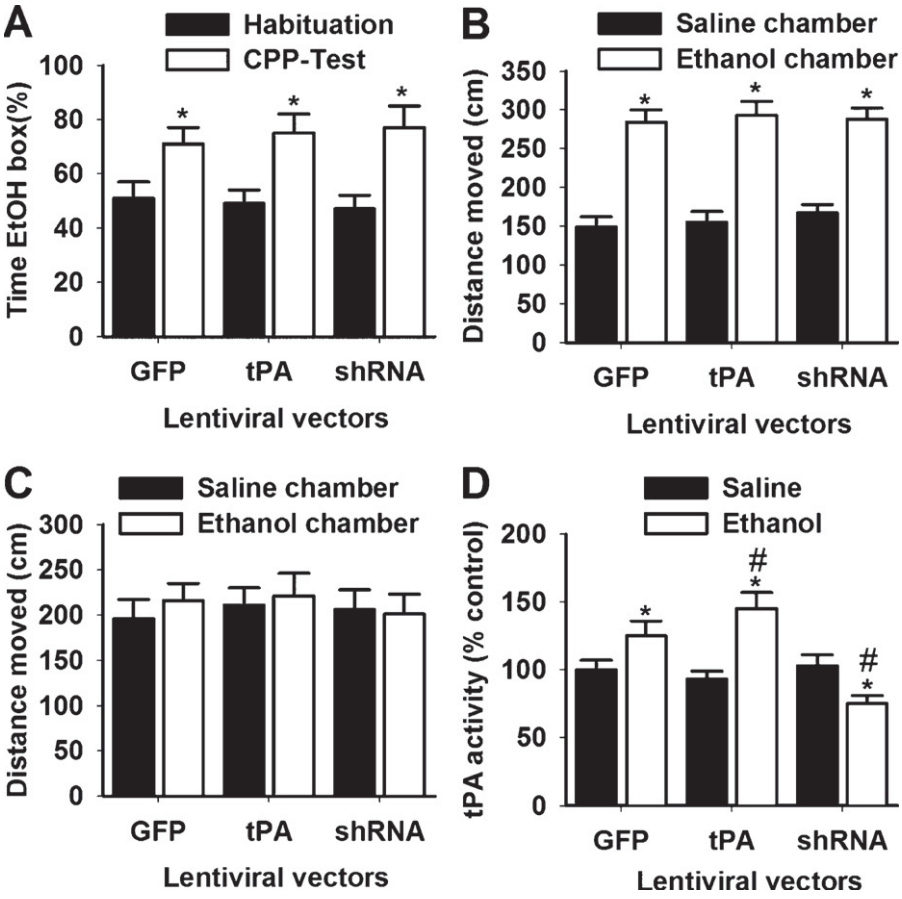

Fig. 4. Ethanol-induced place preference in mice expressing GFP, tPA or shRNA in the dorsal striatum. (A) Mice were tested for their baseline and steretoaxically injected in the dorsal striatum with either LV-GFP, LV-tPA or LV-shRNA. Mice were then conditioned with ethanol (1 g/kg, i.p.) and tested for CPP. Values indicate means ± SEM. [LV-GFP ($n = 10$); LV-tPA ($n = 9$); LV-shRNA ($n = 9$).] $^{*}P < 0.05$ compared to habituation. (B) In the same mice, ethanol significantly increased locomotor activity in LV-GFP, LV-tPA, and LV-shRNA injected mice during conditioning. $^{*}P < 0.05$ compared to habituation. (C) In absence of ethanol LV-GFP, LV-tPA and LV-shRNA did not affect locomotor activity during place preference test. (D) After CPP completion, mice were killed and tPA activity in was assessed. [LV-GFP (EtOH, $n = 5$/saline, $n = 5$); LV-tPA (EtOH, $n = 5$/saline, $n = 4$); LV-shRNA (EtOH, $n = 5$/saline, $n = 4$).] $^{*}P < 0.05$ compared to habituation. $^{\#}P < 0.05$ compared to LV-GFP-injected group.

in the ethanol-paired chamber in final preference testing. Although slightly significantly different from the effect of place conditioning with a 2.5 g/kg dose of ethanol, a peak effect was observed with 1 and 2 g/kg ethanol, prompting use of the 1 g/kg dose of ethanol in CPP testing for the remainder of the study.

We then examined the effect of tPA modulation on ethanol-induced CPP expression. The experimental timeline is illustrated in Table 1. Mice were alternately conditioned to ethanol in one compartment and saline in the other compartment. To determine the effects of LV-GFP, LV-tPA and LV-shRNA on ethanol memory, mice were tested for their baseline preference. After the 10-day conditioning sessions, mice were streotaxically injected with the viral vectors in the NAc. After recovery, mice were tested for ethanol-induced CPP as described above. The results are illustrated in Fig. 3B. Analyzing percent of time spent in the ethanol-paired box during the CPP test revealed a significant virus × test interaction [$F_{(2,27)} = 9.864$; $P < 0.05$; LV-GFP ($n = 10$); LV-tPA ($n = 11$); LV-shRNA ($n = 9$)]. Post-hoc tests showed that mice injected with LV-tPA and conditioned to ethanol displayed a significant place preference compared to LV-GFP and pre-conditioning habituation time. In contrast, knocking-down tPA expression with LV-shRNA significantly reduced place preference compared to controls.

As shown in Fig. 3C, locomotor activity data analysis from the mice undergoing acquisition of ethanol CPP, revealed a significant conditioning × virus interaction [$F_{(2,27)} = 4.957$; $P < 0.05$], and the post-hoc evaluation revealed that ethanol significantly stimulated locomotor activity in LV-GFP-injected animals during conditioning. In addition, LV-tPA enhanced ethanol-induced locomotor activity (compared to LV-GFP). In contrast LV-shRNA-injected mice showed a reduced ethanol-induced locomotor activity (compared to LV-GFP). In addition, as shown in Fig. 3D LV-tPA and LV-shRNA did not alter total locomotor activity during the CPP test (saline and ethanol conditioning chambers) [$F_{(2,27)} = 0.967$, $P > 0.05$].

3.4. Ethanol-induced place preference in LV-GFP, LV-tPA and LV-shRNA-injected dorsal striatum

To determine if the effects of LV-GFP, LV-tPA and LV-shRNA on ethanol memory was mediated by the NAc tPA activity, mice were tested for their baseline preference. After the 10-day conditioning sessions, mice were streotaxically injected with the viral vectors in the dorsal striatum. The results are illustrated in Fig. 4A. Analyzing percent of time spent in the ethanol-paired box during the CPP test revealed a significant effect of ethanol [$F_{(1,26)} = 12.945$; $P < 0.05$; LV-GFP ($n = 10$); LV-tPA ($n = 9$); LV-shRNA ($n = 9$)] but no virus × drug interaction was observed [$F_{(2,25)} = 1.957$; $P > 0.05$]. As shown in Fig. 4B, locomotor activity data analysis from the mice undergoing acquisition of ethanol CPP, revealed non-significant conditioning × virus interaction [$F_{(12,93)} = 0.967$; $P > 0.05$], and the post-hoc evaluation revealed that ethanol significantly stimulated locomotor activity in LV-GFP-injected animals during conditioning. In addition, LV-tPA enhanced ethanol-induced locomotor activity but no difference to LV-GFP was found (compared to LV-GFP). Similarly, LV-shRNA-injected mice showed an identical

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ethanol-induced locomotor activity (compared to LV-GFP). In addition, as shown in Fig. 4C, LV-tPA and LV-shRNA did not alter total locomotor activity during the CPP test (saline and ethanol conditioning chambers).

At the end of the experiment we measured tPA activity in dorsal striatum protein extracts (Fig. 4D). Saline treatment had no effect on tPA activity. In contrast, ethanol injection significantly increased tPA activity compared with the saline-treated group [$F_{(1,24)} = 13.674$; $P < 0.05$]. Furthermore, within-subject analysis has revealed an ethanol × virus interaction [$F_{(2,23)} = 9.048$; $P < 0.05$]. Post-hoc evaluation has shown that, compared to control animals injected with LV-GFP, tPA over-expression enhances enzymatic activity. In contrast, knocking down tPA expression by mean of LV-shRNA in the dorsal striatum reduces tPA activity. Overall, these results indicate that ethanol-induced behavioral changes depend on tPA activity in the NAc.

4. Discussion

The present study examined the role of the tissue plasminogen activator "tPA" in ethanol-induced locomotor sensitization and conditioned place preference. The main findings of the present study were that: first, ethanol treatment dose dependently induced tPA activity in the NAc. This effect was mediated by both dopamine D1and D2 receptors. Second, tPA over-expression, but not knock down, enhanced ethanol-induced locomotor stimulation and behavioral sensitization. Finally, the rewarding effects of ethanol were significantly enhanced with tPA over-expression and attenuated with knocking down tPA expression in the nucleus accumbens. Taken together, these data suggest that the initial memory formation of ethanol-seeking behavior did require intact tPA activity. Overall, these findings demonstrate that tPA plays an important role in ethanol reward memory.

tPA is primarily expressed in the brain's motivational circuitry, including the prefrontal cortex, amygdala, NAc, dorsal striatum, thalamus and hippocampus [30–34,38]. Our present study found that ethanol dose dependently induced tPA activity in the NAc. In addition, enhancement of tPA activity was blocked when mice were pretreated with both dopamine D1 receptor antagonist SCH23390 or raclopride, the D2R antagonist. Our observation is in agreement with previously published studies where tPA expression and activity in the nucleus accumbens induced after methamphetamine, morphine and nicotine treatment was blocked when D1 and D2 receptors were antagonized [19,21,22,39]. It has been shown that tPA expression is regulated by cAMP response binding protein (CREB) [30] and when injected with cocaine tPA-KO mice displayed attenuated phosphorylation of ERK, CREB, and dopamine and cAMP-regulated phosphoprotein 32 kDa (DARPP-32) in the NAc [37]. In addition, ethanol treatment enhanced CREB phosphorylation and activity in many brain regions including the NAc [74–79]. Thus, we can speculate that the enhanced ability of ethanol treatment to induce tPA enzymatic activity in the NAc may be due at least in part to the increased CREB function.

Based on sequence analysis and downstream second messenger in the NAc, dopamine has two major receptors, D1 and D2 and despite the opposing effects on cellular signaling via the cAMP/PKA system, D1 and D2 receptor antagonists attenuated the induced behavioral-induced behavioral changes [80–82] leading to reduced levels of functional pCREB [83–86]. Therefore, ethanol-induced tPA activity may be mediated by the activation of both dopamine D1 and D2 receptors, which is associated with the enhanced pCREB function.

To further investigate the physiological significance of the ethanol-induced increase in tPA enzymatic activity in the NAc, we examined the locomotor stimulating and rewarding effects of ethanol in mice. For this purpose we used lentiviral-mediated gene transfer technology to either over-express tPA or knock-down its mRNA expression by mean of shRNA. These technologies have been successfully used to study gene function in the CNS [87–93]. Ethanol induced locomotor stimulation and behavioral sensitization were exacerbated in tPA over-expressing mice but significantly reduced when tPA mRNA was inhibited by mean of specific shRNA-expressing lentiviral vectors in the NAc. These findings correlate with previously published studies in which morphine-induced hyperlocomotion was significantly reduced in tPA-KO mice compared to their wild-type littermates [22]. These same mice displayed impaired locomotor stimulation when treated with either amphetamine, cocaine or nicotine [18,19,21,39]. Also, the tPA target, plasminogen, has been shown to be involved in the stimulating effects of morphine. In fact plasminogen-KO mice exhibit reduced morphine-induced hyper-locomotion similar to those found in tPA-KO mice [22]. Consistent with previous findings, the microinjection of recombinant tPA in shRNA-expressing mice into the NAc dose dependently rescued ethanol-induced locomotor stimulation. Taken together, it is likely that the tPA is crucial in regulating ethanol-induced hyperlocomotion as well as behavioral sensitization in mice.

We also investigated whether tPA is involved in the rewarding properties of ethanol using a paradigm of conditioned place preference. Our findings suggest that the rewarding effects of ethanol are reduced in shRNA-expressing mice but tPA over-expression leads to an exaggerated ethanol response. Ethanol-induced locomotor sensitization and conditioned place preference were significantly attenuated when tPA mRNA was blocked by means of shRNA expressing viral vectors compared with GFP mice, and the attenuation of locomotor sensitization in shRNA mice was significantly reversed by microinjections of exogenous recombinant tPA into the NAc. These findings suggest that tPA activity in NAc plays a crucial role in ethanol-induced behavioral sensitization and conditioned reward. It is well known that ethanol-induced locomotor sensitization and CPP include learning and memory processes [94,95]. In addition, early studies suggested that tPA plays an important role in the processes of learning and memory as tPA-KO mice have shown a reduced exploratory inhibition "high impulsivity" and an impaired acquisition of hippocampal-dependent spatial learning [96–99]. Furthermore, tPA gene ablation prevented corticostriatal long-term potentiation "LTP" [100]. The demonstration that tPA interferes with the induction of corticostriatal LTP in tPA-deficient mice might provide new insights into the molecular mechanisms underlying synaptic plasticity in the striatum. Our hypothesis is that, when blocking tPA expression in the NAc, mice failed to associate the ethanol rewarding effects with the environmental context during the conditioning period.

Ethanol treatment produces structural and morphological changes of neurons. In fact, two-photon microscopy analysis indicated that chronic alcohol treatment modified and disoriented dendrites of medium spiny neurons in the nucleus accumbens [101]. Also baseline decreases in dendritic length and spine density in the agranular insular region of frontal cortex were observed in ethanol-exposed rats. Furthermore rat pups exposed to 5.25 g/kg/day of ethanol have an altered basilar dendritic complexity due to a significant decrease in both length and number of intersections in proximity to the neuronal soma of the prefrontal cortex [102–105]. Thus, tPA may play a role in ethanol-induced structural changes, which may underlie the sensitization and conditioned rewarding effects of alcohol. Although the cellular and molecular mechanisms remain to be further clarified, it is suggested that the effects of tPA might be due to long lasting neuronal and structural changes in ethanol induced drug dependence.

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5. Conclusion

We have demonstrated that ethanol induces tPA enzymatic activity in the brain, and that tPA blockade by mean of shRNA-expressing viral vectors attenuates locomotor sensitization as well as the rewarding effects of ethanol. Taken together with our previous findings that tPA is involved in the rewarding effects of amphetamine and morphine, we propose that tPA plays an important role in long-lasting neuronal and structural changes related to drug addiction.

Disclosure

The authors report no financial relationships with commercial interests.

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