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Research report

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

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ABSTRACT

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.

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