The tissue plasminogen activator–plasmin system participates in the rewarding effect of morphine by regulating dopamine release

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Tissue plasminogen activator (tPA) is a serine protease that catalyzes the conversion of plasminogen (plg) to plasmin, which in turn functions to degrade extracellular matrix proteins in the central nervous system. The tPA-plasmin system plays a role in synaptic plasticity and remodeling. Here we show that this protease system participates in the rewarding effects of morphine by acutely regulating morphine-induced dopamine release in the nucleus accumbens (NAcc). A single morphine treatment induced tPA mRNA and protein expression in a naloxone-sensitive manner, which was associated with an increase in the enzyme activity in the NAcc. The acute effect of morphine in inducing tPA expression was diminished after repeated administration. Morphine-induced conditioned place preference and hyperlocomotion were significantly reduced in tPA$^{-/-}$ and plg$^{-/-}$ mice, being accompanied by a loss of morphine-induced dopamine release in the NAcc. The defect of morphine-induced dopamine release and hyperlocomotion in tPA$^{-/-}$ mice was reversed by microinjections of either exogenous tPA or plasmin into the NAcc. Our findings demonstrate a previously undescribed function of the tPA-plasmin system in regulating dopamine release, which is involved in the rewarding effects of morphine.

Extracellular proteases are expressed by neurons in the central nervous system (1–4), and their function can vary from potentiating neurotransmitter receptor function (5) to structural alterations associated with long-lasting forms of synaptic plasticity (6, 7). Tissue plasminogen activator (tPA) is a serine protease that catalyzes the conversion of plasminogen (plg) to plasmin and plays a role in fibrinolysis. In addition, tPA is abundantly expressed in the central nervous system (8–10), where this protease is stored in synaptic vesicles (11, 12), released into the extracellular space by a depolarization stimulus (11, 12), and then the expression of its mRNA is up-regulated (8, 12). Recent studies have demonstrated that tPA regulates a cascade of extracellular proteolytic activities involved in neurite outgrowth (13), cell migration (14, 15), long-term potentiation and depression (6, 16–18), learning and memory (9, 17, 18), excitotoxic cell death (19, 20), and regeneration or recovery from injury in the nervous system (21). These findings suggest that tPA is involved in the regulation of numerous aspects of synaptic plasticity and remodeling.

It is well known that drugs of abuse, including morphine, acutely modulate the activity of mesolimbic dopaminergic neurons, projecting from the ventral tegmental area (VTA) of the midbrain to the nucleus accumbens (NAcc) (22–24). Morphine increases dopaminergic neurotransmission in the NAcc via the activation of dopamine cells in the VTA, an area that possesses a high density of μ-opioid receptors. This activation results mainly from the disinhibition of inhibitory GABAergic (GABA, γ-aminobutyric acid) interneurons in the VTA (25, 26). The rewarding effects of morphine that are associated with enhanced dopamine release in the NAcc are related to its abuse. It has been proposed that activity-dependent synaptic plasticity and remodeling of the mesolimbic dopaminergic system play a crucial role in the development of drug dependence (27).

In the present study, we examined the role of the tPA-plasmin system in the rewarding effects of and dependence on morphine in mice with a targeted deletion of the tPA (tPA$^{-/-}$ mice) (28) and plg (plg$^{-/-}$ mice) genes (29). Our findings suggest that the tPA-plasmin system participates in the rewarding effects of morphine by acutely regulating morphine-induced dopamine release in the NAcc.

Materials and Methods

Animals. Male Wistar rats (7 weeks old) were obtained from Charles River Breeding Laboratories (Yokohama, Japan). Wild-type (C57BL/6J), tPA$^{-/-}$, and plg$^{-/-}$ mice were from The Jackson Laboratory. All animal care and use were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Nagoya University.

Real-Time RT-PCR. For the single morphine treatment, animals were given morphine hydrochloride (Shionogi Pharmaceutical, Osaka) at a dose of 10 mg/kg s.c., whereas for repeated treatment, they were subjected to a 5-day regimen in which increasing doses of morphine (10, 20, 30, 40, and 50 mg/kg s.c.) were injected twice a day and then challenged with morphine (10 mg/kg s.c.) on day 6. The levels of tPA mRNA were determined by real-time RT-PCR by using an ABI PRISM 7700 sequencer detector (PE Applied Biosystems). The primers used were as follows: 5′-AAGGAGGCTCACGTCAGACTGTA-3′ (forward), 5′-CCTGCACACAGCATGTTGCT-3′ (reverse), TaqMan probe, 5′-CAGCCGCTGTACCTCACAGCATCTGTTTAA-3′.

Immunoprecipitation and Immunoblot. Brain tissues were homogenized, and the supernatants were incubated with polyclonal goat anti-tPA antibodies (Santa Cruz Biotechnology), followed by protein G-Sepharose. The resulting immune complexes were resuspended in Laemli sample buffer. Immunoblots were probed with polyclonal rabbit anti-tPA antibodies (American Diagnostica, Greenwich, CT), followed by horseradish peroxidase-linked

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Abbreviations: plg, plasminogen; tPA, tissue plg activator; NAcc, nucleus accumbens; VTA, ventral tegmental area.

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anti-rabbit secondary antibodies. The enhanced chemiluminescence method (Amersham Pharmacia Biosciences) was used.

Zymography. Gel zymography was adapted from a procedure described previously (30). Ten percent polyacrylamide SDS gels were copolymerized with casein (1 mg/ml; Sigma) and plasminogen (15 μg/ml; Chromogenix, Molndal, Sweden). Brain tissues were homogenized in lysis buffer (50 mM Tris·HCl, pH 6.8/0.05% Triton X-100/2 mM EDTA) and assayed for protein content. Serial concentrations of the homogenate were loaded onto the gels and electrophoresed. After electrophoresis, the SDS was extracted from the gel by using 2.5% Triton X-100, and the gel was incubated for 1 h in 0.1 M Tris·HCl, pH 8.1, at 37°C, followed by staining with 0.125% Coomassie brilliant blue in 50% methanol/10% acetic acid. Destaining with the same solvent revealed a transparent zone of lysis against the dark protein background at 65 kDa corresponding to tPA. The enzymatic activity of tPA was analyzed with the ATTO Densitograph Software Library Lane Analyzer (Atto Instruments, Tokyo).

***In Situ* Hybridization.** Frozen sections (10 μm) were thawed on coverslips and used for *in situ* hybridization (31). pAtlas1A plasmid with rat tPA cDNA (BD Biosciences, Palo Alto, CA) was linearized with *Sac*I or *Kpn*I and used as a template for production of digoxigenin-labeled antisense cRNA (335 base) or sense cRNA (328 base) with T3 or T7 RNA polymerase (Promega).

Immunohistochemistry. Sections (14 μm) were incubated with polyclonal rabbit anti-tPA (1:1,000, Molecular Innovations, Southfield, MI) and monoclonal mouse antimicrotubule-associated protein 2 (MAP2) antibodies (1:5,000, Sigma), in blocking serum containing goat anti-rabbit Alexa Fluor 546 (1:500, Molecular Probes) and goat anti-mouse Alexa Fluor 488 (1:1,000, Molecular Probes). Samples were observed with AXIOVISION 3.0 systems (Zeiss).

***In Vivo* Microdialysis.** Animals were anesthetized with sodium pentobarbital, and a guide cannula (AG-8, EICOM, Kyoto) was implanted in the NAcc (AP +1.1, ML +1.0 from bregma, DV –3.6 from the skull) according to the atlas (32). Two days after the operation, a dialysis probe (AI-8–1; 1-mm membrane length, EICOM) was inserted through a guide cannula and perfused with an artificial cerebrospinal fluid (aCSF; 147 mM NaCl/4 mM KCl/2.3 mM $CaCl_2$) at a flow rate of 1.0 μl/min. The outflow fractions were collected every 20 min. After the collection of three baseline fractions, mice were treated with morphine (10 mg/kg s.c.). For depolarization stimulation, 60 mM KCl containing aCSF was delivered through the dialysis probe for 20 min. Dopamine levels in the dialysates were analyzed as described (33). For the rescue study with tPA and plasmin, a dialysis probe equipped with a microinjection tube (MIA-8–1; 1 mm membrane length, EICOM) was used (34). After the collection of baseline fractions, a 100-ng dose of human recombinant tPA (provided by Eisai, Tokyo) or 0.01 unit dose of human plasmin (Chromogenix) dissolved in 1 μl of aCSF solution was injected during a 10-min period through the microinjection tube into the NAcc. Ten minutes after the microinjection, mice were treated with morphine.

Behavioral Analysis. For the acute locomotor-stimulating effect, $tPA^{-/-}$ and wild-type mice were injected with saline or morphine (10 mg/kg s.c.) on day 1, and the locomotor activity was measured for 180 min. For the sensitization, mice were injected with morphine (10 mg/kg s.c.) twice per day for 5 days from days 2 to 6, and morphine-induced locomotor activity was measured on day 16. For the rescue study with tPA and plasmin, a guide cannula was implanted in the NAcc (AP +1.1, ML ±1.0 from bregma, DV –3.6 from the skull). After recovery from the operation, tPA (100 ng) or human plasmin (0.01 units) was injected through the microinjection tube into the NAcc. Ten minutes after the microinjection, mice were treated with morphine (10 mg/kg s.c.), and the locomotor activity was measured for 180 min.

For the conditioned place preference test (35), a mouse was allowed to move freely between transparent and black boxes for 15 min once per day for 3 days (days 1–3) in the preconditioning. On day 3, the time the mouse spent in each box was measured. On days 4, 6, and 8, the mouse was treated with morphine and confined in either the transparent or black box for 30 min. On days 5, 7, and 9, the mouse was given saline and placed opposite to the morphine-conditioning box for 30 min. On day 10, the postconditioning test was performed without drug treatment, and the time the mouse spent in each box was measured for 15 min.

For the hot-plate test (36), nociceptive latency was assessed as the response time to the hot plate (55 ± 1°C). To avoid tissue damage, an artificial maximum time for exposure was imposed, which prevented the animal from making contact with the plate for >30 sec.

Statistical Analysis. Statistical analysis was performed by using ANOVA and the Bonferroni test. The Mann–Whitney *U* test was used to compare two sets of data. Data were expressed as the mean ± SE. *P* values of <0.05 were considered statistically significant.

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Results

Morphine-Induced tPA mRNA Expression in the Brain. The effects of single and repeated administration of morphine on the expression of tPA mRNA in various regions of the rat brain were measured 2 h after the final morphine treatment by a real-time RT-PCR method. Single morphine treatment remarkably induced the tPA mRNA expression compared with saline treatment in the frontal cortex (FC, 257% of control, $P < 0.01$), NAcc (772%, $P < 0.01$), striatum (STR, 535%, $P < 0.01$), hippocampus (HIP, 799%, $P < 0.01$), VTA (337%, $P < 0.01$), and amygdala (AMY, 332%, $P < 0.01$) (Fig. 1*A*). Although the effect of morphine on tPA mRNA expression was significantly reduced after repeated treatment, expression levels were significantly higher in the repeated morphine-treated group than control group in the FC (146%, $P < 0.05$), NAcc (150%, $P < 0.05$), STR (143%, $P < 0.05$), and HIP (248%, $P < 0.01$) (Fig. 1*A*). Because it is known that there are some species differences in the effect of morphine, we also examined the effect of morphine on tPA expression in mice. The levels of tPA mRNA were significantly increased in the NAcc of mice 1 h (158% of control, $P < 0.01$), 2 h (179%, $P < 0.01$), and 6 h (140%, $P < 0.05$) after morphine treatment and then returned to the control value 24 h later [$F_{(6, 35)} = 4.401$, $P < 0.01$, Fig. 1*B*]. The protein levels of tPA determined by Western blotting were also significantly increased 6 h (147%, $P < 0.05$), 24 h (155%, $P < 0.01$), and 48 h (143%, $P < 0.05$) after single morphine treatment and then returned to the control value 1 week after the treatment [$F_{(6, 42)} = 3.384$, $P < 0.01$, Fig. 1*C*]. The enzymatic activity of tPA, which was assayed by gel zymography (30), was significantly increased compared with the saline-treated group 1 h (145%, $P < 0.01$), 2 h (139%, $P < 0.01$), 6 h (126%, $P < 0.05$), and 48 h (128%, $P < 0.05$) after the morphine treatment [$F_{(6,28)} = 3.733$, $P < 0.01$, Fig. 1*D*]. No enzymatic activity of tPA was detected in $tPA^{-/-}$ mice (Fig. 1*D*). Morphine-induced tPA activity was completely inhibited by pretreatment with naloxone (1 mg/kg i.p.), although naloxone itself had no effect, suggesting the involvement of opioid receptors in the morphine-induced increase in tPA activity (Fig. 1*E*).

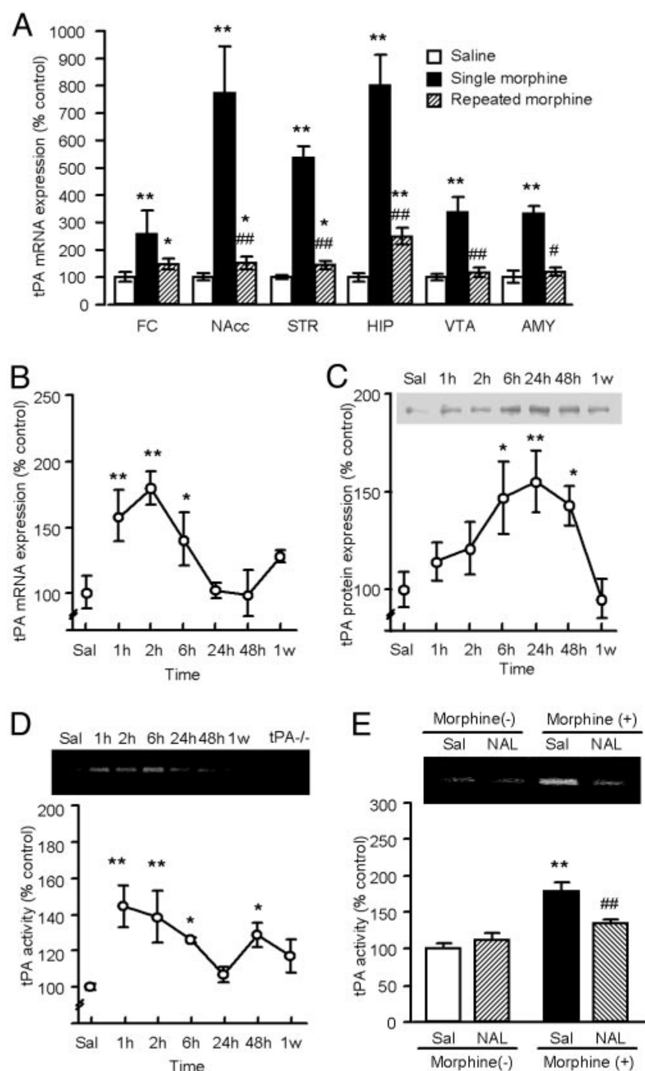

Fig. 1. Increased tPA expression after morphine treatment. (*A*) Changes in tPA mRNA expression after single (10 mg/kg s.c.) and repeated (10–50 mg/kg × 2 per day s.c.) morphine treatment in the rat brain. (*B*) Time course changes in tPA mRNA expression after single morphine (10 mg/kg s.c.) treatment in the NAcc of mice. (*C*) Immunoprecipitation/immunoblot analysis of tPA protein. (*D*) Zymographic analysis of tPA activity. (*E*) Effect of naloxone on single morphine-induced tPA activity. Values indicate means ± SE (n = 9–10 for *A*, n = 6 for *B*, n = 7 for *C*, n = 6 for *D*, and n = 4 for *E*). *, $P < 0.05$ and **, $P < 0.01$ compared with the saline (Sal)-treated group. #, $P < 0.05$ and ##, $P < 0.01$ compared with the single morphine-treated group.

Morphine-Induced tPA Is Produced in Neurons of the NAcc. It has been reported that tPA mRNA is present in both neurons and microglia but not oligodendrocytes or astrocytes (10, 19). To determine the cell types in which tPA expression is induced by acute morphine treatment, *in situ* hybridization with antisense tPA digoxigenin-labeled RNA probes as well as immunohistochemistry with specific tPA antibodies was performed (Fig. 2). The tPA mRNA was detected in cells of the NAcc in both saline- and morphine-treated wild-type mice (Fig. 2 *A* and *B*), but the signals in morphine-treated animals were apparently more intense than those in saline-treated animals. No signals were detected in the brain sections of $tPA^{-/-}$ mice (Fig. 2*C*). Immunohistochemistry revealed that tPA immunoreactivity was localized to cells positive for MAP2, a marker of neuronal cells (Fig. 2 *D–F*), indicating that tPA is produced in neuronal cells after morphine treatment.

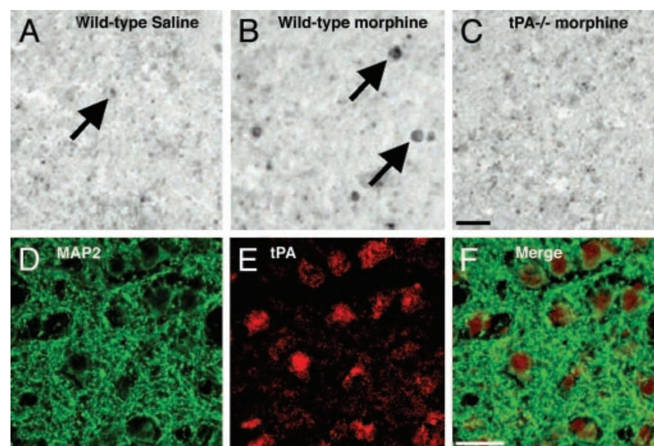

Fig. 2. tPA expression in the NAcc after morphine treatment. *In situ* hybridization analysis of tPA mRNA expression in the NAcc of saline-treated wild-type mice (*A*), morphine-treated wild-type mice (*B*), and morphine-treated $tPA^{-/-}$ mice (*C*). (*D–F*) Immunohistochemical analysis of morphine-induced tPA expression in the NAcc of mice. (*D*) MAP2. (*E*) tPA. (*F*) Merge. (Bar = 20 μm.)

No Changes in Antinociceptive Effects of Morphine and Tolerance in $tPA^{-/-}$ Mice. To investigate the physiological significance of the morphine-induced increase in tPA expression, we examined the antinociceptive effect of morphine and the development of its tolerance (36) in $tPA^{-/-}$ mice. There were no differences in the basal antinociceptive threshold between wild-type and $tPA^{-/-}$ mice in the hot-plate test (wild-type, 4.4 ± 0.5 sec; $tPA^{-/-}$, 4.6 ± 0.5 sec). No differences were observed either in the acute morphine-induced antinociceptive effect between wild-type and $tPA^{-/-}$ mice. Repeated injections of morphine (10 mg/kg × 2 per day s.c.) for 5 days resulted in a gradual loss of the antinociceptive effects of morphine in both wild-type and $tPA^{-/-}$ mice, and there were no differences at all in the time course (Fig. 3*A*). These results suggest that tPA has little effect on morphine-induced antinociception and its tolerance after repeated administration.

Morphine-Induced Behavioral Effects in $tPA^{-/-}$ and $plg^{-/-}$ Mice. Because we observed marked changes in tPA expression and activity in the NAcc, a brain area important for the rewarding effects of drugs of abuse (24, 27), we focused on the role of the tPA-plasmin system in the rewarding effects of morphine, which can be assessed by using the conditioned place-preference test (35). Morphine induced a dose-dependent conditioned place preference in wild-type mice, whereas saline treatment had no effect on place preference [$F_{(2, 48)} = 11.811$, $P < 0.01$, Fig. 3*B*]. Interestingly, the rewarding effects of morphine were markedly reduced in $tPA^{-/-}$ mice at the conditioning doses of 3 and 10 mg/kg ($P < 0.05$, Fig. 3*B*). Moreover, morphine (10 mg/kg s.c.) failed to induce place preference in $plg^{-/-}$ mice ($P < 0.05$, Fig. 3*C*), suggesting a role for the tPA-plasmin system in the rewarding effects of morphine.

The NAcc is involved not only in rewarding effects but also in locomotor-stimulating effects of morphine (22). Therefore, we measured the locomotor-stimulating effects of morphine in $tPA^{-/-}$ mice. Single morphine (10 mg/kg s.c.) treatment induced a hyperlocomotion in wild-type mice and $tPA^{-/-}$ mice, but the magnitude was significantly reduced in $tPA^{-/-}$ mice compared with wild-type mice ($P < 0.05$, Fig. 3*D*). Although repeated morphine treatment significantly potentiated the locomotor-stimulating effects of morphine in wild-type mice ($P < 0.01$), it failed to potentiate the hyperlocomotion in $tPA^{-/-}$ mice (Fig. 3*D*). We also examined whether exogenous tPA and plasmin can reverse the defect of loco-

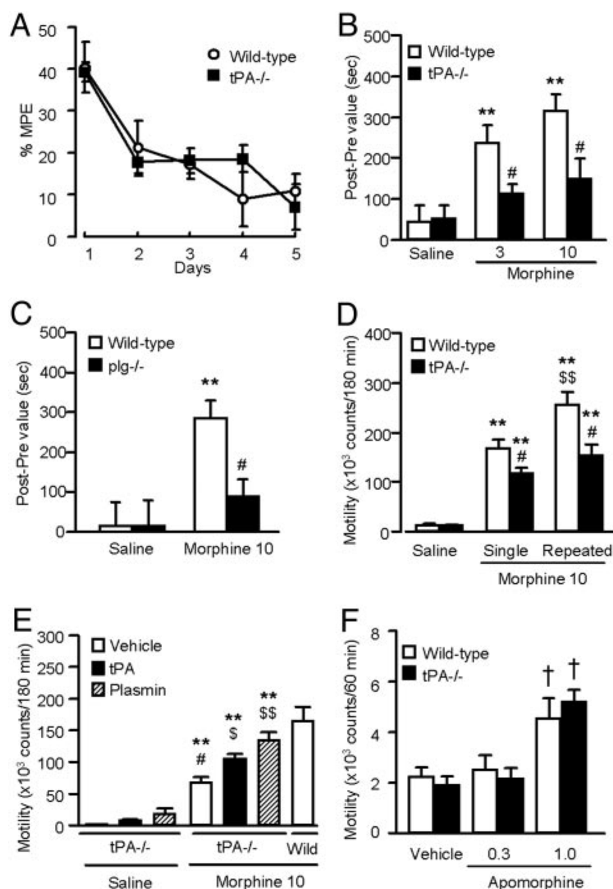

Fig. 3. Morphine-induced behavioral effects in $tPA^{-/-}$ and $plg^{-/-}$ mice. (*A*) Development of tolerance to the antinociceptive effect of morphine (10 mg/kg s.c.) in $tPA^{-/-}$ mice. (*B* and *C*) Morphine (3 and 10 mg/kg s.c.)-induced place preference in $tPA^{-/-}$ mice (*B*) and $plg^{-/-}$ mice (*C*). (*D*) Locomotor activity and locomotor sensitization induced by morphine (10 mg/kg s.c.) treatment. (*E*) Effect of tPA and plasmin microinjection into the NAcc on locomotor activity induced by morphine (10 mg/kg s.c.) in the $tPA^{-/-}$ mice. (*F*) Apomorphine (0.3 and 1.0 mg/kg s.c.)-induced hyperlocomotion. Values indicate means ± SE (n = 4–5 for *A*, n = 8–21 for *B*, n = 6–12 for *C*, n = 10 for *D*, n = 8 for *E*, and n = 8 for *F*). *, $P < 0.05$ and **, $P < 0.01$ compared to corresponding saline-treated mice. #, $P < 0.05$ compared to corresponding morphine-treated wild-type mice. \$, $P < 0.05$ and \$\$, $P < 0.01$ compared to vehicle plus morphine-treated $tPA^{-/-}$ mice. †, $P < 0.05$ compared to corresponding vehicle-treated mice.

motor-stimulating effect of morphine in $tPA^{-/-}$ mice. The attenuation of hyperlocomotion in $tPA^{-/-}$ mice was significantly reversed by microinjections of either exogenous tPA (100 ng, $P < 0.05$) or plasmin (0.01 units, $P < 0.01$) into the NAcc [$F_{(6, 49)} = 32.824$, $P < 0.01$, Fig. 3*E*].

The reduction of these behavioral effects of morphine in $tPA^{-/-}$ and $plg^{-/-}$ mice might be due to the alteration of dopamine and/or opioid receptor sensitivity. To test this possibility, we studied locomotor responses to different doses of apomorphine, a direct dopamine D1/D2 receptor agonist. In wild-type mice, apomorphine (0.3 and 1.0 mg/kg s.c.) significantly increased locomotor activities in a dose-dependent manner [$F_{(2, 21)} = 4.389$, $P < 0.05$], and there were no differences in apomorphine-induced hyperlocomotion between wild-type and $tPA^{-/-}$ mice (Fig. 3*F*). We also measured dopamine- and morphine-induced stimulation of [^{35}S]GTPγS binding in membrane preparation *in vitro*. The dopamine- and morphine-induced [^{35}S]GTPγS binding in $tPA^{-/-}$ mice did not differ from those in wild-type mice (Fig. 6, which is published as supporting information on the PNAS web site). These results indicate that the tPA-plasmin system plays a crucial role in morphine-induced rewarding and locomotor stimulating effects, and that these behavioral changes in $tPA^{-/-}$ mice are not due to alterations of dopamine and opioid receptor sensitivity.

Role of tPA in Morphine-Induced Dopamine Release in the NAcc. It has been suggested that the enhancement of dopamine release in the NAcc is an essential process related to the morphine-induced rewarding effect (37). To clarify the mechanisms by which $tPA^{-/-}$ mice exhibited the reduced morphine-induced rewarding and locomotor-stimulating effects, we measured morphine-induced dopamine release in the NAcc. *In vivo* microdialysis revealed that basal levels of dopamine in the NAcc were not different between wild-type and $tPA^{-/-}$ mice (wild-type, 0.41 ± 0.13 nM, n = 4; $tPA^{-/-}$, 0.53 ± 0.01 nM, n = 4). The dopamine levels in the NAcc were markedly increased by s.c. injection of morphine at 10 mg/kg in wild-type mice (Fig. 4*A*). This morphine-induced dopamine release was markedly diminished in $tPA^{-/-}$ mice [$F_{(1, 6)} = 13.061$, $P < 0.05$, Fig. 4*A*]. Similarly, although basal levels of dopamine in the NAcc of $plg^{-/-}$ mice did not differ from those in wild-type mice (wild-type, 0.38 ± 0.10 nM, n = 6; $plg^{-/-}$, 0.38 ± 0.05 nM, n = 6), morphine-induced dopamine release in the NAcc was significantly reduced in $plg^{-/-}$ mice compared to wild-type mice [$F_{(1, 10)} = 25.147$, $P < 0.01$, Fig. 4*B*].

We investigated the mechanisms underlying the defect of morphine-induced dopamine release in $tPA^{-/-}$ mice and $plg^{-/-}$ mice. Microinjection of tPA (100 ng) into the NAcc slightly but significantly increased basal levels of extracellular dopamine in $tPA^{-/-}$ mice [$F_{(1, 8)} = 7.941$, $P < 0.05$, Fig. 4*C*]. Further, this pretreatment dramatically increased morphine-induced dopamine release in $tPA^{-/-}$ mice as observed in wild-type mice [$F_{(1, 8)} = 5.428$, $P < 0.05$, Fig. 4*D*]. In contrast, microinjection of tPA (100 ng) into the VTA failed to increase morphine-induced dopamine release in the NAcc of $tPA^{-/-}$ mice (Fig. 7, which is published as supporting information on the PNAS web site). These results suggest that the defect of morphine-induced dopamine release in $tPA^{-/-}$ mice is due to the deficiency of tPA in the NAcc, not to a developmental malfunction. Microinjection of plasmin (0.01 units) into the NAcc caused a transient but significant increase in basal dopamine levels in $tPA^{-/-}$ mice [$F_{(1, 8)} = 6.612$, $P < 0.05$, Fig. 4*E*]. Moreover, this pretreatment markedly increased morphine-induced dopamine release in $tPA^{-/-}$ mice [$F_{(1, 8)} = 13.121$, $P < 0.01$, Fig. 4*F*]. These results suggest that tPA modulates morphine-induced dopamine release probably by converting plg to plasmin in the NAcc.

Because tPA is released into the extracellular space by a depolarization stimulus (11, 12), we investigated the depolarization (60 mM KCl)-evoked dopamine release in the NAcc of $tPA^{-/-}$ mice. The lack of tPA significantly attenuated the depolarization-evoked dopamine release in the NAcc [$F_{(1, 10)} = 6.846$, $P < 0.05$, Fig. 5*A*]. There were no differences in the protein content of tyrosine hydroxylase in the NAcc and lower midbrain, a rate limiting enzyme of dopamine synthesis, between $tPA^{-/-}$ and wild-type mice (Fig. 5*B*).

Discussion

It has been demonstrated that tPA takes part in the proteolysis of extracellular matrix proteins in the central nervous system and is involved in neuronal plasticity. In the present study, we demonstrated a previously undescribed function of the tPA-plasmin system in regulating dopamine release in the NAcc. Furthermore, the tPA-plasmin system is involved in the rewarding effects of morphine by regulating dopamine release.

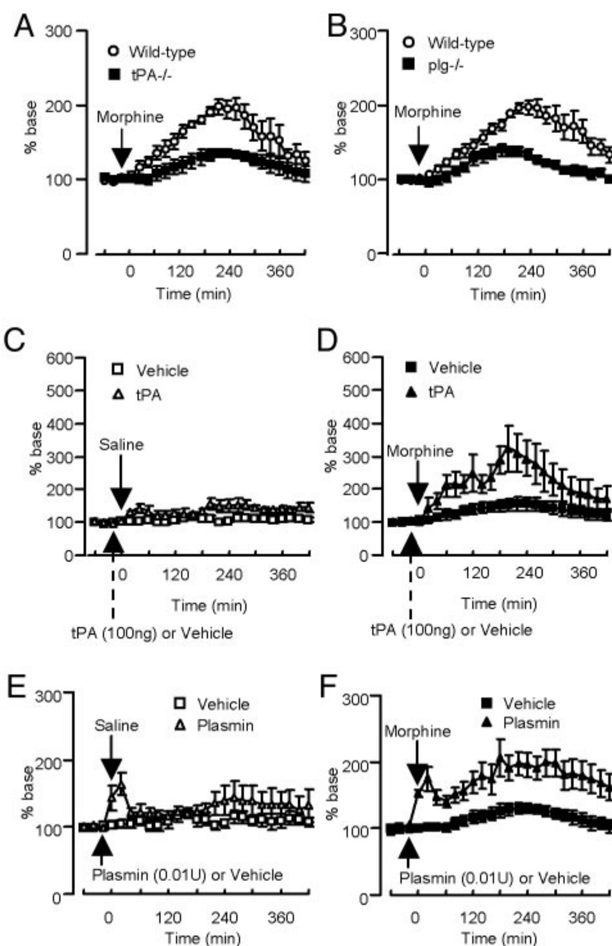

Fig. 4. Defect of morphine-induced dopamine release in $tPA^{-/-}$ mice and $plg^{-/-}$ mice. (*A*) Basal levels of dopamine in the NAcc did not differ between wild-type and $tPA^{-/-}$ mice (wild-type, 0.41 ± 0.13 nM, $n = 4$; $tPA^{-/-}$, 0.53 ± 0.01 nM, $n = 4$). Morphine (10 mg/kg s.c.)-induced dopamine release was markedly diminished in $tPA^{-/-}$ mice [$F_{(1, 6)} = 13.061$, $P < 0.05$]. (*B*) Basal levels of dopamine in the NAcc of $plg^{-/-}$ mice did not differ from those in wild-type mice (wild-type, 0.38 ± 0.10 nM, $n = 6$; $plg^{-/-}$, 0.38 ± 0.05 nM, $n = 6$). Morphine-induced dopamine release in the NAcc was significantly reduced in $plg^{-/-}$ mice compared to wild-type mice [$F_{(1, 10)} = 25.147$, $P < 0.01$]. (*C* and *D*) Effect of tPA on dopamine release in the NAcc of $tPA^{-/-}$ mice. Microinjection of tPA significantly increased basal levels of extracellular dopamine [$F_{(1, 8)} = 7.941$, $P < 0.05$] and morphine-induced dopamine release [$F_{(1, 8)} = 5.428$, $P < 0.05$) in $tPA^{-/-}$ mice]. (*E* and *F*) Effect of plasmin on dopamine release in the NAcc of $tPA^{-/-}$ mice. Microinjection of plasmin significantly increased basal levels of extracellular dopamine [$F_{(1, 8)} = 6.612$, $P < 0.05$] and morphine-induced dopamine release [$F_{(1, 8)} = 13.121$, $P < 0.01$] in $tPA^{-/-}$ mice. Values indicate means ± SE ($n = 4$ for *A*, $n = 6$ for *B*, and $n = 5$ for 4 *C*–*F*).

Single morphine treatment induced tPA mRNA expression in various regions of the rat brain. Although repeated morphine treatment reduced the ability of morphine to induce tPA mRNA expression in the brain, levels of tPA mRNA remained significantly higher in the frontal cortex, NAcc, striatum, and hippocampus. It is reported that the level of tPA is regulated by cAMP response element-binding protein (CREB) (8), and that CREB levels are reduced in the NAcc after chronic morphine treatment (38). Therefore, the reduced ability of morphine after repeated treatment to induce tPA mRNA expression in the brain may be due at least in part to the decrease in CREB levels.

Morphine-induced conditioned place preference was significantly attenuated in $tPA^{-/-}$ and $plg^{-/-}$ mice as compared with wild-type mice, suggesting that the tPA-plasmin system participates in the rewarding effects of morphine. However, because $tPA^{-/-}$ mice show impaired learning and memory in the context fear conditioning and two-way active avoidance test (18), we cannot exclude the possibility that $tPA^{-/-}$ mice failed to associate morphine-induced rewarding effects with the context during the conditioning. The mesolimbic dopaminergic pathway projecting from the VTA to the NAcc is thought to play a major role in mediating the rewarding effects of many stimuli, such as electrical brain stimulation and drugs of abuse (24). This dopamine system is important not only for rewarding effects but also locomotor-stimulating effects of morphine, the behavior being unaffected by the learning and memory function. The attenuation of both morphine-induced place preference and hyperlocomotion in $tPA^{-/-}$ and $plg^{-/-}$ mice suggests that the tPA-plasmin system plays a role in morphine-induced dopamine release in the NAcc.

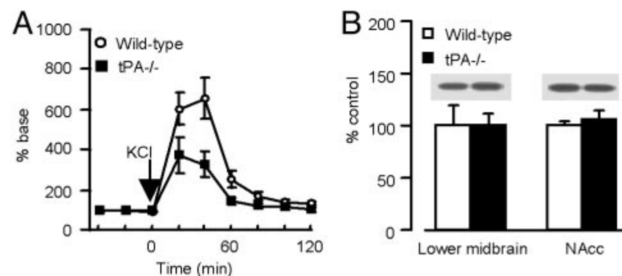

Fig. 5. Depolarization-evoked dopamine release and tyrosine hydroxylase content of $tPA^{-/-}$ mice. (*A*) High KCl (60 mM)-induced dopamine release in the NAcc of wild-type and $tPA^{-/-}$ mice. The lack of tPA significantly attenuated the depolarization-evoked dopamine release in the NAcc [$F_{(1, 10)} = 6.846$, $P < 0.05$]. (*B*) Immunoblot analysis of tyrosine hydroxylase of $tPA^{-/-}$ mice. There were no differences in the protein content of tyrosine hydroxylase in the lower midbrain and NAcc between $tPA^{-/-}$ and wild-type mice. Values indicate means ± SE ($n = 6$ for *A*, and $n = 4$ for *B*).

Electrophysiological, biochemical, and behavioral data have suggested that tPA interacts extensively with dopamine D1 receptor-mediated responses in the brain. For instance, inactivation of the gene encoding tPA prevents the electrophysiological effects of D1 receptor agonists and mimics the effects of D1 receptor antagonists on the late phase of CA1 hippocampal long-term potentiation (39). The sensitivity of striatal cholinergic interneurons to dopamine D1 receptor stimulation is lost in $tPA^{-/-}$ mice (40). In contrast, we found that there were no differences in dopamine and morphine-induced increases in [^{35}S]GTPγS binding between wild-type and $tPA^{-/-}$ mice. No differences were evident in apomorphine-induced hyperlocomotion and tyrosine hydroxylase protein levels between the two types of mice. Accordingly, it is unlikely that the alterations of rewarding and locomotor-stimulating effects of morphine in $tPA^{-/-}$ mice are mainly due to the dysfunction of dopamine and opioid receptors in $tPA^{-/-}$ mice.

In vivo microdialysis and electrophysiological studies have provided evidence that the enhancement of dopamine release in the NAcc may be an essential process related to the morphine-induced rewarding effect (37, 41). In the present study, we demonstrated that morphine-induced dopamine release was attenuated in the NAcc of $tPA^{-/-}$ and $plg^{-/-}$ mice, and that microinjection of either exogenous tPA or plasmin into the NAcc, but not into the VTA, restored the morphine-evoked dopamine release in $tPA^{-/-}$ mice. The reduction of morphine-induced hyperlocomotion in $tPA^{-/-}$ mice was also reversed by microinjections of either exogenous tPA or plasmin into the NAcc. Western blotting of tyrosine hydroxylase contents of the brain revealed there are no differences be-

tween $tPA^{-/-}$ and wild-type mice, which is consistent with the immunohistochemical data (40). Therefore, plasmin that is converted from plg by tPA may have a role in regulating morphine-induced dopamine release in the NAcc.

The molecular mechanisms by which the tPA-plasmin system regulates morphine-induced dopamine release in the NAcc remain to be determined. However, it is known that the tPA-plasmin system degrades several extracellular matrix proteins (42), including laminin (43). Laminin in the synaptic cleft localizes calcium channels to the sites of active zones (44) and induces a small but significant increase in calcium levels in ciliary ganglion neurons when applied in soluble form to the culture medium (45). Accordingly, it is possible that a defect of the tPA-plasmin system may result in a malfunction of calcium channel activity, which leads to the reduction of depolarization-evoked dopamine release.

Conclusion

We have demonstrated that morphine increases tPA expression, and its enzyme activity in neuronal cells of the NAcc, by activating opioid receptors. The tPA-plasmin system plays a crucial role in regulating morphine-induced dopamine release in the NAcc and thereby is involved in the rewarding and locomotor-stimulating effects, without affecting the antinocipeptive effects.

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