Tissue plasminogen activator modulates the cellular and behavioral response to cocaine

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Cocaine exposure induces long-lasting molecular and structural adaptations in the brain. In this study, we show that tissue plasminogen activator (tPA), an extracellular protease involved in neuronal plasticity, modulates the biochemical and behavioral response to cocaine. When injected in the acute binge paradigm, cocaine enhanced tPA activity in the amygdala, which required activation of corticotropin-releasing factor type-1 (CRF-R1) receptors. Compared with WT mice, tPA−/− mice injected with cocaine displayed attenuated phosphorylation of ERK, cAMP response element binding protein (CREB), and dopamine and cAMP-regulated phosphoprotein 32 kDa (DARPP-32) and blunted induction of immediate early genes (IEGs) c-Fos, Egr-1, and Homer 1a in the amygdala and the nucleus accumbens (NAc). tPA−/− mice also displayed significantly higher basal preprodynorphin (ppDyn) mRNA levels in the NAc in comparison to WT mice, and cocaine decreased ppDyn mRNA levels in tPA−/− mice only. Cocaine-induced locomotor sensitization and conditioned place preference (CPP) were attenuated in tPA−/− mice. Cocaine exposure also had an anxiolytic effect in tPA−/− but not WT mice. These results identify tPA as an important and novel component of the signaling pathway that modulates cocaine-induced changes in neurotransmission and behavior.

Results

Acute Binge Cocaine Exposure Increases tPA Activity in the Amygdala. To determine if cocaine regulates tPA activity in the brain, WT mice were injected with cocaine in the acute binge paradigm to mimic the abuse pattern of cocaine in humans (18, 19). Mice were killed 30 min or 6 h after the final injection of cocaine, and extracellular tPA activity in the amygdala was measured by in situ zymography. A 2.1-fold increase in extracellular tPA activity was observed in the amygdala 30 min after the final cocaine injection in comparison to saline-injected mice (Fig. 1A and B). However, no change in extracellular tPA activity was observed in the NAc or caudate putamen (CP, data not shown). This increase in tPA activity was no longer observed 6 h after the final cocaine injection (Fig. 1A and B). In-gel zymography showed that total tPA activity increased in the amygdala 30 min after the last injection of cocaine and returned to basal levels after 6 h. tPA protein levels, measured by ELISA, were unchanged at either of


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Acute cocaine increases tPA activity in the amygdala. (A) Extracellular tPA activity was measured by in situ zymography 30 min and 6 h after the final cocaine injection and quantified by measuring area of lysis (dark lytic zones). tPA activity was significantly increased in the amygdala of WT mice 30 min but not 6 h after acute binge cocaine exposure compared to saline-injected mice. (B) Quantification of results in A. Two-way ANOVA analysis revealed a significant main effect of time \( F(1, 12) = 9.01, P < 0.05 \), treatment \( F(1, 12) = 25.53, P < 0.005 \), and treatment \( \times \) time interaction \( F(1, 12) = 9.01, P < 0.05 \); \( * * P < 0.005 \), cocaine vs. saline, \( n = 4–5 \)/group. (C) Acute cocaine exposure increased PAI-1 levels in the amygdala 6 h but not 30 min after the last cocaine injection compared to saline-injected control samples. \( ** P < 0.005 \), t test, vs. saline control, \( n = 4–5 \)/group. Sal, saline; Coc, cocaine.

To explore the mechanism behind this regulation of tPA, we examined levels of PAI-1 (plasminogen activator inhibitor-1), an inhibitor of tPA, by ELISA. Acute binge cocaine enhanced PAI-1 levels 1.3-fold 6 h after the final cocaine injection in the amygdala of WT mice (Fig. 1C), which may explain the reduction in tPA activity observed at this time point. These changes were not found after saline injections. Changes in neuroserpin levels were not observed at either of these time points (data not shown).

Acute Cocaine Increases tPA Activity in the Amygdala in a CRF/CRF-R1-Dependent Manner. Acute cocaine is a potent activator of the hypothalamic pituitary adrenal (HPA) axis and increases plasma corticosterone (CORT) and CRF levels in hypothalamic and extrahypothalamic brain regions (20, 21). Previous work showed that CRF regulates tPA activity in the amygdala (10). To determine whether cocaine-induced increases in tPA activity occur in a CRF-dependent manner, we examined whether antalarmin, a specific inhibitor of the CRF-R1, blocked cocaine-induced increases in tPA activity. Mice were injected with antalarmin 30 min before the acute binge cocaine injection and were killed 30 min after the final cocaine injection. Control groups included mice treated with vehicle plus saline, vehicle plus cocaine, and antalarmin plus saline. tPA activity was measured in the amygdala by in situ zymography. In mice that received injections of vehicle, cocaine increased tPA activity 1.6-fold in the amygdala. In contrast, mice that received antalarmin before cocaine injections failed to show significant enhancement of tPA activity (Fig. 2A and B). Differences in tPA activity were not observed between the vehicle plus saline and antalarmin plus saline groups. These results suggest that cocaine enhances tPA activity in the amygdala in a CRF/CRF-R1-dependent manner.

We next determined cocaine-induced activation of the stress responsive CRF system in WT and tPA\( -/- \) mice. Cocaine increased plasma CORT levels in WT and tPA\( -/- \) mice (Fig. 3A). Cocaine also significantly increased CRF and CRF-R1 mRNA levels in the amygdala of WT and tPA\( -/- \) mice 30 min after the final injection of cocaine, while these levels remained unchanged in saline-injected mice (Fig. 3B and Fig. S2). These
results demonstrate that there are no differences in cocaine-induced activation of the CRF system in the amygdala of WT and tPA−/− mice.

Effects of Acute Binge Cocaine on Preprodynorphin (ppDyn) mRNA in WT and tPA−/− Mice. Basal levels of ppDyn mRNA in the NAc of tPA−/− mice were significantly higher than those in that of WT mice (Fig. 4). However, ppDyn mRNA levels were significantly decreased in tPA−/− but remained unchanged in WT mice after cocaine injection in comparison to saline-injected tPA−/− mice (Fig. 4). mRNA levels of KOP-r and prepro-enkephalin remained unchanged in the NAc between genotypes before and after treatment (data not shown). It has been demonstrated that NAc dialysate DA levels are decreased after stimulation of KOP-r receptors, which may lead to a decreased dopaminergic tone (22). On the basis of this evidence, we hypothesize that increased basal levels as well as cocaine-induced alterations of ppDyn may lead to altered DAergic signaling in the NAc.

Cocaine-Induced Neuronal Signaling Is Altered in the Amygdala and NAc of tPA−/− Mice. Acute cocaine initiates a sequence of signaling events, including phosphorylation of several intracellular signaling molecules and transcription factors, and culminates in the induction of immediate early genes (IEGs) (1). We compared signaling events in WT and tPA−/− mice by Western blot analysis. Mice were injected in the acute binge cocaine paradigm and killed 30 min after the final injection. Cocaine significantly increased ERK phosphorylation in the amygdala of WT but not tPA−/− mice (Fig. 5A and B). The expression of c-Fos and Egr-1 IEGs was examined 2 h after the final cocaine injection. Cocaine induced a significant increase in c-Fos and Egr-1 expression in the amygdala of WT mice, while it caused no change in c-Fos and a decrease in Egr-1 expression in tPA−/− mice (Fig. 5A and B). Significant increase in basal expression of Egr-1 was also observed in the amygdala of tPA−/− mice (Fig. S3).

In the NAc, dopamine and cAMP-regulated phosphoprotein 32 kDa (DARPP-32) phosphorylation (at Thr-34) was significantly increased 5 min after the final cocaine injection in WT but not tPA−/− mice. Acute cocaine also increased phosphorylation of cAMP response element binding protein (CREB) 15 min following acute cocaine exposure in WT but not tPA−/− animals (Fig. 5 C and D). Induction of c-Fos expression was also observed 2 h after the final cocaine injection in the NAc of WT but not tPA−/− mice (Fig. 5C and Fig. S2). Acute cocaine increased the expression of Homer 1a in WT but not tPA−/− mice. Our results also revealed an increase in basal level of expression of Homer 1a in the NAc of tPA−/− mice in comparison to WT mice (Fig. 5 C and D). No changes in total DA D1 receptor levels were observed in the NAc of WT and tPA−/− mice (Fig. S4).

Locomotor Stimulation and Behavioral Sensitization to Cocaine in tPA−/− Mice. Our results suggest altered cocaine-induced neuronal activation in the NAc and amygdala of tPA−/− mice. Since the development of behavioral sensitization to cocaine requires neuroadaptation in these two brain regions (1), we compared this behavior between WT and tPA−/− mice. Mice were injected with repeated binge cocaine for 5 days, were not disturbed on day 6, and were challenged with acute binge cocaine on day 7. Cocaine-induced locomotor activation was compared on days 1 and 7. Differences were not observed in acute (day 1) cocaine-induced locomotor stimulation between WT and tPA−/− mice (Fig. 6A). However, WT mice showed significant sensitization to the locomotor stimulating effects of cocaine on day 7 while tPA−/− mice did not (Fig. 6A and Fig. S5).
Cocaine-induced WT mice spent significantly more time (250 s) in the cocaine-paired side of the chamber compared to their saline-injected counterparts (Fig. 6B). However, cocaine-injected tPA−/− mice spent only 100 s more time in the cocaine-paired side of the compartment, which is not significantly different from saline-injected tPA−/− mice. These results demonstrate that cocaine-induced CPP is attenuated in tPA−/− mice.

**Cocaine Induces Anxiolytic Behavior in tPA−/− Mice.** Acute binge cocaine increases CRF mRNA levels and tPA activity in the amygdala, a brain region known to facilitate the development of anxiogenic responses (21). Cocaine also causes a decrease in Dyn levels in the NAc of tPA−/− mice, which may lead to decreased KOP-1 activation and reduced anxiety (23). Hence, we determined anxiety levels in WT and tPA−/− mice following repeated binge cocaine administration. On day 7, mice were subjected to the elevated plus maze 30 min after the final cocaine injection. WT mice displayed very few open arm entries (OAEs) after either saline or cocaine injections, indicating a high basal level of anxiety (Fig. 6C). The number of OAEs in saline-injected tPA−/− mice was not significantly different from that in WT mice. However, tPA−/− mice displayed significantly more OAEs after cocaine injection compared to their saline-injected counterparts. These results suggest that cocaine induces anxiolytic behavior in tPA−/− but not WT mice (Fig. 6C).

**Discussion**

It is hypothesized that addiction is in part due to drug-induced changes in neuronal processes that underlie learning and memory (1). Cocaine is thought to produce long-lasting changes in synaptic strength, structure, and behavior by regulating intracellular signaling and gene expression (1). Here we show that tPA is a critical component of the signaling cascade initiated by cocaine and is required for cocaine-induced molecular and behavioral adaptations.

In this study, we show that acute binge cocaine administration enhanced extracellular tPA activity in the amygdala. tPA activity returned to basal levels 6 h following the final cocaine injection. A concomitant increase in PAI-1 levels was observed 6 h after cocaine injection, suggesting that PAI-1 could serve as a homeostatic mechanism to counter cocaine-induced increases in tPA activity. It has been reported that the effect of cocaine on synaptic DA and CRF levels is negligible 3 h after its administration (18, 24). Hence, it is possible that the eventual decrease in tPA activity may reflect a decrease in cocaine activation of the CRF/CRF-R1 pathway. One previous study reported increased tPA mRNA levels in the PFC and NAc after single injections of cocaine (13, 16). However, acute binge cocaine did not alter total tPA activity in the PFC or NAc at 30 min or 6 h after the final cocaine injection (data not shown).

Acute binge cocaine is a potent activator of the HPA axis. Cocaine enhances plasma CORT levels and induces expression of CRF mRNA, an important component of the behavioral response to stress in the hypothalamus (20, 21). Cocaine-induced expression of CRF in extrahypothalamic regions, such as the amygdala, is thought to modulate the development of locomotor sensitization, reward, and reinstatement to cocaine (3, 25, 26). In our study, acute binge cocaine injections activated the CRF system in both WT and tPA−/− mice. Antalarmin, a specific inhibitor of CRF-R1, attenuated cocaine-induced increase in tPA, suggesting that cocaine enhances tPA activity in the amygdala in a CRF/CRF-R1-dependent manner. This result provides further evidence that tPA is a downstream effector protein for CRF in the amygdala.

Cocaine-induced increases in extracellular DA lead to a sequence of signaling events initiated by activation of DA D1 receptors. The subsequent increase in intracellular cAMP levels and protein kinase A (PKA) activation results in initiation of intracellular signaling pathways (27). In the amygdala, activation of PKA leads to phosphorylation of ERK and activation of IEGs (28). Cocaine induces phosphorylation of DARPP-32 at Thr-34 in a subset of neurons in the NAc (27, 28). This in turn leads to phosphorylation of the transcription factor CREB and its subsequent translocation to the nucleus where it initiates a program of gene expression (29). Interfering with these cocaine-induced signaling events leads to an altered behavioral response to cocaine. For example, inhibiting ERK phosphorylation using a specific inhibitor or genetic deletion of Egr-1 reduces cocaine reward (30, 31). Recent studies suggest that mice in which an alanine mutation is introduced at position Thr-34 of DARPP-32 have a delayed acquisition of cocaine self-administration. However, mutant mice self-administer cocaine at a higher level after cocaine self-administration behavior is established, suggesting that phosphorylation of Thr-34 is critical in modulating the reinforcing effects of cocaine (32). In tPA−/− mice, these signaling events were attenuated both in the NAc and in the amygdala, which strongly suggests that tPA is an important...
component of this signaling pathway initiated by cocaine. The lack of these signaling events may underlie deficits in the behavioral effects of cocaine observed in tPA−/− mice.

Acute binge cocaine induces the expression of Homer 1a, which interacts with metabotropic glutamate receptors to regulate glutamatergic synaptic transmission (33). In contrast to WT mice, cocaine decreased Homer 1a levels in the NAc of tPA−/− mice (although this effect did not reach statistical significance, P = 0.06; Fig. 5 C and D). Increased basal levels of Homer 1a expression were also observed in the NAc of tPA−/− mice in comparison to WT mice. These suggest that tPA is necessary for cocaine regulation of Homer 1a expression and may play a role in modulating drug-induced neuronal plasticity by modulating glutamatergic signaling in the NAc.

Basal ppDyn mRNA levels were increased in the NAc (but not CP) of tPA−/− mice in comparison to WT mice (Fig. 4 and Fig. S6). Pretreatment with Dyn A (1–17) is effective in decreasing striatal DA neurotransmission and attenuating cocaine-induced CPP in mice (34). Overexpression of CREB with resulting increases in ppDyn gene expression in the NAc has been shown to decrease the rewarding effects of cocaine (29). Further, both Dyn and KOP−/− mice display enhanced behavioral sensitization (4). Hence, the lack of behavioral sensitization and reward in tPA−/− mice could also be due to an increased basal level of dynorphin. In agreement with other reports (4, 5) acute cocaine did not alter the ppDyn gene expression in the NAc of WT animals (Fig. 4), but it significantly decreased ppDyn mRNA levels in the NAc of tPA−/− mice.

How might tPA modulate cocaine-induced neuroadaptation?

A previous study revealed that there are no differences in tyrosine hydroxylase levels between WT and tPA−/− mice (12). Furthermore, no differences in acute locomotor stimulatory effects of cocaine were observed in WT and tPA−/− mice (Fig. 6). These results suggest that there are likely no differences in cocaine-induced increases in DA levels in tPA−/− mice.

Cocaine-induced activation of ERK and IEGs in the amygdala is dependent on the D1 receptor (25, 35). D1 receptor expression is similar between WT and tPA−/− mice in the amygdala (data not shown) and NAc (Fig. S4). However, cell surface expression of D1 receptors may be altered in tPA−/− mice. Several studies suggest a role for tPA in regulating D1 receptor function (36, 37). Therefore, it is possible that tPA may be required for proper D1 receptor signaling in the amygdala (Fig. 7).

In the NAc, coincident signaling by NMDA and D1 receptors is thought to mediate cocaine-induced changes in signaling and gene expression (28). Because tPA is required for proper assembly and signaling through the NMDAR complex (8), we hypothesize that tPA may regulate signaling through both the D1 and the NMDA receptors in the NAc. Our results indicate that cocaine does not alter extracellular and total TPA activity in the NAc (data not shown), suggesting that tPA could be an essential upstream component of the D1 signaling pathway that is activated by cocaine (Fig. 7). It is also possible that the lack of tPA during development initiates compensatory changes in the NAc and amygdala, such as increased basal expression of Egr-1, Homer 1a, and Dyn that may lead to altered cocaine-induced neuroadaptation.

Because cocaine-induced neuroplasticity in the amygdala and NAc is critical for the behavioral effects of cocaine (1), we examined some of these behaviors in tPA−/− mice. Differences were not observed in acute binge cocaine-induced locomotor stimulation between WT and tPA−/− mice. However, unlike their WT counterparts, tPA−/− mice failed to develop significant behavioral sensitization to cocaine and showed attenuated CPP. Consistent with this finding, rats in which tPA expression in the NAc was knocked down using siRNA strategies also displayed deficits in locomotor sensitization (13). However, an earlier study showed that tPA−/− mice display enhanced sensitization to cocaine (17). Different from our cocaine treatment, this study examined locomotor sensitization after 10 sessions (10 mg/kg cocaine per session) over a 4-week period.

Acute cocaine potently activates the HPA axis and increases CRF mRNA and TPA activity in the amygdala, all key players involved in the development of anxiogenic responses (9, 10). Our results demonstrate that binge cocaine reduces Dyn levels in the NAc of tPA−/− mice, which may in turn lead to reduced activation of KOP−/−. It has been demonstrated that blockade of KOP−/− results in anxiolytic behavior (23). Hence, anxiety levels were measured in WT and tPA−/− mice after cocaine injection. Consistent with our biochemical findings, cocaine had an anxiolytic effect in tPA−/− but not WT mice.

In summary, our results indicate a role for tPA in modulating cocaine-induced molecular and behavioral adaptations. Given the role of tPA in regulating the behavioral response to stress, one interesting hypothesis that stems from this study is that tPA may be an important link between environmental stress and drug abuse. These data provide a rationale for the development of tPA-based pharmacotherapies for the treatment of compulsive cocaine use in humans.

Methods

Binge Cocaine Administration. tPA−/− mice (8–12 weeks of age on CS7 background) were obtained from Jackson Labs and bred at The Rockefeller University’s animal care facility (SI Methods). WT (CS7) mice were also obtained from Jackson Labs. In the acute binge paradigm, mice received 3 i.p. injections of cocaine at a total dose of 45 mg/kg/day (3 × 15 mg/kg/injection) in their home cages at 1-h intervals. For the repeated binge cocaine paradigm, mice were injected with 3 × 15 mg/kg/injection for 5 or 7 days in their home cages.

Materials. The following antibodies were used: anti-phospho (P)-ERK1/2 and anti-ERK1/2 (Cell Signaling), anti-P-CREB and anti-CREB (Cell Signaling), anti-Homer 1a (Santa Cruz Biotechnology), anti-C-Fox (Santa Cruz Biotechnology), anti-Egr-1 (Santa Cruz Biotechnology), anti-P-DARPP-32 (Phosphosolutions), anti-D1 Receptor, and DARPP-32 (Cell Signaling). The PAI-1 total antigen kit from Molecular Innovations was used. Plasma CORT levels were determined by an RIA (MP Biomedicals).
Plasmids. See SI Methods.

Antalarmin Experiments. Antalarmin (Sigma) was prepared in DMSO. WT mice were injected i.p. at a concentration of 20 mg/kg 30 min before acute binge cocaine injection.

Western Blotting. See SI Methods.

Solution Hybridization Ribonuclease (RNase) Protection Assays. Tissues were homogenized in guanidinium thiocyanate buffer and extracted with acidic phenol and chloroform. Protection assay was performed as described previously (20) (see SI Methods).

In Situ Zymography. In situ zymography was performed as described previously (9) (see SI Methods).

Behavioral Analysis. See SI Methods.

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