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Silencing urokinase in the ventral tegmental area *in vivo* induces changes in cocaine-induced hyperlocomotion

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Abstract

Serine proteases in the nervous system have functional roles in neural plasticity. Among them, urokinase-type plasminogen activator (uPA) exerts a variety of functions during development, and is involved in learning and memory**.** Furthermore, psychostimulants strongly induce uPA expression in the mesolimbic dopaminergic pathway. In this study, doxycycline-regulatable lentiviruses expressing either uPA, a dominant-negative form of uPA, or non-regulatable lentiviruses expressing small interfering RNAs (siRNAs) targeted against uPA have been prepared and injected into the ventral tegmental area (VTA) of rat brains. Overexpression of uPA in the VTA induces doxycycline-dependent expression of its receptor, uPAR, but not its inhibitor, plasminogen activator inhibitor-1 (PAI-1). uPAR expression in the VTA is repressed upon silencing of uPA with lentiviruses expressing siRNAs. In addition, overexpression of uPA in the VTA promotes a 15-fold increase in locomotion activity upon cocaine delivery. Animals expressing the dominant-negative form of uPA did not display such hyperlocomotor activity. These cocaine-induced behavioural changes, associated with uPA expression, could be suppressed in the presence of doxycycline or uPA-specific siRNAs expressing lentiviruses. These data strongly support the major role of urokinase in cocaine-mediated plasticity changes.

Keywords: addiction, *in vivo* gene transfer, lentivirus, plasticity, small interfering RNA, urokinase-type plasminogen activator.

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Proteolytic enzymes play a role in normal and pathological events in the brain, including plasticity. Plasminogen activators are important mediators of extracellular metabolism, involved in remodelling events during development and regeneration in the nervous system. The generation of plasmin from its inactive precursor plasminogen is mediated by serine enzymes known as tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA), and contributes to the turnover of the extracellular matrix in the central nervous system. tPA plays a role in cognitive memory, mediates reverse occlusion plasticity of the visual cortex, and promotes neurodegeneration. tPA is synthesized by neurons of most brain regions, in particular within the hippocampus and hypothalamus (Sappino *et al.* 1993). tPA-catalyzed proteolysis in neural tissues is not limited to ontogeny, but may also contribute to adult central nervous system physiology, for instance by influencing neuronal plasticity and synaptic reorganization (Sappino *et al.* 1993; Nakagami *et al.* 2000). The identification of an extracellular proteolytic system active in the adult central nervous system may also help gain insights into the pathogeny of neurodegenerative disorders associated with extracellular protein deposition.

Alternatively, the uPA is an inducible secreted serine protease traditionally linked to blood clot dissolution and initiates an extracellular proteolytic cascade implicated in a broad spectrum of events related to cell adhesion and tissue remodelling, but its function in the brain is poorly understood. Transgenic mice producing high levels of uPA specifically in nerve cells in the brain showed uPA involvement in learning-related plasticity, but these mice were

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Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix components; GFP, green fluorescent protein; HEK, human embryonic kidney; NAc, nucleus accumbens; siRNA, small interfering RNA; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator; uPAR, receptor of uPA; PAI-1, inhibitor-1 of plasminogen activator; VTA, ventral tegmental area.

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impaired in tasks of spatial, olfactory and taste-aversion learning while displaying normal sensory and motor capabilities (Meiri *et al*. 1994). Furthermore, in the normal mouse brain, treatment with the seizure-inducing agents kainic acid and metrazol activated the uPA gene in the hippocampus and amygdala (Sharon *et al*. 2002; Miskin and Abramovitz 2005). The gene encoding the specific inhibitor of uPA, PAI-1, was also strongly activated. The genes of uPA and PAI-1 thus respond to neuronal triggering similarly to plasticity-related genes (Dent *et al*. 1993; Yoshida and Shiosaka 1999; Nakagami *et al*. 2000; Miskin *et al*. 2005). Induction of uPA receptor (uPAR) promotes cell adhesion through its interaction with vitronectin in the extracellular matrix, and facilitates cell migration and invasion by localizing uPA to the cell surface, the balance between cell adhesion and cell detachment being governed by PAI-1 (Yu *et al*. 2001; Deng *et al*. 2003).

Thus, expression of uPA and its receptor, uPAR, may play an important role in synaptogenesis, remodelling, and reactive processes other than for cell migration in developing mouse brain (Del Bigio *et al*. 1999). Therefore, its expression is a major determinant of the basal level of activated ERK/MAP kinase (Zhong *et al*. 2001), a process that may inhibit apoptosis during development. Furthermore, uPA, under the control of uPAR, is responsible for activating hepatocyte growth factor, which plays an important role during development for the movement of nerve cells to their correct location in the brain network (Powell *et al*. 2001). Mice lacking uPAR have an increased susceptibility to seizure, an altered electroencephalogram 'EEG' profile, increased anxiety-like behaviour and significant loss of social interaction, many features which are shared with individuals with autism (Powell *et al*. 2001). Therefore, uPA appears to be involved in complex brain functions and to play a major role in learning-related plasticity (Miskin *et al*. 1990; Masos and Miskin 1996; Yu *et al*. 2001).

In previous studies, we have shown that uPA is strongly induced upon psychostimulant delivery. Cocaine induced 2–6-fold increase of uPA mRNA in the mesolimbic dopaminergic pathway, including the ventral tegmental area (VTA), the nucleus accumbens (Nac) and the hippocampus (Bahi *et al*. 2004a). Furthermore, local over-expression of uPA in these brain area, by stereotaxic injection of a doxycycline-regulated uPA-expressing lentivirus, showed a ~12-fold increase in locomotor activity after cocaine treatment compared with saline injection, an effect completely abolished with doxycycline or when a dominant-negative form of uPA had been delivered (Bahi *et al*. 2004a). In order to further clarify the role of uPA in psychostimulant-induced behaviour, lentiviruses expressing small interfering RNAs (siRNAs) targeted against uPA mRNA have been prepared and tested both *in vitro* and *in vivo*. Our data clearly establish that uPA induces strong behavioural changes associated with drug delivery. The physiological function of these observations is discussed.

Experimental procedures

Lentiviral vectors

Design of uPA-specific siRNA followed published guidelines (Bahi *et al*. 2005a,b) and was performed using the Internet application of Hannon's design criterion http://katahdin.cshl.org:9331/RNAi/html/rnai.html; uPA-Sil1: 21–45; uPA-Sil2: 1270–1293 and uPA-Sil3: 673–692. In order to minimize off-target effects, a BLAST homology search (based on sense and antisense sequences) was systematically performed to be sure that a single mRNA sequence was targeted.

Briefly, an *Xho*I restriction site was 3′ added to each oligo. Using the pSilencer 1.0-U6 (Ambion, Austin, TX, USA) as a template and a U6 promoter-specific forward primer containing the *Bam*HI restriction site, each target was added to the mouse U6 promoter by PCR. The PCR product was digested with *Bam*HI and *Xho*I, cloned into similar sites in pTK431 and sequenced to verify the integrity of each construct (Bahi *et al*. 2005a,b).

Lentivirus construction of Lenti-uPA and Lenti-GFP

Briefly, the rat uPA cDNA was amplified by reverse transcription using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The cDNA was then PCR amplified, 6His-tagged, digested with *Bam*HI and *Xho*I and cloned into similar sites in pTK431 (Bahi *et al*. 2004a).

A control vector construct, pTK433 in which green fluorescent protein (GFP) expression is regulated by a tetracycline inducible promoter, was generated by cloning a *Bam*HI/*Bgl*II DNA fragment containing the GFP gene into a *Bam*HI site in pTK431 (Bahi *et al*. 2004a,b; 2005a,b).

The mutated form of uPA used in this study was described previously (Bahi *et al*. 2004a). Briefly, the uPA cDNA sequence was point-mutated in two active site-composing amino acids, H225G and S377A using the QuickChange site-directed mutagenesis kit according to the manufacturer's instructions (Stratagene, La Jolla, CA, USA). PCRs were performed with wild-type pCRII-uPA-His6 cDNA as template. The PCR product was then digested with *Bam*HI/*Xho*I and cloned into pTK431 transfer vector. cDNA inserts of the mutant were sequenced to ensure that the correct mutations were introduced. All plasmids were $CsCl_2$ purified.

Vesicular stomatitis virus G pseudotyped lentiviruses were produced by the transient calcium phosphate co-transfection of human embryonic kidney (HEK)293T cells with pTK vectors, together with pMDG-VSV-G and pΔNRF as previously described (Naldini *et al*. 1996; Bahi *et al*. 2004a,b; 2005a,b). Lentiviral vector quantifications were performed according to the p24 ELISA (KPL, Gaithersburg, MA, USA) in accordance with the manufacturer's instructions.

Cell culture

HEK293T cells were maintained under standard conditions in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine. For infections, 10^5 293T cells were plated, 24 h later lentiviruses were mixed with 8 μg/mL

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Polybrene (Sigma, St Louis, MO, USA), added to the cells and incubated at 37°C.

For doxycycline regulation, briefly HEK293T cells were infected with 3, 6 or 9 μL from viral stock of Lenti-uPA or Lenti-uPA-Mut, together with 3 μL of Lenti-CD81 (Bahi *et al.* 2004b; 2005b). Culture medium was supplemented with 30 ng/mL of doxycycline.

To test the knock-down effect of uPA with Lenti-uPA-siRNAs *in vitro*, control cells were infected with 3 μL of Lenti-uPA, together with 3 μL of Lenti-CD81, knock-down was performed by adding either 3 μL of Lenti-uPA-Sil1, 3 μL of Lenti-uPA-Sil2, 3 μL of Lenti-uPA-Sil3 or 3 μL of the three targets together.

For all infections, after 48 h medium was changed (with or without 30 ng/mL doxycycline) and cells were kept in culture for a further 48 h. Cells were than processed for total RNA extraction (Bahi *et al.* 2005a).

Quantitative RT-PCR

To analyse uPA mRNA levels, total RNA was extracted from HEK293T cells and the VTA region of brains stereotaxically injected with lentiviral vectors using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Retrotranscription step was performed using Superscript II Reverse Transcriptase (Invitrogen) following the manufacturer's instructions. Real-time quantitative PCR was performed using the iCycler with an IQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) for DNA detection. Expression levels of the housekeeping gene encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were also quantified and used for normalization. Primers for rat uPA were 5′-CAGATCCGATGCTCTTAGCC-3′ and 5′-TAGAGCCTTCTGGC-CACACT-3′. Primers for rat uPA receptor (uPA-R): 5′-GCTGC-AACTTCACCCAATGC-3′ and 5′-ACTCCGGTTTCCCAGCA-CAT-3′. Primers for rat uPA inhibitor (uPA-I): 5′-TTCCTTGAATG-TGCCAATGA-3′ and 5′-GTTTTCCACCGTCCTTTGAA-3′. Primers for rat CD81 were 5′-TGATCCTGTTTGCCTGTGAG-3′ and 5′-CAGTTGAGCGTCTCATGGAA-3′. Primers for GAPDH were 5′-ATGACTCTACCCACGGCAAG-3′ and 5′-CATACTCAGCACC-AGCATCAC-3′. Primers for β-actin were 5′-AGCCATGTACGTA-GCCATCC-3′ and 5′-CTCTCAGCTGTGGTGGTGAA-3′. All primers were designed using PRIMER3 software (Bahi *et al.* 2004a,b; 2005a,b). The 199-base pair fragment for rat uPA was amplified using the following PCR conditions: 3 min at 95°C (initial denaturation), a 20°C/s temperature transition rate up to 95°C for 30 s and 64°C for 30 s, repeated 40 cycles (amplification). The PCR reaction was evaluated by melting curve analysis following the manufacturer's instructions and checking the PCR products on 2% agarose gel.

Animal work

Animals used in this experiment were male Wistar rats weighing 225–250 g (BRL, Füllinsdorf, Switzerland). All animal experiments were carried out in accordance with the guidelines and regulations for Animal Experimentation, BAG, Bern, Switzerland. The animals were housed in threes in clear plastic cages with wire grid lids. Access to food and water was unrestricted. The animals were kept in the animal facility maintained on a 12-h light : 12-h dark cycle (lights off at 07.00 h).

At the end of the behavioural tests, rats were decapitated and brains were quickly removed. Various regions including the VTA were rapidly dissected out, frozen in Trizol reagent and stored in a freezer at −80°C until assayed.

Surgery

All surgical procedures were performed as previously described (Bahi *et al.* 2004a,b; 2005a,b). Briefly, rats were anaesthetized with a mix of ketamine/xylazine (100 mg/kg/10 mg/kg, i.p.). Using a 5-μL Hamilton syringe, 4 μL of concentrated lentiviral solutions mix (c.a. 200 000 ng of p24 antigen/mL) per site were bilaterally injected into the VTA, at the corresponding coordinates (anterior −6; lateral ±0.6; ventral −8; Paxinos and Watson 1998), with a rate of 1 μL/min, in a stereotaxic frame. The needle was then left in place for an additional 5 min and gently withdrawn. After surgery, animals were injected subcutaneously with a single dose of caprofen (5 mg/kg) to limit inflammatory reaction resulting from the surgery and 5 mL of pre-warmed saline to avoid animal dehydration. Animals were left 7 days for recovery.

Measurement of locomotor activity

This test was performed as previously described (Bahi *et al.* 2004a,b; 2005a,b). Briefly, after surgery animals were left for recovery for 7 days, and then chronically injected with cocaine (15 mg/kg i.p.) over 15 days. Chronic cocaine administration was subdivided into three 5-day sessions.

Session A, without doxycycline

Initially, animals ($n = 9$) were daily injected over 5 days under a normal regimen, with saline (1 mL/kg, i.p.), placed individually in MED-OFA-RS cage (MED Associates Inc., Georgia, VT, USA) and locomotor activity was measured every 5 min for baseline. After 30 min, the session automatically paused and, during this interval, each rat received an intraperitoneal injection of cocaine-HCl (15 mg/kg) and was then placed back into the locomotor activity-monitoring cage for 60 min. At the end of session A, three rats were killed for RNA extraction.

Session B, with doxycycline

Over the next five consecutive days, remaining animals ($n = 6$) were given water supplemented with 0.02% doxycycline and 5% sucrose (Sigma) and tested for locomotor activity every day with daily cocaine administration as in session A. At the end of session B, three animals were killed.

Session C, without doxycycline

The remaining animals ($n = 3$) were then fed doxycycline-free water over 5 days with daily monitoring for locomotor activity with daily cocaine administration as in session A, and finally killed at the end of this last session.

After death, brains were dissected out (including the VTA) and used for total RNA extraction. After reverse transcription, mRNA expression levels were measured by means of quantitative real time-PCR.

To test uPA-induced locomotor activity upon cocaine delivery, B428, a specific uPA inhibitor (Towle *et al.* 1993; Evans and Sloan-Stakleff 2000; Todaro *et al.* 2003) was used. A group of animals was bilaterally injected into the VTA with Lenti-uPA using a stereotaxic frame as previously described. After recovery, rats were

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injected with B428 (30 mg/kg, i.p.). Thirty minutes later rats were injected with saline (1 mL/kg, i.p.) and placed in open-field cages for the habituation phase. After this period, animals received cocaine (15 mg/kg) and then were placed back into the locomotor activity-monitoring cage for 60 min.

Statistical analysis

All data were expressed as the means ± SE. The statistical evaluation was performed as previously described (Bahi *et al.* 2004a,b; 2005a,b).

Immunohistochemistry

Immunohistochemistry was performed according to previously published procedures (Bahi *et al.* 2004a).

Results

Lentivirus-mediated uPA expression *in vitro* in HEK293T cells

For this study, lentiviruses expressing either uPA, a dominant-negative form of uPA (uPA-mut), or siRNAs targeted against uPA have been prepared and tested *in vitro*. HEK293T cells were infected with various amounts of Lenti-uPA or Lenti-uPA-Mut, together with Lenti-CD81 (used as a control, see Bahi *et al.* 2004a) and expression was quantified by means of quantitative RT-PCR, normalized against GAPDH. As shown in Fig. 1, expression of uPA by Lenti-uPA or its mutated form Lenti-uPA-Mut are titre-dependent. Increases (~2.7-, ~5.6- and ~10.7-fold) were observed with 3, 6 or 9 μL viral stock in cells infected with Lenti-uPA (Fig. 1a), whereas cells infected with Lenti-uPA-Mut displayed a ~3.2-, ~6.0- and ~12.0-fold increase, respectively, at equivalent titres (Fig. 1c). Under these conditions, Lenti-CD81 also induced the expression of CD81 transcripts (~3-fold increase), but its transcript level was not affected by Lenti-uPA or Lenti-uPA-Mut titre changes. Endogenous β-actin mRNA was not affected under these conditions and a constant ratio of ~2.6 to 2.8 was found under all conditions. This expression of uPA, uPA-Mut and CD81 is regulated by low doses of doxycycline (30 ng/mL). Lenti-uPA-mediated uPA transcript expression was completely abolished at low virus concentrations (Fig. 1b) and uPA expression reached almost basal levels (ratio ~1.3), similar to levels observed in control, non-infected cells; however, when higher virus titre is used (9 μL of Lenti-uPA), a residual but significant uPA transcript level was observed (ratio ~4.3), because the expression blockade by 30 ng/mL doxycycline was not complete under these conditions, because of a slight leakiness of the cytomegalovirus (CMV) promoter. The same observations were made with cells infected with Lenti-uPA-Mut, expressing the dominant-negative, mutated form of uPA (Fig. 1d). Under these conditions, lentivirus-mediated expression of CD81 transcripts was also fully blocked by doxycycline, in agreement with previous data (Bahi *et al.* 2004a, 2005b) whereas β-actin levels was not affected (Figs 1b and d).

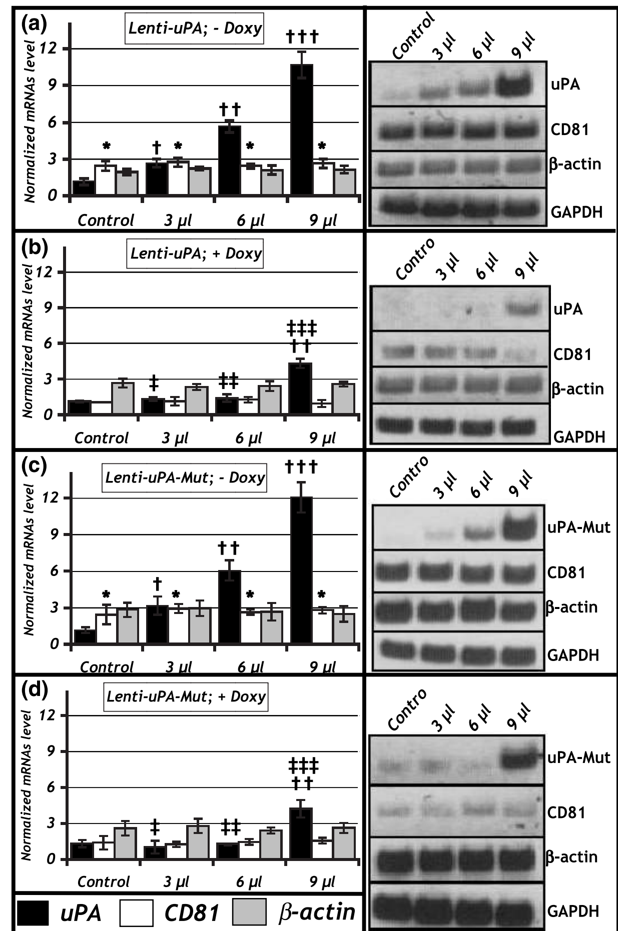

Fig. 1 Changes in urokinase plasminogen activator (uPA) mRNA expression after cell infection. HEK293T cells were infected with 0 μL (control), 3, 6 or 9 μL of Lenti-uPA (a, b) or Lenti-uPA-Mut (c, d), together with 3 μL of Lenti-CD81. Cells were maintained in standard Dulbecco's modified Eagle's medium (DMEM; a, c) or in DMEM supplemented with 30 ng/mL of doxycycline (b, d). Total RNA was extracted, reverse transcribed and used for quantitative real-time PCR using specific primers. Values indicate means ± SEM. PCR products were loaded on 2% agarose gel. $^*p < 0.05$ compared with doxycycline-treated cells; $^\dagger p < 0.05$, $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.005$ compared with control cells; $^\ddagger p < 0.05$, $^{\ddagger\ddagger}p < 0.01$, $^{\ddagger\ddagger\ddagger}p < 0.005$ compared with doxycycline-free DMEM cultured cells.

The effects of the three silencing lentiviruses, that express different siRNAs (targeting bp 21–45, 1270–1293 and 673–692 of the mRNA of uPA and uPA-mut) were tested in HEK293T cells co-infected with 3 μL of either Lenti-uPA (Fig. 2a) or Lenti-uPA-mut (Fig. 2b), together with Lenti-siRNAs (either Lenti-uPA-Sil1, Lenti-uPA-Sil2, Lenti-uPA-Sil3 or all together). These silencing lentivirus constructs are not doxycycline-regulated. Target specificity was controlled by co-infections of HEK293T cells with Lenti-CD81 (Bahi

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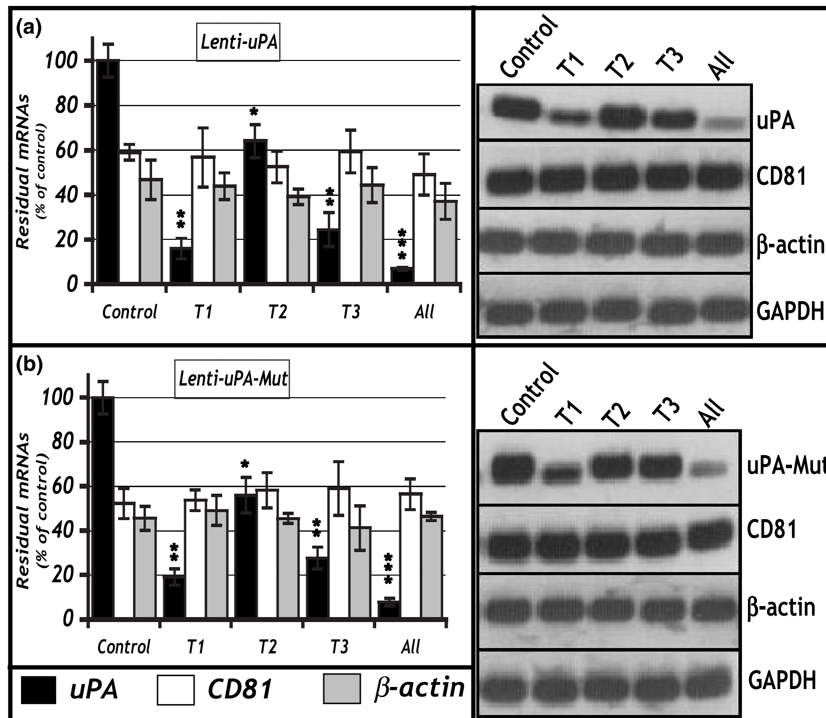

Fig. 2 Quantitative real-time PCR of uPA expression in HEK293T cells transfected with Lenti-uPA-siRNA. HEK293T cells were infected with 3 μL of Lenti-uPA (a) or Lenti-uPA-Mut (b), together with 3 μL of Lenti-CD81 (as control) with or without Lenti-uPA-siRNAs. Total RNA was extracted, reverse transcribed and used for real-time PCR using specific primers. Values indicate means ± SEM. PCR products were loaded on 2% agarose gel. Control, 3 μL of Lenti-uPA or Lenti-uPA-Mut + 3 μL of Lenti-CD81; T1, 3 μL of Lenti-uPA or Lenti-uPA-Mut + 3 μL of Lenti-CD81+ 3 μL of Lenti-uPA-Sil1; T2, 3 μL of Lenti-uPA or Lenti-uPA-Mut + 3 μL of Lenti-CD81+ 3 μL of Lenti-uPA-Sil2; T3, 3 μL of Lenti-uPA or Lenti-uPA-Mut + 3 μL of Lenti-CD81+ 3 μL of Lenti-uPA-Sil3; All, 3 μL of Lenti-uPA or Lenti-uPA-Mut + 3 μL of Lenti-CD81+ 3 μL of the three targets of Lenti-uPA-Sil. *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$ compared with control cells.

et al. 2004b, 2005b). Cells were harvested 96 h after infection, total RNA was extracted and transcripts levels were measured by means of quantitative RT-PCR using specific primers for each candidate. All normalizations were performed against GAPDH. qRT-PCR showed 85, 26 and 76% reduction of uPA mRNA in cells co-infected with Lenti-uPA, under these conditions (Fig. 2a), and 81, 45 and 73% reduction of uPA-Mut mRNA in cells co-infected with Lenti-uPA-Mut (Fig. 2b). When cells were co-infected with all three Lenti-siRNAs simultaneously (Lenti-uPA-Sil1, Lenti-uPA-Sil2 and Lenti-uPA-Sil3), almost 92% reduction of uPA or of uPA-Mut mRNA was observed. Lenti-siRNAs had no effects on other mRNA targets, e.g. CD81 or β-actin used as controls – under all conditions, the expression levels of CD81 or β-actin mRNAs were not affected by Lenti-siRNAs infection, indicating that these targets are highly uPA-specific. The expression levels of control genes were ~56 and ~45%.

In vivo uPA silencing

Six groups of lentivirus-infected animals ($n = 9$ each) were used for assessing the levels of uPA expression in the VTA, as measured by qRT-PCR (Figs 3a and b). First, behavioural evaluation was performed in three 5-day sessions: session A (no doxycycline, full gene expression), session B (animals fed doxycycline, inducing local gene suppression) and then session C (no doxycycline, recapitulating both gene expression of session A and initially observed behaviour). Chronic cocaine administration was continued daily throughout each session A, B and C. This protocol enables for behavioural evaluation of effects of local gene expression changes on the very same animals (Bahi *et al.* 2004a,b, 2005a,b).

When animals were injected with Lenti-uPA in the VTA, cocaine induced strong expression of uPA transcripts in doxycycline-free, with a ratio of ~12.5 (session A) and 11.7-fold (session C), but only 3.6 in the presence of doxycycline (session B), when normalized against GAPDH. Under these conditions, the ratios of expression levels for CD81 or β-actin were ~3.0 and ~1.0, respectively, and remained unchanged by switch of regimen. Evaluation of CD81 expression was used as a control, as it is well established that CD81 is endogenously induced in this brain area upon chronic cocaine under the same conditions (Bahi *et al.* 2004a, 2005a). In full agreement with the present data, we

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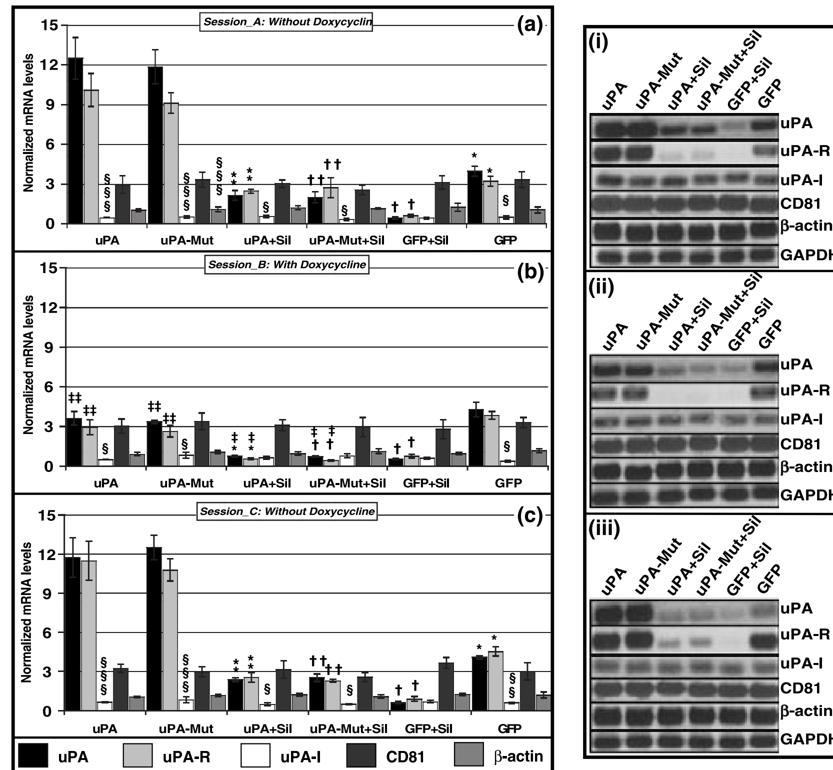

Fig. 3 Quantitative real-time PCR of uPA, uPA receptor (uPA-R) and uPA inhibitor (uPA-I) expression *in vivo*. Six groups of rats ($n = 9$) were stereotaxically injected into the VTA with 4 μL lentiviral vector mix (at ~2 to 10^5 ng/mL p24 each lentivirus). After recovery, animals were chronically injected with cocaine (daily injection of 15 mg/kg i.p.) under a different regimen and locomotor activity was measured daily. Session A: animals were fed with normal water over 5 days; session B: the same animals were then switched to the doxycycline regimen for a further 5 days; session C: finally the same animals were re-switched again to a normal doxycycline-free regimen for a further 5 days. Chronic cocaine administration was continued daily throughout each of sessions A, B and C. At the end of each session, three animals were killed by decapitation, brains were removed and various regions including the VTA were dissected out. Total RNA was extracted, reverse transcribed and used for quantitative PCR using specific primers. (a) Quantitative mRNA levels normalized against GAPDH. (b) PCR products were loaded on 2% agarose gel uPA: Lenti-uPA alone; uPA-Mut: Lenti-uPA-Mut alone; uPA-Sil: Lenti-uPA + Mix of Lenti-uPA-Sil; uPA-Mut + Sil: Lenti-uPA-Mut + Mix of Lenti-uPA-Sil; GFP-Sil: Lenti-GFP + Mix of Lenti-uPA-Sil: GFP: Lenti-GFP alone. *$p < 0.05$, **$p < 0.01$ compared with uPA group; †$p < 0.05$, ††$p < 0.01$ compared with uPA-Mut group; ‡$p < 0.05$ compared with GFP group; ‡$p < 0.05$, ‡‡$p < 0.01$ compared with doxycycline-free water-fed animals (sessions A and C). §$p < 0.05$, §§$p < 0.01$, §§§$p < 0.001$ compared with uPA-R expression.

previously reported that chronic cocaine delivery induces uPA (~10.5-fold; Bahi *et al*. 2004b) and CD81 (~3.2-fold; Bahi *et al*. 2004a) into the VTA. Furthermore, we observed a 10.1-fold endogenous expression of uPAR in this brain area. When animals are fed doxycycline in session B, down-regulation of uPA is accompanied by a similar down-regulation of uPAR to 2.9-fold, that is fully reversed later in session C, when animals are again fed without doxycycline. This effect was not observed for PAI-1, whose expression remained relatively constant under all conditions and seemed not affected by the levels of uPA expression.

When animals were infected with Lenti-uPA-Mut, expressing a dominant-negative form of uPA in the VTA, the expression ratios were almost similar to the previous group in all sessions ($p > 0.21$, session C vs. session A). Surprisingly, expression of the dominant-negative uPA-Mut also induces a 9- and 11.8-fold uPAR expression (not PAI-1), as observed in both sessions A and C, respectively, but not in session B, when uPA-Mut is not expressed.

When animals, infected either with Lenti-uPA or with Lenti-uPA-Mut, were co-infected with a mix of Lenti-uPA-siRNAs, drastic inhibition of uPA and, respectively, uPA-Mut expression, was observed in session A, resulting in a ratio of ~2.1 in both groups, i.e. a ~6-fold inhibition ($p < 0.01$ vs. Lenti-uPA- or Lenti-uPA-Mut-infected animals, respectively). Lenti-uPA-siRNAs had no significant effects on β-actin and CD81 expression ($p > 0.25$ vs. Lenti-uPA- or Lenti-uPA-Mut-infected animals). In session B, uPA or uPA-Mut expression are strongly inhibited, with ratios ~0.7 ($p < 0.005$ vs. Lenti-uPA- or Lenti-uPA-Mut-infected

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animals). Doxycycline blocks exogenous, lentiviral-mediated uPA or uPA-Mut over-expression, but not the endogenously expressed uPA, while the silencers also block endogenous uPA. Under these conditions, the other targets, CD81 or β-actin, were not modified at all ($p > 0.25$ vs. Lenti-uPA-infected animals). Removal of doxycycline in session C restores the levels back to those observed during session A. Interestingly, suppression of uPA or uPA-Mut expression by the siRNAs expressing lentiviruses also results in strong down-regulation of uPAR (respectively, 2.4- and 2.7-fold expression in session A, 0.5- and 0.4-fold expression in session B and, again, 2.5- and 2.3-fold expression in session C).

A group of animals was infected with Lenti-GFP only (a mock control, to evaluate endogenous uPA expression), and a last group was injected Lenti-GFP together with Lenti-uPA-siRNAs mix (for silencing of endogenous uPA). When infected with Lenti-GFP only, uPA expression was ~3.9 in session A, reflecting cocaine-mediated induction of endogenous uPA by chronic cocaine, in full accordance with our previous finding (Bahi *et al*. 2004b). When Lenti-uPA-siRNAs were co-infected with Lenti-GFP, uPA mRNA expression level was only ~0.5 in session A, indicating that a ~90% knock down of the endogenous uPA had been achieved with a mix of Lenti-uPA-siRNAs ($p < 0.05$ vs. Lenti-GFP-infected animals). In this same group, β-actin and CD81 mRNAs were unchanged. In session B, the ratios remained unchanged in Lenti-GFP-treated animals ($p > 0.23$ vs. Lenti-GFP-infected animals in session A). Animals treated with Lenti-GFP and Lenti-uPA-siRNAs also displayed lower uPA expression in session B, with a ratio ~0.5 ($p < 0.01$ vs. Lenti-GFP-infected animals), whereas other targets, e.g. β-actin and CD81, were not modified ($p > 0.25$ vs. Lenti-uPA-infected animals). Removal of doxycycline (in session C) restored uPA levels back to those observed during session A ($p > 0.23$ session A vs. session C) in both groups (~4.1- vs. ~0.6-fold, $p < 0.05$), but control genes remained unmodified (a ~1.2- and ~3.0-fold increase for β-actin and CD81, respectively, in agreement with previous observations, Bahi *et al*. 2004a,b, 2005a,b) and an increase of uPAR was observed in animals treated with Lenti-GFP only.

Behavioural changes induced upon uPA expression

Animals infected with only Lenti-GFP served as a control group expressing GFP in the VTA in a doxycycline-regulated way. After surgery, animals were fed water, enabling full expression of GFP in the target area. One week after surgery, chronic drug delivery was started and locomotor activity was monitored. At each daily session, animals received saline injections before the habituation period (30 min) followed by cocaine delivery (i.p. 15 mg/kg) and the locomotor activity was monitored over 60 min immediately after drug injection. During session A, saline injection induced low levels of locomotor activity (~46 counts), while cocaine delivery produced a significant induction of locomotor activity (~241 counts; means over a 5-day session). But no significant changes were observed when the animals were changed regimen (i.e. fed doxycycline after 5 days, session B, or fed back without doxycycline after 10 days, session C). Also, no significant behavioural sensitization was observed under these conditions (Fig. 4b).

Animals infected with doxycycline-regulatable Lenti-uPA into the VTA underwent similar treatment. Saline delivery before the habituation period induced locomotor activity comparable with the GFP control group under all doxycycline regimens (~47 counts, $p > 0.2$ sessions A and C vs. session B). But after cocaine injection, a phenomenal increase in locomotor activity was observed in session A, with a total activity of ~1442 counts. After 5 days, the regimen was switched and the very same animals ($n = 6$) were fed doxycycline in the drinking water (inducing down-regulation of exogenous, lentivirus-induced expression of uPA in the VTA), and their behaviour upon chronic cocaine treatment was further monitored daily for five consecutive days (session B) – under these conditions, the distance travelled during the habituation period was unchanged (average ~50 counts, $p > 0.2$ vs. Lenti-uPA of session A), whereas after cocaine injection a total distance dropped down to ~367 counts. After 5 days under this regimen, doxycycline was removed (session C, enabling re-expression of lentivirus-mediated uPA in the VTA). Under these conditions, no changes were observed during the habituation period (~52 counts, $p > 0.2$ vs. Lenti-uPA of sessions A and B), but after cocaine injection locomotor activity returned back to its initial levels (~1640 counts). The difference between sessions A and C was not significant ($p > 0.2$). Note the very strong and significant sensitization observed during session A, but no more during sessions B and C (Fig. 4b). To check the direct uPA inhibition, a separate group of animals was administered B428, an uPA inhibitor, in the drinking water and locomotor activity was measured (Fig. 4c). In presence of B428, complete inhibition of uPA had been described (Towle *et al*. 1993; Todaro *et al*. 2003). Under these conditions, locomotor activity was completely blocked during the habituation period and negligibly increased after cocaine administration (~10 counts). This compound, however, appears to act as an anaesthetic rather than a true inhibitor of uPA over-expression in this brain region.

Animals injected with Lenti-uPA-Mut, the lentivirus expressing the mutated, dominant-negative form of uPA, display a small but significant inhibition in cocaine-mediated locomotor activity when compared with GFP control groups. The mutated form of uPA is interfering with the endogenous expressed one (Bahi *et al*. 2004b). Note also the absence of significant behavioural sensitization in this group (Fig. 4b).

Animals ($n = 9$) co-injected with the regulatable Lenti-uPA (same concentration as the first group) together with

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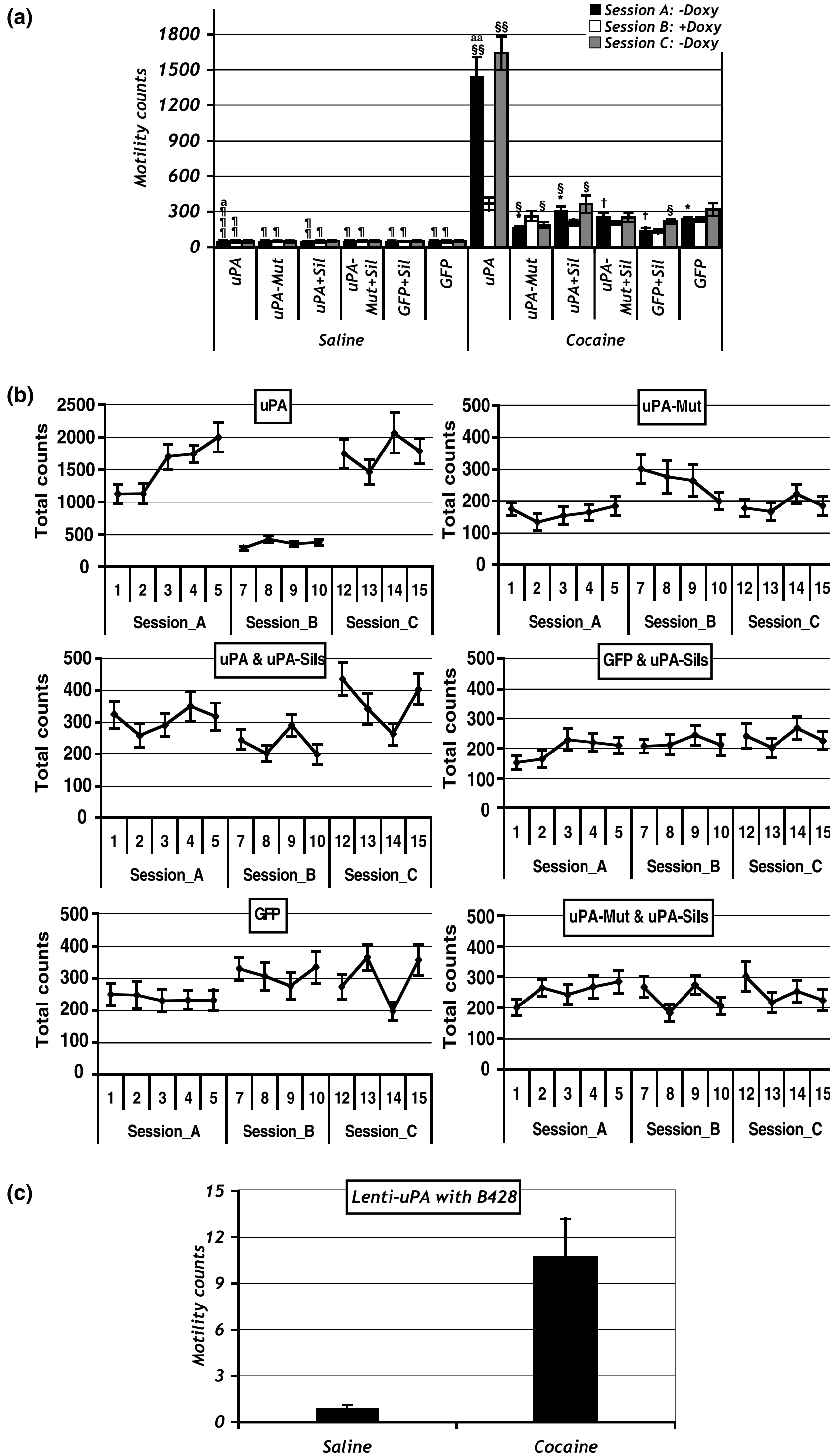

Fig. 4 Cocaine-mediated hyperlocomotion is blocked by silencing uPA expression in the VTA. Daily locomotor activity of animal groups used for Fig. 3. uPA expression in the VTA was silenced locally by bilateral stereotaxic injection of Lenti-uPA-siRNAs as described in Fig. 3. (a) Total mobility counts averaged over each 5-day period. In all sessions, rats were treated with saline (1 mL/kg i.p.), and the locomotor activity was measured for 30 min (habituation period). Rats then received an i.p. injection of 15 mg/kg of cocaine and locomotor activity was assessed for a further 60 min. Initially animals were fed doxycycline-free water for 5 days (session A, –Doxy). Animals were then switched to the doxycycline regimen for a further 5 days (session B, +Doxy). Finally, animals were switched back to a normal, doxycycline-free regimen (session C, –Doxy). Chronic cocaine administration was continued daily throughout each of sessions A, B and C. (b) Differential habituation and sensitization over the three sessions. Daily data from (a) are plotted individually for each group separately. (c) Animals injected with Lenti-uPA into the VTA were injected with B428 (30 mg/kg, i.p.). Thirty minutes later rats were injected with saline (1 mL/kg, i.p.) and placed in testing cages for half an hour. After this period, animals received cocaine (15 mg/kg) and were then placed back into the locomotor activity-monitoring cage for 60 min. *$p < 0.001$ compared with the uPA group; †$p < 0.05$ compared with the uPA-Mut group; ‡$p < 0.05$ compared with the GFP group; §$p < 0.05$, §§$p < 0.001$ compared with doxycycline-fed animals; ¶$p < 0.05$, ¶¶$p < 0.01$, ¶¶¶$p < 0.001$ compared with cocaine-treated animals; $^{a}p < 0.01$, $^{aa}p < 0.0001$ compared with B428-treated animals.

(non-regulatable) Lenti-uPA-siRNAs were submitted to the same regimen and drug treatments. In session A, i.e. when lentivirus-mediated uPA expression is silenced by the Lenti-siRNAs, cocaine delivery induces low locomotor activity (~310 counts), corresponding to a ~4.6-fold decrease of the activity monitored under the same conditions in the absence of siRNAs ($p < 0.05$ vs. Lenti-uPA). After 5 days, doxycycline was added to the regimen (session B), inducing down-regulation of exogenous, lentivirus-mediated uPA expression (but not of lentivirus-siRNAs). Under these conditions, upon cocaine injection a total travelled distance of ~207 counts was observed, significantly lower than in session A and

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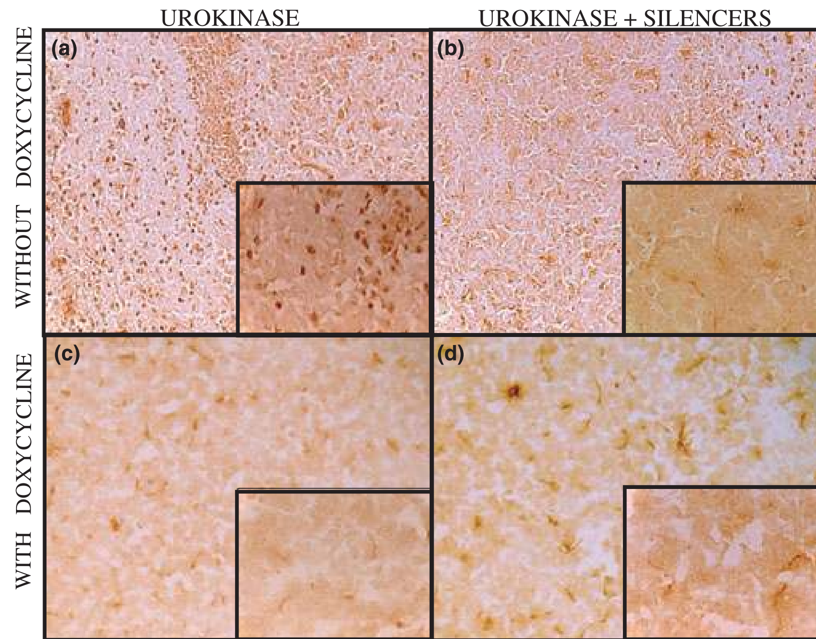

Fig. 5 Immunocytochemistry of uPA expression in the VTA. (a) Animals injected with Lenti-uPA, after session A; (b) animals injected with Lenti-uPA and Lenti-silencers; (c) animals injected with Lenti-uPA, as in (a), fed doxycycline (after session b); (d) animals injected with Lenti-uPA and Lenti-silencers, as in (b), fed doxycycline (after session B). Chronic cocaine administration was continued daily throughout each of sessions A and B. See Experimental procedures. Magnification 10 ×, inserts 40 x.

similar to GFP-treated animals under corresponding conditions ($p < 0.05$ vs. sessions A and C). When doxycycline was finally removed from the water on the very same animals (session C), enabling full expression of the Lenti-uPA in the VTA, the locomotor activity raised back to the level observed in session A (∼361 counts after cocaine delivery, $p > 0.22$ vs. session A). No significant behavioural sensitization was observed in this group of animals where uPA had been silenced by siRNAs in all three sessions (Fig. 4b).

Very similar results were found with the group of animals co-infected with Lenti-uPA-Mut and with Lenti-uPA-siRNAs (Figs 4a and b).

Finally, animals co-infected with Lenti-GFP and Lenti-uPA-siRNAs served to assess effects of endogenous uPA. In the absence of doxycycline (session A), animals displayed the same locomotor activity during the habituation period (∼48 counts, $p > 0.2$ vs. Lenti-uPA), but a high locomotor activity after cocaine delivery (∼138 counts, $p < 0.05$ vs. Lenti-GFP). When switched to the doxycycline regimen, either in session B or later in session C, no significant changes were observed ($p > 0.2$).

At the end of each session, expression of uPA was assessed by means of immunocytochemistry on the brain section from one of the animals which had been killed. As displayed in Fig. 5, doxycycline treatment, as well as treatment with Lenti-uPA-siRNAs, results in >95% decrease in uPA-positive cells in the targeted area.

Discussion

uPA is strongly over-expressed after cocaine delivery under various paradigms (chronic, acute or binge) and strongly affects locomotor behaviour (Bahi *et al*. 2004b). The present study firmly establishes these data and yields a 15-fold change in locomotor activity upon cocaine delivery (compared with saline injection). This considerable change is very selective and may be systematically manipulated through doxycycline, in full agreement with our previous study. This study points to an important role of the plasmin system in chronic cocaine. Other studies showed already that the tPA-plasmin system releases dopamine in the NAc upon methamphetamine, which activates long-term synaptic plasticity and remodelling, and acutely participates in the rewarding effects of methamphetamine or morphine (Ripley *et al*. 1999; Iwata *et al*. 2004; Nagai *et al*. 2004; Yamada *et al*. 2005). Activity-dependent synaptic plasticity and remodelling of the mesolimbic dopaminergic system play a crucial role in the development of drug dependence (Koob *et al*. 1998; Nestler 2001).

Plasminogen activators, tPA and uPA, both convert plasminogen to plasmin (Vassalli *et al*. 1991), which in turn function to degrade extracellular matrix (ECM) components (Werb 1997; Flumelli *et al*. 1999). Its involvement in the fibrinolysis of the blood clot is well known, but a large body of evidence has shown that this system also functions in the CNS. Synaptic reorganization takes place continuously as neurons undergo stimulation and requires remodelling of ECM through the action of extracellular proteases, including plasminogen activators (Tsirka *et al*. 1995, 1997; Werb 1997; Tsirka 2002). Plasmin plays an important role in neurite extension and synaptic remodelling by altering the cell–ECM interaction (McGuire and Seeds 1990). The role of uPA, for dendritic spine dynamics regulated by ECM degradation, is well established (Fiorillo *et al*. 1998; Oray *et al*. 2004).

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Plasmin degrades several ECM proteins, including laminin (Goldfinger *et al*. 2000), which localizes calcium channels to the sites of active zones in the synaptic cleft (Bixby *et al*. 1994; Sunderland *et al*. 2000). Therefore, a uPA- or tPA-mediated modulation of plasminogen activation may result in a malfunction of calcium channel activity and leads to the reduction of depolarization-evoked dopamine release in dopaminergic neurons (Nagai *et al*. 2004).

Furthermore, our investigation gives evidence that local over-expression of uPA in the VTA induces endogenous expression of its receptor uPAR, but not its inhibitor PAI-1. Both uPAR expression and cocaine-induced behavioural changes are regulated upon uPA silencing. uPAR is highly up-regulated in the presence of uPA, but also when the dominant-negative uPA mutant is expressed, in agreement with the well-established fact that signalling functions of uPA do not require its proteolytic activity (Tarui *et al*. 2003). uPA is secreted as an inactive pro-enzyme, which, after binding to its cell surface high-affinity receptor, is rapidly activated to active uPA by plasmin. By uPA binding to uPAR, it can localize enzyme activity at the cell surface and contact other molecules on the cell surface and in the extracellular matrix (de Bock and Wang 2004). The mechanisms of cocaine-mediated uPAR expression remain to be established. Cocaine activates several pathways, among them redox-sensitive transcription factors, e.g. nuclear factor-kappa-B (NF-κB), activator protein-1 (AP-1), and TNF-α gene expression (Ang *et al*. 2001). NF-κB in turns activates uPAR transcription, binding to its promoter region at position −45, and AP-1 presents binding motifs located at −184 bp of the uPAR promoter (Wang *et al*. 2000). uPAR in turn appears to be finely tuned in various environments by complex mechanisms controlling its gene expression. uPAR mRNA is highly inducible and unstable, but it is strongly stabilized by interaction with the integrin LFA-1 (CD11a/CD18), which has a major stabilizing effect on the 3′ UTR of uPAR mRNA (Wang *et al*. 1998), enhancing uPAR mRNA half-life and its engagement into cell adhesion integrin-mediated cascade. Integrins, through interactions with matrix ligands, influence gene expression largely through kinase cascades modulating transcription factor complexes, which synergize with growth factor responses and also lead to increased synaptic plasticity, as observed upon cocaine sensitization. However, at least two forms of uPAR have been described, which engage different signalling cascades and multiple interactions with G protein-coupled receptors (Mazzieri *et al*. 2005).

In view of the facts that uPAR is highly up-regulated in the presence of both uPA, or its dominant-negative mutant form, that both forms bind the receptor, and that no proteolytic activity is needed for the cellular signalling, why are the observed phenotypes then so different? In our studies, only the active form of uPA induces significant cocaine-mediated behavioural changes, which probably implies that plasmin activation plays a significant role in this process. This is supported by the full behavioural inhibition observed after cocaine in the presence of B428, an uPA inhibitor.

In certain tumour and stromal cells, uPA activity may be neutralized and regulated by PAI-1. Models of self-regulation of the uPA-uPAR-PAI-1 system have been proposed (reviewed by de Bock and Wang 2004). Interestingly, this type of mechanism seems not to occur in the cocaine paradigms under investigation in our study. No changes in PAI-1 expression were observed under any circumstance, despite reports that cocaine administration may be associated with an increase in plasma PAI-1 activity, which may play a role in vascular thrombosis by recreational users of the drug (Moliterno *et al*. 1994).

Plasmin regulates a cascade of extracellular proteolytic activities involved in neurite outgrowth, cell migration (Moonen *et al*. 1982; Seeds *et al*. 1999), long-term potentiation and depression (Frey *et al*. 1996; Calabresi *et al*. 2000), learning and memory (Madani *et al*. 1999; Calabresi *et al*. 2000), excitotoxic cell death (Tsirka *et al*. 1997; Nicole *et al*. 2001), and regeneration or recovery from injury in the nervous system (Siconolfi and Seeds 2001; Wolfer *et al*. 2001). These findings suggest that plasmin is involved in the regulation of numerous aspects of synaptic plasticity and remodelling (Shimizu *et al*. 1998). tPA and uPA are synthesized in most brain regions (Sappino *et al*. 1993; Salle and Strickland 2002) and their localized expression during neuronal development suggests that plasmin-mediated proteolysis facilitates neurite outgrowth and cell migration (Sumi *et al*. 1992; Ware *et al*. 1995).

In addition, tPA is induced as an immediate-early gene accompanying seizure, kindling, or LTP, and contributes to structural changes observed during activity-dependent synaptic plasticity (Pang *et al*. 2004; Pawlak *et al*. 2005). tPA-deficient mice are resistant to excitotoxin-induced neuronal degeneration in the hippocampus and have an elevated threshold for seizure (Tsirka *et al*. 1995; Tsirka *et al*. 1997) and a reduction in the maintenance of LTP (Frey *et al*. 1996). In contrast, mice over-expressing tPA show an enhanced LTP (Madani *et al*. 1999), whereas mice over-expressing uPA display impaired learning (Meiri *et al*. 1994). In organotypic hippocampal cultures, the maintenance of LTP is impaired by degradation of laminin, indicating that laminin-mediated cell–ECM interaction may be necessary for the maintenance of LTP (Nakagami *et al*. 1997, 2000). Together, these data show that plasmin promotes synaptic plasticity as an acute effect.

However, uPA may also regulate synaptic plasticity through mechanisms independent of plasmin or laminin, in analogy to mechanisms proposed for tPA, such as mediating an interaction between microglia and dopaminergic neurons (Nakajima *et al*. 1994). Microglia activated by secreted tPA affect mossy fibre pathfinding and outgrowth and other proteases released by microglia and then promote neurite growth (Bednarski *et al*. 1997; Patton *et al*. 1998; Wu *et al*.

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2000). It may also induce a plasminogen-independent mechanism (Tsirka *et al*. 1997) or activate other substrates, such as hepatocyte growth factor (Powell *et al*. 2001), or yet another physiological target of plasmin, e.g. DSD-1-PG/phosphacan, an extracellular matrix component associated with neurite reorganization (Wu *et al*. 2000). Plasminogen-deficient mice exhibit DSD-1-PG/phosphacan deposition, as tPA functions acutely, both through and independently of plasmin, to mediate mossy fibre reorganization and remodelling of neuronal connections, a mechanism implicated in seizure episodes (Wu *et al*. 2000). Seizure in addicts is very common and, from our present data, may well involve uPA-mediated plasminogen activation. More studies will be required to clarify these different possible pathways and the role of uPA and uPAR in response to cocaine.

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