

Tissue-type plasminogen activator requires a co-receptor to enhance NMDA receptor function

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Abstract

Glutamate is the main excitatory neurotransmitter of the CNS. Tissue-type plasminogen activator (tPA) is recognized as a modulator of glutamatergic neurotransmission. This attribute is exemplified by its ability to potentiate calcium signaling following activation of the glutamate-binding NMDA receptor (NMDAR). It has been hypothesized that tPA can directly cleave the NR1 subunit of the NMDAR and thereby potentiate NMDA-induced calcium influx. In contrast, here we show that this increase in NMDAR signaling requires tPA to be proteolytically active, but does not involve cleavage of the NR1 subunit or plasminogen. Rather, we demonstrate that enhancement of NMDAR function by tPA is mediated by a member of the low-density lipoprotein receptor (LDLR) family.

Hence, this study proposes a novel functional relationship between tPA, the NMDAR, a LDLR and an unknown substrate which we suspect to be a serpin. Interestingly, whilst tPA alone failed to cleave NR1, cell-surface NMDARs did serve as an efficient and discrete proteolytic target for plasmin. Hence, plasmin and tPA can affect the NMDAR via distinct avenues. Altogether, we find that plasmin directly proteolyzes the NMDAR whilst tPA functions as an indirect modulator of NMDA-induced events via LDLR engagement.

Keywords: low-density lipoprotein receptor family, NMDA receptor, plasmin, serine protease inhibitor, tissue-type plasminogen activator.

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The serine protease, tissue-type plasminogen activator (tPA), is known for its ability to cleave the pro-enzyme plasminogen into the potent protease plasmin, which can in turn lyse blood clots via the digestion of fibrin. In addition to this vascular role, tPA is now recognized to perform important roles within the brain (Melchor and Strickland 2005; Samson and Medcalf 2006). Neurons and glia secrete tPA upon appropriate stimulation (Vincent *et al.* 1998; Fernandez-Monreal *et al.* 2004b; Lochner *et al.* 2006; Polavarapu *et al.* 2007). Extracellular tPA activity is balanced by inhibitors and spatially targeted by association with cell-surface receptors. tPA^{-/-} mice display cognitive deficits, alterations in addiction and stress, and shifts in the response to pathological situations including seizure and ischemia (Wang *et al.* 1998; Yepes *et al.* 2002). Notably, recombinant tPA is used as a thrombolytic agent in patients with ischemic stroke (NINDS 1995). Altogether, a multitude of physiological and pathological roles have been ascribed to tPA. Despite the

functional diversity of tPA within the brain, two recurrent themes exist: (i) the physiological actions of tPA are contextual with synaptic plasticity processes; (ii) pathologically, tPA operates as an injurious excitotoxic factor. The paradigms of synaptic plasticity and excitotoxicity are both

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Abbreviations used: $\Delta[\text{Ca}^{2+}]_i$, change in free intracellular calcium; DIV, days *in vitro*; HBSS, Hanks' Balanced Salt Solution; LDLR, low-density lipoprotein receptor; LRP-1, low-density lipoprotein receptor-related protein 1; NBM, neurobasal media; NMDAR, NMDA receptor; PBS, phosphate-buffered saline; PDGF-C, platelet-derived growth factor-C; PN-1, protease nexin-1; RAP, receptor-associated protein; ROI, regions of interest; SDS, sodium dodecyl sulfate; tPA, tissue-type plasminogen activator.

reliant upon the NMDA receptor (NMDAR). As a result, several recent studies which show interplay between tPA and the NMDAR have received considerable attention (Nicole *et al.* 2001; Kvajo *et al.* 2004; Medina *et al.* 2005; Pawlak *et al.* 2005a,b; Benchenane *et al.* 2007; Norris and Strickland 2007; Martin *et al.* 2008; Park *et al.* 2008).

A persuasive body of evidence suggests that tPA can directly (i.e. in a plasmin-independent manner) cleave the NR1 subunit and thereby increase the Ca^{2+} -permeability of the NMDAR (Nicole *et al.* 2001; Fernandez-Monreal *et al.* 2004a; Benchenane *et al.* 2007). Our previous analyses have demonstrated that tPA does indeed augment NMDA-induced calcium flux ($\Delta[\text{Ca}^{2+}]_i$; Reddrop *et al.* 2005). Indicative of a novel effector substrate, here we show that the potentiating effect of tPA is dependent upon its proteolytic activity and independent of plasmin(ogen). Moreover, we find that tPA does not directly cleave the NR1 subunit. Hence, the mechanism by which tPA increases NMDAR signaling appears to be more complicated than first hypothesized. In line with this notion, we show that the ability of tPA to augment NMDA-induced $\Delta[\text{Ca}^{2+}]_i$ requires a member of the low-density lipoprotein receptor (LDLR) family. Thus, the potentiating effect of tPA on NMDAR signaling requires a co-receptor and an unknown substrate. In support of a multifactorial mechanism, we find that tPA does not alter NMDAR-mediated currents in an expressed heterologous *Xenopus* oocyte system, suggesting that additional cellular factors present in neuronal cultures are necessary for tPA to modulate NMDAR function. Lastly, distinct from the potentiation of NMDAR function, our analyses uncover the unique capacity of plasmin to discretely cleave the NR1 subunit. Thus, tPA has a dual influence on the NMDAR: one being the indirect potentiation of calcium flux via LDLR engagement, and the other being the plasmin-dependent proteolysis of NR1.

Materials and methods

Animals

C57Black/6 mice between 2 and 6-month old were used in this study. Experiments adhered to NH&MRC of Australia guidelines for live animal use. Experiments were approved by the appropriate Institutional Animal Ethics Committee.

Materials

Unless stipulated all reagents were from Invitrogen (Carlsbad, CA, USA). Recombinant human tPA used was Actilyse® (Boehringer Ingelheim, Ingelheim am Rhein, Germany). For Figs 1, 2(b), 5 and 7 the Actilyse® had been dialysed against 0.35 M HEPES-KOH (pH7.4). No difference in the effect of dialysed versus undialysed tPA was seen in this study. Aprotinin, human plasmin, NMDA and MK-801 were obtained from Sigma, St Louis, MO, USA. Cyanogen bromide-digested human fibrinogen was from American Diagnostica Stamford, CT, USA. Human thrombin was from Calbiochem, San Diego, CA, USA. ctPA was kindly provided by PAION

Deutschland GmbH (Aachen, Germany). ctPA was generated from Actilyse® by covalently coupling a 10-fold molar excess of D-phenyl-prolyl-arginine chloromethyl ketone into the reactive centre of the tPA molecule. Receptor-associated protein (RAP) and anti-human low-density lipoprotein receptor-related protein 1 (LRP-1) antibody were kind gifts from Prof. Dudley Strickland (University of Maryland, USA). Human LRP-1 was provided by Prof. Phil Hogg (University of New South Wales, Australia). NeuN and Glial fibrillary acidic protein antibodies were donated by Dr Gabriel Liberatore (University of Melbourne, Australia).

Preparation of primary cortical neuron cultures

Cultures were prepared from E15–16 mice (Reddrop *et al.* 2005). In brief, cortices were removed in ice-cold HBSS+: Hanks' Balanced Salt Solution with 1 mM Na pyruvate, 10 mM HEPES-KOH (pH 7.3), 3 g/L bovine serum albumin and 1.2 mM MgSO_4 . The isolated cortices were centrifuged (900 g, 5 min, 4°C), supernatant discarded, and tissue pellet incubated in HBSS+ with 0.2 g/L trypsin and 80 U/mL DNase I (5 min, 37°C with agitation). Trypsinization was stopped by the addition of HBSS+ with 0.5 g/L trypsin inhibitor, centrifuged (900 g, 5 min, 4°C), the supernatant discarded and 10 mL of HBSS+ with 0.5 g/L trypsin inhibitor and 2.1 mM MgSO_4 was added. The pellet was triturated through an 18-gauge blunted needle. The resultant single cell suspension was centrifuged (900 g, 5 min, 4°C) and the pellet resuspended in Neurobasal media with $1 \times \text{B27}$, 10% dialysed fetal calf serum, 0.5 mM L-glutamine and 50 U/mL penicillin/streptomycin (P/S). The cell suspension was seeded onto poly-D-lysine (BD Transduction Laboratories, Lexington, KY, USA) coated 24- or 12-well plates (\pm glass coverslips) at 150 000 cells/cm² and maintained in a humidified 37°C incubator under 5% CO_2 and 8% O_2 . Twenty four hours after seeding (DIV1; 'days *in vitro*'), the serum-containing media was aspirated and replaced with NBM+ media (Neurobasal media with $1.25 \times \text{B27}$, 0.5 mM L-glutamine and 50 U/mL P/S). At DIV5, an equal volume of NBM+ media was added. All experiments were performed with either DIV5 or DIV12–13 cultures (as indicated) in a humidified 37°C incubator under 5% CO_2 , 20% O_2 . Figure S5 demonstrates the cellular constituents of these cultures.

Measurement of $\Delta[\text{Ca}^{2+}]_i$

Neurons cultured on coverslips were incubated in phenol red-free NBM+ media containing 1 μM Oregon Green® 488 BAPTA-1 AM, for 45 min at 37°C. The media was replaced with fluorophore-free NBM+ media and incubated for a further 45 min. The coverslips were then assembled into a perfusion chamber (Warner Instruments, Hamden, CT, USA, Model RC-20H) on the stage of a Leica DM-IRBE confocal microscope (Leica, North Ryde, Australia) which was encased in a Perspex incubator and held at 37°C by an electric air heater. A single field of neurons (typically 15–30 neurons) was selected and Flow Buffer (phenol red-free HBSS with 2 mM CaCl_2 and 0.6 mM MgCl_2) perfused over the cells at 0.5 mL/min. For the assessment of the modulation of NMDA-induced $\Delta[\text{Ca}^{2+}]_i$, a perfusion protocol involving three stimulations was employed. $\Delta[\text{Ca}^{2+}]_i$ was monitored in 1.8-s intervals. The first and second stimulations involved two identical 45-s exposures to either 25 or 50 μM NMDA (separated by 10 min), whilst the third stimulation was a 75 mM KCl exposure (third stimulation data not shown). Vehicle/Buffer alone (control) or various treatments (tPA, ctPA,

RAP, plasmin) were perfused for 5 min over the cells in between the two transient NMDA exposures. A thorough example of this procedure has been published (Weiss *et al.* 2006). The data were analyzed using Leica physiology software with regions of interest (ROI) corresponding to the cell body being selected. Each ROI was assigned a $N = 1$ value and only ROI that displayed a sharp, definitive rise in fluorescence from all three stimulations were analyzed. For each ROI, the $\Delta[\text{Ca}^{2+}]_i$ (i.e. area under the curve) above baseline (i.e. median value of the unstimulated periods) was measured and the second NMDA-induced $\Delta[\text{Ca}^{2+}]_i$ was expressed relative to the first NMDA-induced $\Delta[\text{Ca}^{2+}]_i$. This value was averaged across all ROI within the same treatment group (i.e. % modulation). The % modulation for each 'treated' group was normalized to that of the 'control' group (i.e. % modulation relative to control). Each independently seeded culture was assigned an $n = 1$ value. Differences between treatment groups were tested by one-way ANOVA and *post hoc* correction for multiple comparisons with $p < 0.05$ being considered as statistically significant. Note, none of the modulatory agents were found to discernibly alter basal calcium flux (Fig. S6).

Tissue protein extracts

Unless indicated, all buffers/manipulations were at 4°C. An adult mouse brain was removed and rinsed with phosphate-buffered saline (PBS) (0.137 M NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4 , 1.76 mM KH_2PO_4 pH 7.4). The cortices were dissected, rinsed again in PBS, then homogenized in RIPA buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% Na deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulfonyl fluoride, 5 mg/L aprotinin, 5 mg/L leupeptin, 2 mM imidazole, 1 mM NaF, 1 mM Na_3VO_4]. The homogenate was centrifuged (400 g, 5 min) and the supernatant stored at -80°C. Protein extracts of the liver were prepared in the same manner.

Cell-free cleavage assay

An adult mouse brain was removed and washed with PBS. The cortices were dissected, rinsed again in PBS, then homogenized in PBS containing 2.5% Triton X-100. The resulting homogenate was pelleted by centrifugation (400 g, 5 min), and the protein concentration of the supernatant quantified and adjusted to 3 mg/mL. Proteases (plasmin, tPA, thrombin; 4–25 μL) were added to 0.5 mL aliquots of the cortical lysates and incubated at 37°C for 10 min to 2 h. Fifty μg of each sample was then subjected to immunoblot.

Cell-based cleavage assay

Days *in vitro* 12 culture media (in 24-well plates) was replaced with phenol red-free and B27-free Minimum Essential Medium with Earle's salts (400 μL per well) and incubated for 1 h in a humidified 37°C incubator. Proteases (tPA, plasmin, thrombin; 2–10 μL) were added to the media and incubated for 10 min or 1 h, after which the media was aspirated and ristocetin-induced platelet agglutination buffer added to each well. The lysates were collected, quantified and stored at -80°C.

Immunoblot analyses

Samples were boiled in SDS-loading buffer with dithiothreitol, subjected to SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. The membranes were

probed with primary antibodies [mouse anti-NR1 N-terminal (Chemicon, Temecula, CA, USA, 1:300–1000), goat anti-NR1 C-terminal (Santa Cruz Biotechnology Inc., 1:100–1000), goat anti-tPA (Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:1000), sheep anti-plasminogen (Serotec, Raleigh, NC, USA), rabbit anti-GFAP (Dako, Carpinteria, CA, USA, 1:1000), mouse anti-Glyceraldehyde 3-phosphate dehydrogenase (Chemicon Int., 1:5000), 4B3 mouse monoclonal anti-rat protease nexin-1 (PN-1) (Meier *et al.* 1989)] followed by appropriate horseradish peroxidase-conjugated secondary antibody [sheep anti-mouse IgG (Chemicon Int., 1:5000), rabbit anti-goat IgG (Sigma, 1:5000), rabbit anti-sheep IgG (Chemicon Int., 1:5000)]. Signals were revealed by chemiluminescence (Supersignal, Thermo Scientific, Waltham, MA, USA).

Electrophysiology

RNA preparation, oocyte preparation and expression of NMDAR subunits in *Xenopus* oocytes were performed as described previously (Kloda and Adams 2005). Plasmids with cDNA encoding the rat NR1a and NR2A NMDAR subunits were kindly provided by Dr. J. Boulter (UCLA, Los Angeles, CA, USA). All oocytes were injected with a 1 : 3 ratio of 5 ng NR1a and 15 ng NR2A cRNA, respectively and kept at 18°C in ND96 buffer (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl_2 , 1 mM MgCl_2 and 5 mM HEPES pH 7.4) supplemented with 50 mg/L gentamycin and 5 mM pyruvic acid 2–5 days before recording. Membrane currents were recorded from *Xenopus* oocytes using a two electrode voltage virtual ground circuit on a Gene clamp 500B amplifier or an OpusXpress™ 6000A workstation, (Molecular Devices, Sunnyvale, CA, USA), as previously described (Clark *et al.* 2006). Electrodes were filled with 3 M KCl and had resistances of 0.2–1.3 Mohm. All recordings were conducted at 20–23°C using a Ca^{2+} and Mg^{2+} -free solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM BaCl_2 and 10 mM HEPES at pH 7.3). Current amplitudes were determined by the steady-state plateau response elicited by 30 μM glutamate and 10 μM glycine, in the absence and presence of 1 μM tPA and 250 nM Plasmin at a holding potential of -70 mV. Membrane currents were sampled at 500 Hz and filtered at 200 Hz.

Results

Potentiation of NMDA-induced $\Delta[\text{Ca}^{2+}]_i$ is dependent upon proteolytic activity

Tissue-type plasminogen activator has previously been shown to potentiate NMDA-induced $\Delta[\text{Ca}^{2+}]_i$ (Nicole *et al.* 2001; Reddrop *et al.* 2005; Park *et al.* 2008). To ascertain whether the ability of tPA to augment NMDA-induced $\Delta[\text{Ca}^{2+}]_i$ was dependent on its proteolytic capacity, we utilized a novel inactive variant of tPA termed 'ctPA' (PAION GmbH, Germany). Relative to tPA, ctPA retained < 0.01% activity by amidolytic assay (data not shown), was 100 to 1000-fold less active by fibrin zymography (Fig. 1a), and was equivalent in molecular weight (Fig. S1) and receptor binding (Table S2). As shown in Fig. 1(b) and (c), while 500 nM tPA enhanced NMDA-induced $\Delta[\text{Ca}^{2+}]_i$, ctPA failed to elicit any potentiation. Rather, 500 nM ctPA appeared to

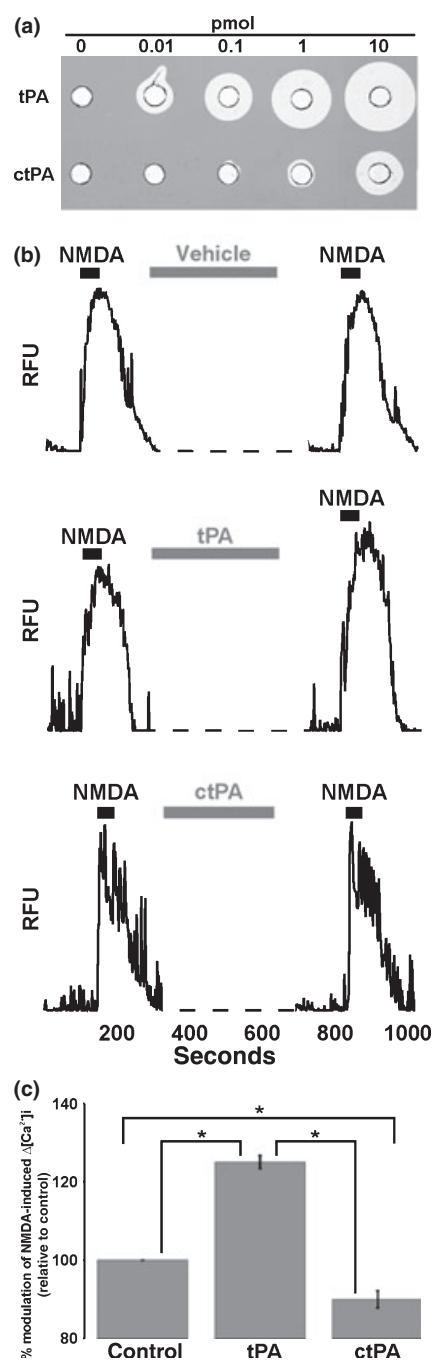
Fig. 1 Tissue-type plasminogen activator (tPA) requires its proteolytic activity to potentiate NMDA-induced $\Delta[\text{Ca}^{2+}]_i$. (a) Increasing amounts of tPA or ctPA were loaded into wells of a fibrin:plasminogen:agarose matrix (Granelli-Piperno and Reich 1978) and incubated at 37°C until proteolyzed zones appeared. (b) Representative single cell traces of 25 μM NMDA-induced $\Delta[\text{Ca}^{2+}]_i$ before and after 5 min perfusion with vehicle, 500 nM tPA or 500 nM ctPA. (c) Collated data demonstrating that 500 nM ctPA does not enhance 25 μM NMDA-induced $\Delta[\text{Ca}^{2+}]_i$. Data represent mean \pm SEM of the second NMDA-induced $\Delta[\text{Ca}^{2+}]_i$ relative to the first NMDA-induced $\Delta[\text{Ca}^{2+}]_i$ (i.e. % modulation). All data were normalized to control values. The data were pooled from $N = 61$ cells (control), $N = 84$ cells (tPA), $N = 75$ cells (ctPA) over three independent cultures ($n = 3$; $*p < 0.05$).

slightly suppress this calcium response. The basis of this suppression is unknown. Notably, 1 μM ctPA also failed to augment NMDA-evoked $\Delta[\text{Ca}^{2+}]_i$ (data not shown). Thus, tPA potentiates NMDA-triggered $\Delta[\text{Ca}^{2+}]_i$ in a manner dependent on its proteolytic activity.

Plasmin, but not tPA, can cleave the NR1 subunit

The requirement for proteolytic capacity implies the existence of a tPA-sensitive substrate. Previous studies have identified the NR1 subunit of the NMDAR as the operative substrate in this setting (Nicole *et al.* 2001; Fernandez-Monreal *et al.* 2004a; Benchenane *et al.* 2007). To determine whether tPA can cleave NR1 we performed ‘cell-based’ cleavage assays. In these assays, 500 nM tPA was added to the media of neuronal cultures for 10 min. As a control, cultures were also treated with 250 nM plasmin or 500 nM thrombin – two other trypsin-like serine proteases. Cell lysates were then prepared and NR1 content assessed by immunoblot analysis. As shown in Fig. 2(a), only plasmin was able to cleave the NR1 subunit producing a 90 kDa fragment. Incubation of cultures with the indicated proteases for 1 h resulted in no additional effects (data not shown). This finding suggests that the plasmin-mediated cleavage of full-length NR1 to the 90 kDa fragment utilizes portions of the NMDAR that are appropriately exposed on the cell-surface. To account for the possibility that cleavage of the NMDAR by tPA required NMDA engagement, similar cleavage experiments were also performed in the presence of NMDA. Under these conditions, tPA still failed to cleave NR1 (data not shown).

Our cell-based cleavage assays also show that plasmin treatment does not deplete full-length NR1. This finding reflects the fact that bath-applied proteases cannot access the full complement of cellular NMDARs [as a proportion of NMDARs exist intracellularly (Xia *et al.* 2001)]. To circumvent this, we utilized a ‘cell-free’ cleavage assay, whereby protein lysates of the adult tPA^{-/-} mouse cortex were incubated with plasminogen, tPA, and aprotinin (a reversible plasmin inhibitor). To ensure that tPA was fully active, we also supplemented lysates with cyanogen-digested fibrinogen (CNBr-F); a co-factor that potently enhances tPA activity towards certain substrates (Verheijen *et al.* 1982; Schaefer



et al. 2006). Lysates were then incubated for 15 min at 37°C and the NR1 content assessed by immunoblotting. To accommodate for the possibility that NR1 cleavage products contain epitopes that are not recognized by a single antibody, we used two different anti-NR1 antibodies – one directed against the extracellular N-terminal domain and one directed against the intracellular C-terminal domain of NR1 (Fig. 2c). As shown in Fig. 2(b), immunoblotting with either anti-NR1 antibody revealed that whilst tPA or tPA + cyanogen bromide-digested fibrinogen were unable to proteolyse

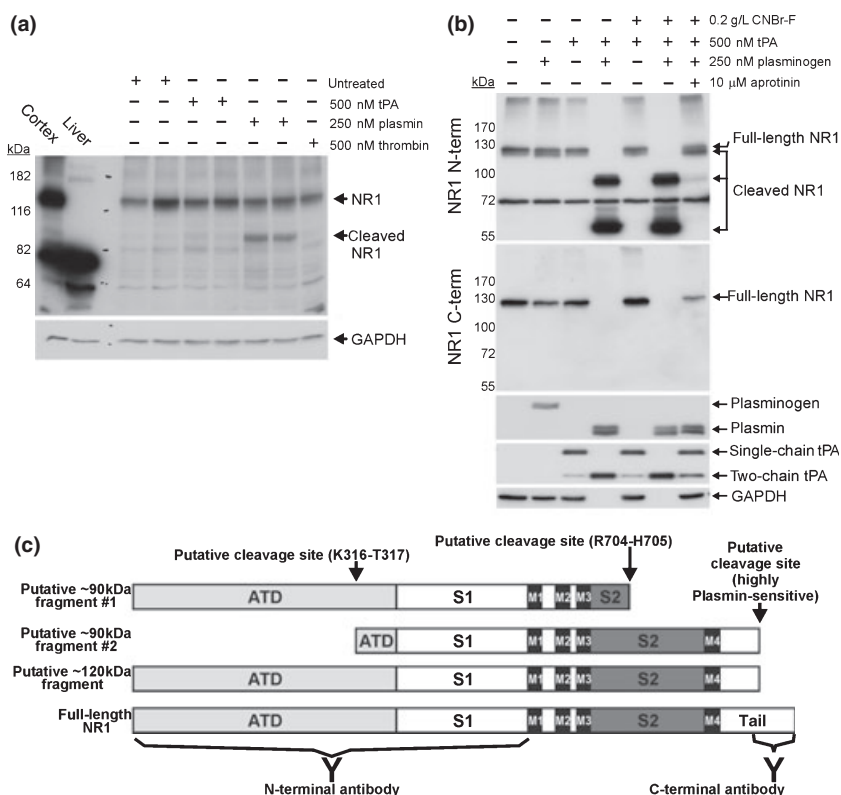


Fig. 2 The NR1 subunit of the NMDAR is a substrate for plasmin, but not tPA. (a) Representative cell-based cleavage assay demonstrating that plasmin, but not tPA, can cleave the NR1 subunit of the NMDAR. Cultures were incubated with 500 nM tPA, 250 nM plasmin or 500 nM thrombin at 37°C for 10 min. The NR1 content was assessed by immunoblot using the anti-NR1 N-terminal antibody. The membrane was re-probed for GAPDH as a loading control. A total of six independent cell-free cleavage assays demonstrated that tPA cannot cleave the NR1 subunit ($n = 6$). Note, the anti-NR1 N-terminal antibody is a 'pan' antibody as it recognizes all NR1 variants. (b) Representative cell-free cleavage assay demonstrating that plasmin, but not tPA, can cleave the NR1 subunit. tPA^{-/-} mouse cortical lysates were supplemented with CNBr-F, 500 nM tPA, plasminogen and aprotinin and incubated at 37°C for 15 min. The NR1 content was assessed by immunoblot using both anti-NR1 N-terminal and C-terminal antibodies. To assess the proteolytic actions of tPA and plasmin, membranes were re-probed for plasmin(ogen) and tPA, respectively. The membranes were re-probed for GAPDH as a loading control. Note, the generation of plasmin under cell-free conditions leads to a loss of GAPDH, presumably as a consequence of proteolytic degradation. A total of seven independent cell-free cleavage assays demonstrate that plasmin, but not tPA, can cleave the NR1 subunit ($n = 7$). (c) The NR1 subunit is comprised of an amino-terminal domain (ATD), a bi-lobed ligand binding domain (S1 and S2), four transmembrane segments (M1–M4) and a C-terminal tail. Note, the anti-NR1 N-terminal antibody is raised against the ATD and S1 regions of NR1, whilst the anti-C-terminal antibody is raised against the C-terminal tail. Our cell based

cleavage assays indicate that a 90 kDa fragment is generated from the naturally presented NR1 molecule, while additional fragments of 120 and 60 kDa are generated when cryptic cleavage sites are exposed following cell disruption (i.e. as seen using the cell free cleavage assay). The schematic indicates how these fragments may be generated from the native NR1 molecule. It appears that a highly-plasmin sensitive cleavage site resides near the C-terminus of NR1. Proteolysis at this point yields the 120 kDa fragment. The 120 kDa fragment is further proteolysed into a 90 kDa and a 60 kDa fragment that are recognized solely by the N-terminal antibody. Proteolytic generation of the 90 kDa fragment is best explained by cleavage at one of two putative cleavage sites: (1) a site within the S2 domain yielding 'fragment #1', or (2) a site within the ATD yielding 'fragment #2'. The specific cleavage site yielding the 60 kDa fragment is unclear and is not shown in the diagram. Several studies have determined the site specificity of plasmin (Backes *et al.* 2000; Harris *et al.* 2000; Gosalia *et al.* 2005; Xue and Seto 2005). This consensus substrate specificity was used to scan the S2 domain for putative plasmin-sensitive cleavage sites. Any putative cleavage sites were then located within the crystal structure of the NR1 : NR2A dimeric complex (PDB Identifier # 2A5T) to ensure surface exposure. Via this method, Arginine⁷⁰⁴-Histidine⁷⁰⁵ was highlighted as a putative plasmin-sensitive cleavage site with the S2 domain. Using a similar approach, Lysine³¹⁶-Tyrosine³¹⁷ was identified as a putative plasmin-sensitive cleavage site with the ATD. In this instance, as the crystal structure of the NR1 ATD has not been resolved, the ATD of the closely related mGluR1 was used [PDB Identifier # 1EWK; (Huggins and Grant 2005)].

NR1, co-incubation with tPA+plasminogen resulted in complete NR1 cleavage (see Fig. S2 for quantitation of Fig. 2b). Aprotinin inhibited NR1 degradation caused by

tPA + plasminogen incubation. As expected, under these cell-free conditions, a loss of full-length NR1 coincided with the appearance of three NR1 fragments: a ~120, 90 and

60 kDa species. The 90 kDa fragment is presumably the same as that detected in our cell-based assays, whilst the 60 kDa is most likely a consequence of the cell-free conditions whereby regions of NR1 which are spatially hidden become available for ectopic proteolysis. Notably, aprotinin blocked the conversion of single-chain to two-chain tPA (a plasmin-dependent process), but did not inhibit conversion of plasminogen to plasmin (a tPA-dependent process). Thus, plasmin inhibition, rather than tPA inhibition, was responsible for the blockage of NR1 proteolysis by aprotinin. Figure 2(b) also shows that the anti-NR1 C-terminal antibody failed to detect any cleaved NR1 products. Interestingly, whilst immunoblotting with the anti-NR1 N-terminal antibody revealed that aprotinin potently inhibited plasmin-mediated NR1 degradation, immunoblotting with the anti-NR1 C-terminal antibody revealed only slight inhibition of NR1 degradation. This discrepancy suggests that NR1 has at least two plasmin-sensitive cleavage sites: one that is highly plasmin-sensitive (poorly inhibited by aprotinin) and another that is moderately plasmin-sensitive (effectively inhibited by aprotinin).

Our cleavage data best fit a model where a highly plasmin-sensitive cleavage site resides within the short intracellular C-terminal tail. Cleavage at this C-terminal site produces the 120 kDa fragment. Removal of the intracellular C-terminal tail would explain why all cleavage fragments are not detected by the anti-NR1 C-terminal antibody (Fig. 2c). Cleavage at two separate sites yields the 90 and 60 kDa fragments.

Given the results obtained from the cell-based cleavage assays, the 90 kDa plasmin-generated fragment is the only species that can occur under physiological conditions. Figure 2(c) provides a schematic of how this 90 kDa fragment can be generated from full length NR1 by plasmin.

To confirm the identity of the NR1 cleavage fragments, immunoprecipitations with the anti-NR1 C-terminal antibody were performed from neuronal culture lysates. The immunoprecipitated material was then plasmin-digested and subjected to anti-NR1 N-terminal immunoblot. The 120, 90 and 60 kDa species appeared following plasmin-digestion (Fig. S3). Thus, plasmin can generate the appropriate NR1 cleavage fragments from immunoprecipitated full-length native NR1. Bioinformatic analysis highlights either Arg⁷⁰⁴-His⁷⁰⁵ or Lys³¹⁶-Tyr³¹⁷ as the putative plasmin-sensitive cleavage site responsible for the 90 kDa fragment (Fig. 2c).

Additional cell-free cleavage assays using wild-type mouse cortical lysates incubated with 1 μ M tPA, 250 nM plasmin or 500 nM thrombin for 10 min and 2 h support our conclusion that plasmin, but not tPA, can cleave NR1 (data not shown). Thrombin, albeit with markedly lower efficiency, was also capable of producing the 90 and 60 kDa NR1 fragments under cell-free conditions, suggesting utilization of the same cleavage sites (data not shown) (Gingrich *et al.* 2000).

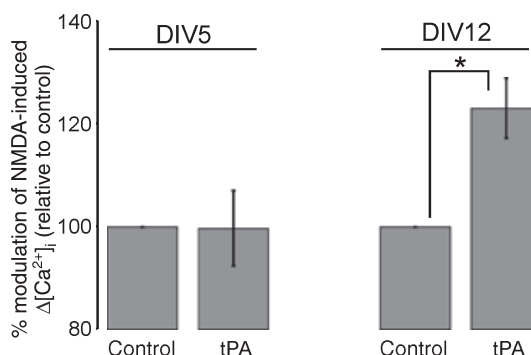


Fig. 3 Tissue-type plasminogen activator (tPA) potentiation of NMDA-induced $\Delta[Ca^{2+}]_i$ is a function of *in vitro* culture age. Collated data demonstrating the potentiating effect of 500 nM tPA on 50 μ M NMDA-induced $\Delta[Ca^{2+}]_i$ only in mature DIV12 culture. Data represent mean \pm SEM of the second NMDA-induced $\Delta[Ca^{2+}]_i$ relative to the first NMDA-induced $\Delta[Ca^{2+}]_i$ (i.e. % modulation). All data were normalized to control values. For DIV5 the data were pooled from $N = 161$ cells (control) and $N = 153$ cells (tPA) over four independent cultures ($n = 4$). For DIV12 the data were pooled from $N = 36$ cells (control) and $N = 56$ cells (tPA) over three independent cultures ($n = 3$; * $p < 0.05$). Figure S4 and Table S1 show the changes in the appearance, NMDA-responsiveness and NMDAR subunit expression that arose during *in vitro* culture maturation.

Potentiation of NMDA-induced $\Delta[Ca^{2+}]_i$ is a function of culture age

NR1 is an obligatory subunit of the NMDAR. Therefore, if NMDAR potentiation involved a direct association between tPA and NR1, then tPA should impact on any neuron with functional cell-surface NMDARs. But, despite NMDA eliciting a classical $\Delta[Ca^{2+}]_i$ in both DIV5 and DIV12 cultures (Fig. S4 and Table S1), tPA only potentiated NMDA-induced $\Delta[Ca^{2+}]_i$ in DIV12 cultures (Fig. 3). Hence, the ability of tPA to modulate NMDA-induced $\Delta[Ca^{2+}]_i$ is a function of *in vitro* culture age. That tPA cannot influence NMDA-induced $\Delta[Ca^{2+}]_i$ in early (DIV5) cultures denotes a requirement for additional cellular factors besides tPA and the NMDAR.

tPA alone does not alter NMDAR currents

To confirm that additional cellular factors were required for the potentiation of NMDAR function by tPA, we measured NMDAR-mediated currents in a heterologous non-neuronal system via two-electrode voltage clamp. Expression of heteromeric NR1a/2A NMDARs in *Xenopus* oocytes generated functional glutamate-activated channels, which were activated by 30 μ M glutamate and 10 μ M glycine. The addition of 1 μ M tPA for 30 s to the activated NMDAR revealed no change to the NMDAR-mediated current amplitude ($101.3 \pm 2.5\%$; $p > 0.05$ by *t*-test, $n = 17$; Fig. 4a). The addition of 250 nM plasmin in the open state of the NMDAR also revealed no change to the NMDAR-mediated current ($100.5 \pm 2.5\%$; $n = 4$; Fig. 4b). As tPA was incapable of

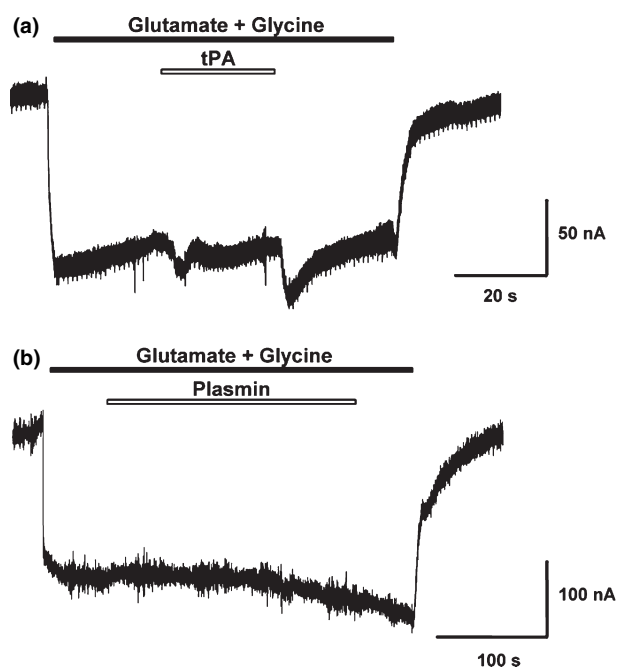


Fig. 4 Tissue-type plasminogen activator (tPA) does not alter recombinant NMDA-mediated currents in expressed heterologous *Xenopus* oocytes. Shown is a representative trace of NR1a/2A NMDA-mediated currents expressed in *Xenopus* oocytes. Cells were voltage clamped at -70 mV. NMDA-mediated currents were first evoked with 30 μ M glutamate and 10 μ M glycine (black bar), then during NMDAR activation either (a) 1 μ M tPA or (b) 250 nM Plasmin (open white bars) were transiently co-applied. Artifact peaks in (a) were because of solution exchange. For tPA, a total of 17 independent experiments were conducted. For plasmin, a total of four independent experiments were conducted.

altering NMDAR-mediated current in this isolated non-neuronal system, we conclude that tPA requires additional cellular factors to potentiate NMDAR function. This finding, in conjunction with our evidence showing that NR1 is not a tPA-sensitive substrate, contradicts the postulate that tPA directly alters NMDAR function via NR1 cleavage (Nicole *et al.* 2001; Fernandez-Monreal *et al.* 2004a).

Potentiation of NMDA-induced $\Delta[\text{Ca}^{2+}]_i$ is plasmin-independent

Having gained strong evidence against NR1 being a tPA-sensitive substrate, we next considered the prototypical substrate for tPA; namely plasminogen. Consequently, we monitored the modulatory effect of 25 nM plasmin on NMDA-induced $\Delta[\text{Ca}^{2+}]_i$. As shown in Fig. 5, unlike tPA, the perfusion of 25 nM plasmin resulted in no enhancement of NMDA-induced $\Delta[\text{Ca}^{2+}]_i$. Therefore, we conclude that tPA potentiates NMDA-induced $\Delta[\text{Ca}^{2+}]_i$ in a manner independent of plasmin(ogen). A concentration of 25 nM plasmin was chosen on the basis that treatment of neuronal

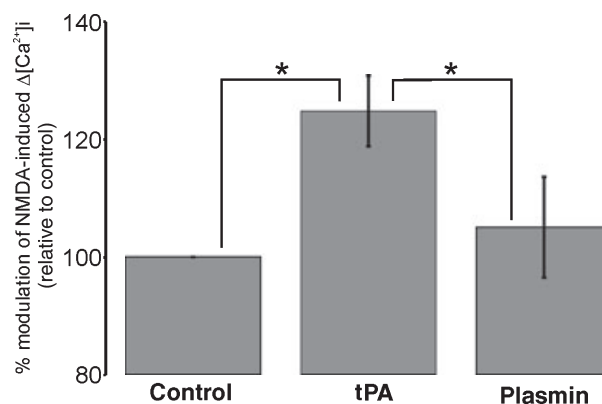


Fig. 5 Tissue-type plasminogen activator (tPA) potentiates NMDA-induced $\Delta[\text{Ca}^{2+}]_i$ in a plasmin-independent manner. Collated data demonstrating that unlike 500 nM tPA, 25 nM plasmin fails to potentiate 25 μ M NMDA-induced $\Delta[\text{Ca}^{2+}]_i$. Data represent mean \pm SEM of the second NMDA-induced $\Delta[\text{Ca}^{2+}]_i$ relative to the first NMDA-induced $\Delta[\text{Ca}^{2+}]_i$ (i.e. % modulation). All data were normalized to control values. The data were pooled from $N = 134$ cells (control), $N = 64$ cells (tPA) and $N = 66$ cells (plasmin) over three independent cultures ($n = 3$; * $p < 0.05$).

cultures with 25 nM plasmin resulted in NR1 cleavage (data not shown), whereas treatment of cultures with tPA failed to result in NR1 cleavage (Fig. 2a). Hence, the concentration of plasminogen in our cultures must be less than 25 nM.

Expression of PN-1 correlates with culture maturity

Besides plasminogen, tPA also displays high proteolytic activity towards several serpins, notably PAI-1, neuroserpin and PN-1 (Lawrence *et al.* 1995; Hastings *et al.* 1997; Rossignol *et al.* 2004). A prior study has shown that PN-1 and tPA together regulate NMDAR function (Kvajo *et al.* 2004). Consequently, we determined whether our neuronal cultures expressed PN-1. As shown in Fig. 6, PN-1 was virtually absent from DIV5 cultures, but was abundant in DIV12 cultures. Whilst we cannot exclude the role of other tPA-sensitive serpins, this strong correlation between PN-1 expression and the ability of tPA to enhance NMDA-induced $\Delta[\text{Ca}^{2+}]_i$ led us to postulate that PN-1 may be an additional cellular factor required for the modulation of NMDAR function by tPA.

Potentiation of NMDA-induced $\Delta[\text{Ca}^{2+}]_i$ by tPA requires LDLR engagement

Protease nexin-1 rapidly forms a complex with tPA, which in turn avidly binds to cell-surface LDLRs, initiating intracellular signaling cascades and tPA:serpin complex internalization (Herz and Strickland 2001). Indeed, LDLRs are the sole recognized receptor for tPA:serpin complexes. Accordingly, we assessed whether LDLR engagement was required for tPA to potentiate NMDA-induced $\Delta[\text{Ca}^{2+}]_i$. For this, we utilized the LDLR pan-ligand blocker, RAP. As shown in Fig. 7, we

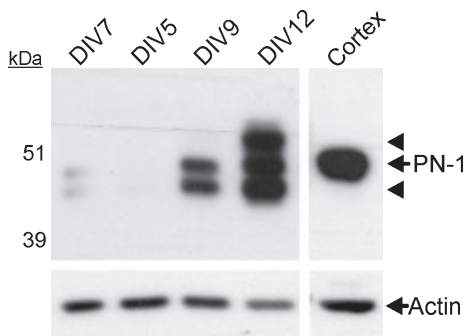


Fig. 6 Protease Nexin-1 (PN-1) expression increases dramatically during *in vitro* culture maturation. Cellular protein lysates were obtained from neuronal cultures at different days of *in vitro* (DIV) maturation. Lysates were collected and analyzed in quadruplicate from two independently-seeded cultures ($n = 2$, $N = 8$). PN-1 expression was assessed by immunoblot analysis. The membrane was re-probed for actin as a loading control. An adult mouse brain cortical lysate was used as a positive control for PN-1 (arrows). Arrowheads indicate probable alternate glycosylated forms of PN-1. Samples were loaded onto the gel in a blinded fashion in the order DIV 7, 5, 9 and 12 as indicated.

observed that whilst the application of RAP alone produced no significant change in NMDA-induced $\Delta[\text{Ca}^{2+}]_i$, it fully ablated the ability of tPA to enhance NMDA-induced $\Delta[\text{Ca}^{2+}]_i$. This observation suggests that tPA-mediated potentiation of NMDA-induced $\Delta[\text{Ca}^{2+}]_i$ is dependent upon LDLR engagement. This finding is consistent with the hypothesis that potentiation of NMDA-induced $\Delta[\text{Ca}^{2+}]_i$ involves tPA:serpin complex formation and subsequent LDLR engagement.

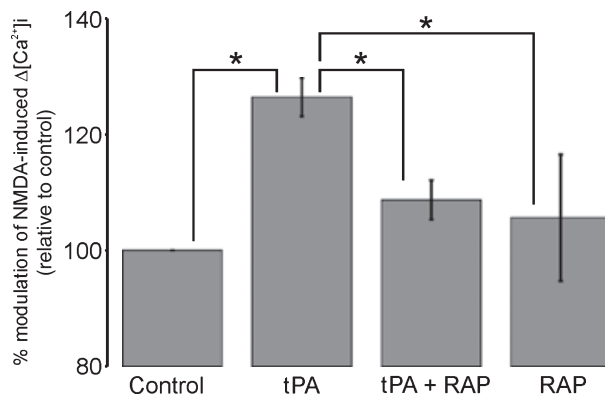


Fig. 7 A low-density lipoprotein receptor mediates the tPA potentiation of 50 μM NMDA-induced $\Delta[\text{Ca}^{2+}]_i$. Collated data demonstrating potentiation of NMDA-induced $\Delta[\text{Ca}^{2+}]_i$ by 500 nM tPA and the ability 500 nM RAP to block this effect. Data represent mean \pm SEM of the second NMDA-induced $\Delta[\text{Ca}^{2+}]_i$ relative to the first NMDA-induced $\Delta[\text{Ca}^{2+}]_i$ (i.e. % modulation). All data were normalized to control values. The data were pooled from $N = 83$ cells (control), $N = 78$ cells (tPA), $N = 64$ cells (tPA + RAP) and $N = 55$ cells (RAP) over three independent cultures ($n = 3$; $*p < 0.05$).

Discussion

Several studies document that tPA potentiates NMDA-induced $\Delta[\text{Ca}^{2+}]_i$ (Nicole *et al.* 2001; Reddrop *et al.* 2005; Park *et al.* 2008). The currently proposed mechanism involves direct cleavage of NR1 by tPA, which in turn increases the Ca^{2+} -permeability of the NMDAR (Nicole *et al.* 2001). In support of a proteolytic event, the reversible tPA inhibitor, tPA-STOP, has been shown to diminish the influence of exogenous tPA on NMDA-induced $\Delta[\text{Ca}^{2+}]_i$ (Liot *et al.* 2004). The low concentration of tPA-STOP (10 nM) relative to exogenous tPA (~ 300 nM), however, queries this interpretation. Consequently, our finding that an inactive tPA fails to enhance NMDA-induced $\Delta[\text{Ca}^{2+}]_i$ conclusively demonstrates that the enhancement of NMDA-induced $\Delta[\text{Ca}^{2+}]_i$ relies upon the proteolytic capacity of tPA.

Implicit in the requirement for proteolytic activity is the existence of an effector substrate. Published evidence from one laboratory defines NR1 as the pertinent substrate (Fernandez-Monreal *et al.* 2004a; Benchenane *et al.* 2007). In an attempt to detect proteolysis of NR1, both cell-free and cell-based cleavage assays were performed. Cleavage times were varied from 10 min to 2 h, and tPA concentrations from 50 nM to 1 μM were tested. Yet, despite trialing these different conditions, no evidence for the proteolysis of NR1 by tPA was found. Cell-based experiments in the presence of NMDA were also conducted and still no tPA-mediated NR1 proteolysis was observed (data not shown). Therefore, we conclude that NR1 is not a tPA-sensitive substrate. This conclusion extends the findings of others (Matys and Strickland 2003; Kvajo *et al.* 2004; Liu *et al.* 2004).

If not NR1, then what is the operative tPA-sensitive substrate? Platelet-derived growth factor-C (PDGF-C) represented a logical candidate (Boucher *et al.* 2003; Fredriksson *et al.* 2004; Su *et al.* 2008). However, we found that PDGF-C expression remained unchanged during neuronal culture development (data not shown) and thus PDGF-C represents an unlikely effector of tPA-mediated NMDAR modulation.

Our data, in conjunction with published data (Nicole *et al.* 2001), also suggests that plasminogen is not the tPA-sensitive substrate in question. Our findings do however, indicate that LDLR engagement is vital for tPA to influence NMDAR function. A direct interaction between tPA and a LDLR could explain how RAP ablates the enhancement of NMDA-induced $\Delta[\text{Ca}^{2+}]_i$ by tPA. Several lines of evidence point against this possibility. First, our surface plasmon resonance experiments (Table S2), together with other studies (Orth *et al.* 1994; Zhuo *et al.* 2000; Hu *et al.* 2006; Martin *et al.* 2008), suggest that tPA cannot proteolyse LDLRs. Second, despite having differential effects on NMDA-induced $\Delta[\text{Ca}^{2+}]_i$, both ctPA and tPA bind to LDLRs with high nanomolar K_d (~ 330 nM; Table S2). Thus, the potentiation

of NMDA-induced $\Delta[\text{Ca}^{2+}]_i$ is unlikely to be explained by the direct association/teolysis of a LDLR by tPA. Lastly, none of the tested LDLR family members (LRP-1, LRP-1B, ApoER2, Megalin and VLDLR) exhibited differences in expression between DIV5 and DIV12 cultures (data not shown). On the other hand, tPA displays potent and specific proteolytic activity towards several serpins, with the resultant tPA:serpin complex strongly binding to numerous LDLRs with low nanomolar K_d (Horn *et al.* 1997; Makarova *et al.* 2003). Additionally, we have observed that PN-1 expression in our cultures increases dramatically from DIV5 to DIV12. Therefore, PN-1 likely represents the tPA-sensitive substrate responsible for the potentiation of NMDA-induced $\Delta[\text{Ca}^{2+}]_i$. We propose a model whereby tPA first complexes with PN-1 or another differentially expressed serpin, then binds to a LDLR and signals for an enhancement of NMDA-induced $\Delta[\text{Ca}^{2+}]_i$. Both the requirement for tPA to be proteolytically active and the ability of RAP to block the influence of tPA on NMDA-induced $\Delta[\text{Ca}^{2+}]_i$ are in keeping with this model. It will be interesting to determine whether addition of PN-1 to DIV5 cultures restores the ability of tPA to potentiate NMDA-induced $\Delta[\text{Ca}^{2+}]_i$.

Further support for a tPA:PN-1 complex being a modulator of NMDAR function stems from the observation that both tPA^{-/-} and PN-1^{-/-} mice have reduced NR1 availability (D. Monard, unpublished data). Other tPA-reactive serpins may also elicit similar effects on NMDAR function. For example, it has been hypothesized that tPA, via complex formation with PAI-1, mediates NMDAR-dependent hyperemia (Park *et al.* 2008).

The links between tPA, LDLRs and the NMDAR are compelling. For instance, tPA facilitates NMDAR-dependent synaptic plasticity via engagement of the prototypical LDLR, LRP-1 (Zhuo *et al.* 2000). And similar to the influence of tPA described here-in, numerous LRP-1 ligands alter NMDA-induced $\Delta[\text{Ca}^{2+}]_i$ (Qiu *et al.* 2002, 2003). LRP-1 also physically associates with the NMDAR (May *et al.* 2004). Lastly, a recent study has demonstrated that tPA may elicit NMDAR activation in a LRP-1-dependent manner (Martin *et al.* 2008). Given these ties, it is noteworthy that NMDAR- and tPA-dependent long-term potentiation remains RAP-blockable despite the absence of neuronal LRP-1 (May *et al.* 2004). One possible explanation for this is that glial LRP-1 expression is critical for tPA to alter NMDAR function. Astrocytes are key mediators of neurotransmission that facilitate LTP (Yang *et al.* 2003). Furthermore, astrocytes are a significant component of our cultures (Fig. S5). Thus, we cannot exclude the involvement of astrocytes in our observations. In fact, a peri-cellular communication mechanism between neurons and astrocytes merits consideration, particularly as astrocytic uptake of tPA is blocked by RAP (Fernandez-Monreal *et al.* 2004b), as tPA triggers LRP-1 shedding from astrocytes (Polavarapu *et al.* 2007), and as astrocytes

are known NMDAR modulators (Wolosker *et al.* 2002). Alternatively, it is possible that other LDLRs besides LRP-1 are central to the potentiation of NMDA-triggered events by tPA.

Distinct from the potentiation of NMDA-induced $\Delta[\text{Ca}^{2+}]_i$, our experiments reveal the novel ability of plasmin to discretely proteolyse NR1. We propose that plasmin can efficiently remove the very distal C-terminal portion of NR1. As a result, antibodies raised against the C-terminal portion of NR1 do not detect the ~120 kDa N-terminal fragment of NR1 (or the subsequent 90 and 60 kDa fragments). This model likely explains why previous cell-free experiments have shown that plasmin, instead of discretely cleaving NR1, can completely degrade NR1 (Matys and Strickland 2003). Notably, whilst no effect on rudimentary NMDAR-mediated ion conductance was observed (Fig. 4), the impact of plasmin-mediated NR1 cleavage on other NMDAR properties such as allosteric modulation, cell-surface location and internalization rate remains unknown. That plasmin could efficiently proteolyse the extracellular domain of NR1 in the context of native cell-surface NMDARs intimates biological significance. Indeed, cleavage of NR1 by plasmin most likely occurs under chronic stress, a condition where plasmin has been shown to drastically decrease hippocampal NR1 levels (Pawlak *et al.* 2005b).

In conclusion, our investigations establish the plasmin-independent potentiation of NMDA-induced $\Delta[\text{Ca}^{2+}]_i$ by tPA. Even though tPA needs to be proteolytically active, we find no evidence that tPA can directly cleave the NR1 subunit. Furthermore, our data suggest that the enhancement of NMDA-induced $\Delta[\text{Ca}^{2+}]_i$ by tPA is mediated by a LDLR co-receptor. A similar set of experimental criteria has been previously described, whereby tPA increases blood-brain barrier permeability in a manner dependent upon proteolysis, independent of plasminogen and reliant upon LRP-1 (Yepes *et al.* 2003). As such, we hypothesize that tPA acts on a non-plasminogen substrate. Subsequent to this cleavage event, a LDLR is engaged, which in turn augments calcium flux downstream of the NMDAR. Given the dramatic increase in PN-1 between DIV5 and DIV12 cultures, PN-1 presents as the operative non-plasminogen substrate in this setting. This multi-factorial mechanism may underlie some of the proteolytic, yet plasmin-independent roles of tPA (Pawlak *et al.* 2002; Yepes *et al.* 2002, 2003; Kumada *et al.* 2005; Schaefer *et al.* 2007; Park *et al.* 2008). Finally, adding to the ways in which the plasminogen activator system can modulate the NMDAR, our analyses uncover the capacity of plasmin to discretely cleave the NR1 subunit of the NMDAR.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Section S1 ctPA and tPA have equivalent molecular weights.

Section S2 Plasmin, but not tPA, cleaves the NR1 subunit of the NMDAR.

Section S3 Plasmin cleaves the NR1 subunit of the NMDAR.

Section S4 Variation in NMDAR properties and expression during *in vitro* culture maturation.

Section S5 Characterization of primary neuronal cultures.

Section S6 Neither tPA, plasmin nor RAP alter basal calcium flux.

Section S7 Both free, active tPA and inactive tPA bind equally to LRP-1.

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