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Competing interests statement

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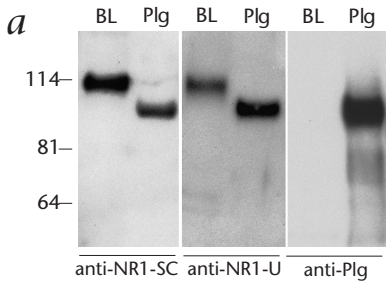
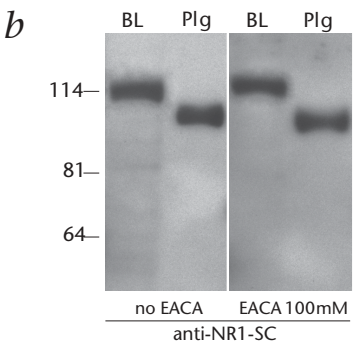
Tissue plasminogen activator and NMDA receptor cleavage

To the editor—It is established that the serine protease tissue plasminogen activator (TPA) and its conventional zymogen substrate plasminogen have an important role in certain central nervous system pathologies related to neuronal death (ref. 1 and references therein). Studies of rodents indicate that TPA and plasminogen synthesized locally[2] or entering brain tissue through a compromised blood-brain barrier[1] can be involved in this phenomenon. Whereas some of these central effects of TPA are associated with the conversion of plasminogen to plasmin, others are independent of plasminogen activation (reviewed in ref. 3). The identity of the substrate(s) for TPA that could mediate the action of this protease in the absence of plasminogen is still a matter of debate.

One possible substrate for TPA could be the *N*-methyl-D-aspartate (NMDA) receptor, as TPA can influence NMDA-mediated effects. Disruption of the gene encoding TPA, *Plat*, in mice interferes with the NMDA-dependent induction of long-term potentiation in the striatum[4], suggesting that endogenous TPA can modulate NMDA receptor signaling. In the January 2001 issue of *Nature Medicine*, Nicole *et al.*[5] reported that TPA potentiates NMDA-induced calcium influx and neuronal death in cortical neuron cultures, and they suggested that TPA can form a complex with and cleave the NR1 subunit of the NMDA receptor. Their conclusion was based on co-immunoprecipitation experiments and detection of the native and cleaved forms of the NR1 subunit using an antibody against NR1 (anti-NR1).

We have found a possible complication in the interpretation of some of the data of Nicole *et al.*[5]. The anti-NR1 antibody used in their experiments (designated here as anti-NR1-SC) is not specific for NR1, but also cross-reacts





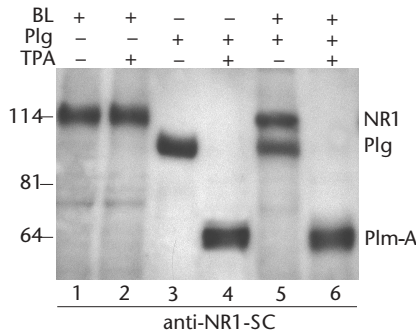


Fig. 1 Binding of anti-NR1 antibodies to plasminogen and NR1 subunit cleavage by plasmin. Male C57BL/6 mice were anaesthetized with Avertin (500 mg/kg) and perfused transcardially with ice-cold PBS (40 ml), and their brains were collected. Hippocampi were dissected and the tissue was homogenized in 100 mM Tris with 0.1% Triton X-100 in the absence of protease inhibitors. ***a***, Immunoblots of brain lysates (1 mg/ml total protein) and plasminogen solution (affinity-purified from human plasma; 50 μg/ml) probed with NMDA-ζ1 (anti-NR1-SC; Santa Cruz Biotechnology, Santa Cruz, California), anti-NR1 alternative C terminus (anti-NR1-U; Upstate Biotechnology, Waltham, Massachusetts) and anti-plasminogen (anti-Plg; Sigma, St. Louis, Missouri) antibodies. ***b***, Immunoblots of brain lysates and plasminogen probed with the anti-NR1-SC antibody in the absence (left) or presence (right) of ε-amino-*N*-caproic acid (EACA, Sigma; 100 mM). ***c***, Immunoblots of brain lysates, plasminogen or both incubated at 37 °C for 30 min with recombinant human TPA (Activase, Genentech, South San Francisco, California; 20 μg/ml) probed with the anti-NR1-SC antibody. NR1 subunit cleavage was observed with plasminogen concentrations as low as 0.2 μg/ml. Although the concentration of plasminogen from perfused brain lysates was insufficient to result in cleavage of the NR1 subunit after TPA addition (lane 2), it is possible that this level could be achieved in specific subregions, especially in conditions in which plasminogen expression is upregulated or the blood-brain barrier is compromised. The numbers on the left indicate molecular weight in kD. BL, brain lysates; Plg, plasminogen; Plm-A, heavy chain of plasmin.

with plasminogen (Fig. 1*a*). We found that another antibody against the NR1 C-terminus, anti-NR1-U, also binds to plasminogen (Fig. 1*a*). In contrast, a plasminogen-specific antibody detected plasminogen but not the NR1 subunit (Fig. 1*a*). The specificity of binding of anti-NR1 antibodies to plasminogen was confirmed in immunoblots in which the primary antibodies were omitted (data not shown). Both of the anti-NR1 antibodies also cross-reacted with a commercial human plasminogen preparation (Roche Diagnostics, Mannheim, Germany; data not shown).

We did not find any amino acid sequence homology between the NR1 subunit and plasminogen, but the binding of two different anti-NR1 antibodies to both proteins indicates that they share similar structural determinants. Since the plasminogen kringle domains have an affinity for lysine[6], plasminogen could bind to lysine residues in the anti-NR1 antibodies. To examine such a possibility, we performed immunoblots in the presence of the lysine analogue ε-amino-*N*-caproic acid (EACA; 0.1–100 mM). Under these conditions, the anti-NR1-SC antibody still bound to plasminogen (Fig. 1*b*), which argues against this mechanism of interaction. The molecular basis and the possible physiological relevance of the similarity between the NR1 subunit and plasminogen remain to be defined.

When we incubated brain lysates with TPA under the same conditions as Nicole *et al.*[5], we did not observe any changes in the density of the NR1 subunit band or appearance of additional bands (Fig. 1*c*, lane 2). We confirmed that TPA at this concentration was active in a zymographic assay (data not shown) and readily converted plasminogen to plasmin (Fig.1*c*, lane 4). The heavy chain (A-chain) of plasmin was also recognized on immunoblots by both anti-NR1-SC (Fig.1*c*, lane 4) and anti-NR1-U (data not shown) antibodies.

When the brain lysate was incubated with TPA in the presence of plasminogen, the NR1 subunit band disappeared (Fig. 1*c*, lane 6). This result indicates that plasmin formed by TPA-mediated activation of plasminogen cleaved the NR1 subunit to fragments that could not be detected by the anti-NR1 antibody. This cleavage was observed at concentrations of plasminogen as low as 0.2 μg/ml (data not shown).

In the experiments of Nicole *et al.*[5], co-immunoprecipitation and identification of the NR1 subunit as a substrate for TPA were done on cultures maintained in serum-supplemented media. Because of this, the presence of plasminogen in these preparations cannot be excluded. If plasminogen were present, binding of the anti-NR1 antibody to plasminogen or plasmin could have influenced the interpretation of the data in several ways. First, it could have led to misidentification of plasminogen or plasmin bands as the NR1 subunit in its native or cleaved form. Second, it could have influenced co-immunoprecipitation experiments; for example, the anti-NR1 antibody could have precipitated TPA in a complex with plasminogen and not, as suggested by the authors, with the NR1 subunit, whereas the TPA-specific antibody could have precipitated a complex of TPA and plasminogen. Such a possibility was proposed in a commentary accompanying the original article[7]. Finally, the decrease in NR1 subunit band density observed by Nicole *et al.*[5] could have been the result of degradation by plasmin formed by TPA-mediated activation of plasminogen.

Our observations, coupled with the results obtained by Nicole *et al.*[5], also raise the question of the functional consequences of NR1 subunit cleavage by blood proteases. In their study, addition of a high concentration of plasmin (100 μg/ml) to neuronal cultures did not influence NMDA-induced neurotoxicity although, as our results show, this protease is able to efficiently cleave the NR1 subunit at much lower concentrations. Similarly, cleavage of the NR1 subunit by another serine protease, thrombin, was not associated with any changes in NMDA receptor signaling[8]. Other reports on the potential association between plasmin(ogen) and NMDA receptor signaling provide conflicting results: whereas plasminogen potentiated an NMDA-evoked calcium concentration increase in cultured rat hippocampal neurons[9], others did not find any change in NMDA receptor–mediated synaptic responses[10].

In conclusion, we emphasize that our findings do not question the ability of TPA to enhance NMDA receptor signaling. That this enhancement is due to cleavage of the NR1 subunit by TPA, however, merits re-evaluation.

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The authors declare that they have no competing financial interests.

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Nicole et al. reply—We showed that TPA, a serine protease known for its fibrinolytic activity, enhances NMDA-mediated calcium signaling and subsequent excitotoxic neuronal injury. This potentiation of excitotoxicity is concomitant with the cleavage of the NR1 subunit of NMDA receptor by TPA[5].

Although Matys and Strickland are not questioning the deleterious influence of TPA on excitotoxic neuronal cell death, they raise an important concern about a possible misinterpretation of our results: does the TPA-induced cleavage of the NMDA receptor NR1 subunit involve the plasminogen-plasmin axis? We believe that we have strong arguments against this possibility.

First, whereas our primary cortical neuronal cultures were maintained in a serum-supplemented solution that may have contained plasminogen, the excitotoxic injury and cleavage experiments were all conducted in serum-free solutions. A casein gel zymography assay did not detect the presence of active plasmin in the culture media or in crude extracts of neuronal cultures, thereby excluding a possible contamination of our samples (data not shown).

Second, we used a bacterial system to produce a recombinant protein corresponding to the N-terminal end of NR1. Treatment of the purified recombinant protein with recombinant TPA (20 μg/ml) led to the appearance of a lower molecular weight product identified by mass-spectrometric analysis as a cleaved form of the NR1 N terminus[11]. These results prove irrefutably that TPA can cleave the N-terminal moiety of the NR1 subunit of the NMDA receptor without the involvement of plasmin.

Our results show that it is very unlikely that plasminogen has a role in the TPA-mediated cleavage of the NR1 subunit. Plasminogen, the preferential target of TPA in blood, seems to have important structural similarities with NR1, as indicated by Matys and Strickland. This raises the possibility that