Antibodies Preventing the Interaction of Tissue-Type Plasminogen Activator With N-Methyl-d-Aspartate Receptors Reduce Stroke Damages and Extend the Therapeutic Window of Thrombolysis

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Background and Purpose—Tissue-type plasminogen activator (tPA) is the only drug approved for the acute treatment of ischemic stroke but with two faces in the disease: beneficial fibrinolysis in the vasculature and damaging effects on the neurovascular unit and brain parenchyma. To improve this profile, we developed a novel strategy, relying on antibodies targeting the proneurotoxic effects of tPA.

Methods—After production and characterization of antibodies (αATD-NR1) that specifically prevent the interaction of tPA with the ATD-NR1 of N-methyl-d-aspartate receptors, we have evaluated their efficacy in a model of murine thromboembolic stroke with or without recombinant tPA-induced reperfusion, coupled to MRI, near-infrared fluorescence imaging, and behavior assessments.

Results—In vitro, αATD-NR1 prevented the proexcitotoxic effect of tPA without altering N-methyl-d-aspartate-induced neurotransmission. In vivo, after a single administration alone or with late recombinant tPA-induced thrombolysis, antibodies dramatically reduced brain injuries and blood–brain barrier leakage, thus improving long-term neurological outcome.

Conclusions—Our strategy limits ischemic damages and extends the therapeutic window of tPA-driven thrombolysis. Thus, the prospect of this immunotherapy is an extension of the range of treatable patients. (Stroke. 2011;42:00-00.)

Key Words: stroke ■ therapeutic window ■ tissue-type plasminogen ■ thrombolysis

Despite tremendous advances in understanding the pathophysiology of stroke and the significant number of clinical trials, the thrombolytic agent recombinant tissue-type plasminogen activator (rtPA) remains the only approved acute pharmacological treatment for ischemic stroke.1 Nevertheless, its use is limited to a short therapeutic window (4.5 hours post-onset, excluding 90% of patients),2,3 and associated with a threat of hemorrhage1 and neurotoxicity.4–8 Thus, there is a critical need for a larger therapeutic window of rtPA and for the development of alternative treatments for patients who are not eligible for thrombolysis.

Vascular tissue-type plasminogen activator (tPA) promotes fibrinolysis by converting plasminogen into active plasmin but can cross the even uninjured blood–brain barrier (BBB).9 Together with tPA released by neurons and glia, it can interact with several substrates within and beyond the neurovascular unit. For instance, tPA might promote BBB leakage through the conversion of pro-platelet-derived growth factor (PDGF-C) into PDGF-CC, the shedding of low-density lipoprotein receptor-related protein, and/or matrix metalloproteinase (MMP-3 and MMP-9) activation.10–12 In the brain parenchyma, the interaction of tPA with N-methyl-d-aspartate receptors (NMDAR), lipoprotein receptor-related protein, annexin II in glial cells and/or neurons activates signaling processes that result in adverse outcomes, including cerebral edema, hemorrhage, and cell death.12–14

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Based on our knowledge of the molecular events through which tPA aggravates NMDAR-mediated neurotoxicity,7 we generated a polyclonal antibody against the interaction site of tPA on the NR1 subunit of NMDAR, which could prevent the proexcitotoxic effect of rtPA in vitro. In a clinically relevant model of thromboembolic stroke in mice,15 a single early or late intravenous administration of the antibody strongly and durably reduced brain lesions and neurological deficits. Moreover, this strategy increased the therapeutic window of rtPA-driven thrombolysis.

Methods

Unless specified, chemicals were from Sigma Aldrich. Experiments complied with the European Directives and the French Legislation on Animal Experimentation and were approved by the local ethical committee. For more detailed protocols, refer to the supplementary methods (http://stroke.ahajournals.org).

Production and Purification of Polyclonal Antibodies

The region of the aminoterminal domain of the NR1–1a subunit (amino acids 19 to 371, a sequence absent from other glutamate receptor subunits) corresponding to the domain of interaction with tPA (designed rATD-NR1), was produced as previously.7 Then, slightly modified from the previous procedure,16 mice were immunized by intraperitoneal injection of immunogenic mixtures: complete Freund’s (first injection) and incomplete Freund's (once a week during 3 weeks) adjuvant alone (control) or containing the rATD-NR1 (30 μg; n=60 per group). Sera collected 2 weeks after the last inoculation were purified on hydroxyapatite columns (Proteogenix) to obtain αATD-NR1 or control polyclonal antibodies. Two different batches of αATD-NR1 were used to conduct experiments.

Thromboembolic Focal Cerebral Ischemia

Male Swiss mice (28 to 30 g; CERJ, Paris, France) or tPA knockout and their wild-type C57BL/6J littermate mice were subjected to focal cerebral ischemia by injection of murine α-thrombin (0.75 UI, 1 μL, Gentaur) into the middle cerebral artery (MCA) under deep anesthesia (isoflurane; induction 5%, maintenance 2% in O2/N2O). Thrombolysis was initiated by rtPA (10 mg/kg; Actilyse; Boehringer Ingelheim; tail vein injection, 10% bolus, 90% perfusion during 40 minutes). Controls received saline under identical conditions. Rectal temperature was maintained at 37±0.5°C throughout the surgical procedure using a feedback-regulated heating system. Cerebral blood flow was continuously measured by laser Doppler flowmetry using an optic fiber probe (Oxford Optronix) affixed on the skull above the MCA downstream of the thrombin injection site. The post-ischemic cerebral blood flow was expressed as the percentage of cerebral blood flow during the last 5 minutes of rtPA or vehicle infusion over the baseline cerebral blood flow before the injection of α-thrombin.

Passive Immunization

A single injection in the tail vein with saline, purified αATD-NR1, or control antibodies (160 μg in 200 μL each) was performed at the time indicated after ischemic challenges. Animals were randomly assigned to the experimental groups. Mortality was null for all procedures/treatments. Also, no evidence of major intracerebral bleeding was observed.

Histological Analysis of Brain Lesions

After 24 hours, brains were collected, frozen in isopentane, cryostat-cut (20-μm sections), stained with thionine, and analyzed (blindly to the group) as described earlier.15

MRI Analyses

MRI analyses were performed on ischemic mice that had been intravenously injected with saline or the αATD-NR1 antibodies 4 hours post-middle cerebral artery occlusion (MCAO) (see also supplementary methods).

Evans Blue Extravasation

Evans blue (200 μL, 2%) was intravenously injected in ischemic mice 3 hours before euthanasia and quantified in perfused cortices as previously described.17 Briefly, ipsilateral and contralateral hemispheres were weighed, homogenized in N,N-dimethylformamide, and centrifuged (21 000 g, 30 minutes). Evans blue was quantified in supernatants at 620 nm minus the background calculated from the baseline absorbance between 500 and 740 nm and divided by the wet weight of each hemisphere.

Post-ischemic Neurological Deficit

A global evaluation of locomotor activity was performed 24 hours, 1 month, and 3 month(s) post-surgery by placing mice in a chamber (detailed in supplementary methods) and recording their movements over 5 minutes. The neurological outcome is expressed as the percentage of freezing time during a 5-minute period.

Cell Culture

Primary cultures of cortical neurons were prepared from fetal mice (E15 to E16) as described previously.5

In Vitro Excitotoxicity

Rapidly triggered excitotoxicity was induced, alone or in combination with rtPA (20 μg/mL) and/or αATD-NR1 or control Igs (0.01 mg/mL), by exposing neurons to 50 μm/L NMDA for 1 hour and transferring cells back to serum-free medium.19 Neuronal death was quantified 24 hours later by lactate dehydrogenase release (Roche).

Calcium Videomicroscopy

NMDA-evoked calcium influx in cultured cortical neurons was recorded by fura-2/AM (5 μmol/L) videomicroscopy and analyzed using Metafluor 4.11 software (Universal Imaging Corporation) as described earlier.5

Zymography Analysis for MMP-2 and MMP-9

Proteins (25 μg) extracted from cerebral hemispheres after ischemia were separated onto 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis containing gelatin (1 mg/mL). Gels were washed in 2.5% Triton X-100, incubated for 36 hours at 37°C, and gelatinase activity quantified after Coomassie blue staining.

Assay of BBB Permeability to Fluorescent Antibodies

αATD-NR1 (8 mg) were complexed (overnight, 4°C in a bicarbonate buffer, pH 8.3) with either Alexa-Fluor 555 or 680 (1 mg) and/or thrombin (0.75 UI, 1 μL) and/or αATD-NR1 or control Igs (0.01 mg/mL) videomicroscopy and analyzed using Metafluor 4.11 software (Universal Imaging Corporation) as described earlier.

Near-Infrared Fluorescence Imaging

Four hours after thromboembolic ischemia, saline or Alexa-labeled αATD-NR1 (160 μg per mouse) were intravenously injected. After 2 hours, mice (n=3 per group) were perfused with heparinized saline and brains were harvested and placed in the planar near-infrared fluorescence imaging system (Photon Imager; Biospace) for a first global picture acquisition. Then coronal brain slices of 1-mm thickness were cut in a matrix. Excitation wavelength was set to 650 nm with a 700-nm high-pass emission filter. Autofluorescence images were acquired using an excitation wavelength of 590 nm with the same emission filter. Fluorescence was quantified on the whole hemispheres using M3Vision software (Biospace).
Statistical Analyses
Results are the mean±SEM. Statistical analyses were performed by the Kruskall-Wallis test followed by post hoc comparison with the Mann-Whitney U test.

Results
Antibodies Against the ATD-NR1 Prevent rtPA-Promoted NMDAR-Mediated Neurotoxicity In Vitro
The aminoterminal domain of the NMDAR NR1 subunit (ATD-NR1) is the site of interaction with tPA, mediating increased NMDAR signaling and the attendant neurotoxicity.7 Accordingly, recombinant ATD-NR1 was used as an immunogen peptide in mice. The resultant purified polyclonal immunoglobulins (termed ATD-NR1) were then characterized and shown to recognize the NR1 subunit of murine and human neurons (Supplemental Figure I). The next step was to validate the ability of ATD-NR1 to prevent the aggravating effect of rtPA on NMDA toxicity.5 In cultured murine cortical neurons exposed to rapidly triggered excitotoxicity, the coapplication of ATD-NR1 prevented rtPA (0.3 μmol/L)-promoted neuronal loss (Figure 1A–B; P<0.01). Consistently, rtPA (0.3 μmol/L) increased NMDA-induced Ca2+ influx in cortical neurons by approximately 30%, an effect prevented by coapplying the ATD-NR1 (Figure 1C–D; P<0.05). The ATD-NR1 alone (0.01 mg/mL) did not influence NMDA-evoked neurotransmission (Figure 1D).

αATD-NR1 Improves Neurological Outcome, Protects the Brain Against Stroke, and Increases the Therapeutic Window of rtPA-Induced Thrombolysis
The therapeutic value of the ATD-NR1 was then investigated in vivo. The ability of antibodies to cross the BBB was investigated. Immunohistology on brain slices harvested 2 hours after an intravenous injection of Alexa555-labeled αATD-NR1 (160 μg) showed positive stainings in both the endothelial vascular wall and brain parenchyma (Figure 2A), arguing for a trans-BBB passage even under normal conditions. Accordingly, in a model of excitotoxicity with no BBB leakage and involving endogenous tPA,9 a single intravenous injection of αATD-NR1 (160 μg) reduced by 47% the lesion size induced by the striatal administration of NMDA compared with mice injected with control antibodies (n=8, P<0.01; data not shown). In sham-operated animals, the proportion of Alexa555-labeled αATD-NR1 (injected after 4 hours) transferred to the brain after 2 hours was 10.4%. Mice

Figure 1. αATD-NR1 prevent rtPA-promoted NMDA receptor-mediated neurotoxicity in vitro. A. Mixed cortical cultures were subjected to rapidly triggered excitotoxicity by exposure to NMDA (50 μmol/L) for 1 hour, alone or in the presence of rtPA (20 μg/mL) and/or the αATD-NR1 (0.01 mg/mL) or control Igs. Neuronal death was quantified after 24 hours (N=3 plates; n=12 wells; *P<0.01, ns indicates not significantly different). B. Illustrative bright field photomicrographs of MAP-2 immunostainings (same conditions as in A-B). Arrows indicate representative dying neurons. C–D. NMDA-evoked Ca2+ increase in cortical neurons measured by fura-2 videomicroscopy. Ci. A 30-second exposure to 25 μmol/L NMDA produced a transient increase in neuronal [Ca2+]i. rtPA treatment (20 μg/mL, 15 minutes) potentiated by 37% the NMDA-evoked Ca2+ influx (D; N=3; n=108; P<0.05). Cii, D. Coapplication of the αATD-NR1 (0.01 mg/mL) completely blocked this potentiating effect of rtPA (N=3; n=108; P<0.05), whereas control Igs (0.01 mg/mL; N=3; n=109; P>0.05) had no such effect Ciii, D). The αATD-NR1 alone (0.01 mg/mL) did not alter NMDA-induced neurotransmission (N=3; n=108; P>0.05). B. Black and white bars represent the 2 consequent stimulations with NMDA, respectively, before and after treatments. *P<0.05 before vs after treatment. NMDA indicates N-methyl-D-aspartate; rtPA, recombinant tissue-type plasminogen activator.
exposed to MCAO showed proportions of 22.8% and 31.8% in the ipsilateral hemisphere when the antibodies were injected 20 minutes and 4 hours post-MCAO, respectively (Figure 2B). Similar observations were obtained using near-infrared fluorescence imaging, showing an increased passage of Alexa630-labeled ATD-NR1 (160 μg, 4 hours post-MCAO) in the ischemic versus contralateral hemisphere (Figure 2C–D).

Because endogenously produced tPA is known to participate in the evolution of ischemic damages,6,16 the use of the ATD-NR1 as a standalone therapy was then tested in a model of thromboembolic stroke in mice. Both early (Figure 3A) and late (Figure 3B) deliveries of the ATD-NR1 alone (160 μg, intravenous, single bolus) conferred brain protection (44% and 41% smaller lesions compared with saline-injected controls when injected 20 minutes and 4 hours post-occlusion, respectively; P<0.001).

Regarding exogenous tPA, although early thrombolysis reduced ischemic lesions by 31.5%, this effect was not improved when rtPA was combined with immunotherapy (Figure 3A). When thrombolysis was performed 4 hours post-clot formation, rtPA still restored blood flow as efficiently as on early administration (Figure 3C), but this time with a significant 33% increase in brain lesion volumes (24.9±1.12 mm^3 for saline compared with 33.03±1.47 mm^3 for rtPA, P<0.002; Figure 3B). Interestingly, the deleterious effect of late rtPA administration was completely suppressed when combined with a single coinjection of ATD-NR1. Importantly, the beneficial effect of the late administration of the antibodies was comparable to that seen without rtPA or on early thrombolysis. This was evident from the resulting lesion volumes, which fell to 55.5% and 37.3% compared with those seen in animals receiving no treatment and only late rtPA, respectively (Figure 3B; P<0.005). Importantly, the coadministration of antibodies did not alter the ability of early or late rtPA injection to induce reperfusion (Figure 3C), consistent with their lack of influence on rtPA-promoted clot lysis in vitro (Supplemental Figure II).

Two further experiments demonstrated that the ATD-NR1 specifically targets the tPA/NMDAR interaction. Control lgs were ineffective against stroke damage (Figure 3A–B) and ATD-NR1 antibodies conferred protection in wild-type but not in tPA-deficient mice (Figure 3D). Confirming previous studies,6 absence of endogenous tPA en-
tailed a significant protection from thromboembolic brain injury. Altogether these data demonstrate two important features of the αATD-NR1: a single injection is sufficient to confer brain protection irrespective of its timing (20 minutes or 4 hours post-MCAO) due to the blockade of the deleterious effects of endogenous tPA and can suppress the injurious effects of rtPA when injected late after stroke onset and thus extends the therapeutic window of rtPA-induced thrombolysis.

Late Immunotherapy Targeting the ATD-NR1 Is Sufficient to Improve Neurological Outcome and Protects the Brain Against Stroke

The clinical relevance of targeting the potentiating effect of endogenous tPA on NMDAR neurotoxicity, even by a delayed immunotherapy, was further confirmed by an MRI-based longitudinal follow-up of animals treated 4 hours post-clot onset. ADC sequences, recorded 24 hours post-ischemia, as well as T2 MRI analyses (Figure 4A) confirmed late immunotherapy targeting the ATD-NR1 is sufficient to improve neurological outcome and protects the brain against stroke.

Figure 3. αATD-NR1 protect the brain against stroke and increase the therapeutic window of rtPA-induced thrombolysis. A–B. Effect of a single early (20 minutes, A) or late (4 hours, B) intravenous injection of αATD-NR1 (160 µg) on brain lesion volume after thromboembolic stroke in mice, with or without early or late rtPA (10 mg/kg; n=8 to 10 mice per group). Individual values are signified for each bar. *P<0.01 vs the corresponding control; †P<0.001 vs control mice (no rtPA, no antibodies); ‡A, P<0.005 vs control mice (no rtPA, no antibodies). ‡B, P<0.005 vs rPA alone. C. The normalized cerebral blood flow (CBF) measured by laser Doppler: no effect of αATD-NR1 on early (20 minutes) or late (4 hours) reperfusion by rtPA (n=8 to 10 per group, *P<0.01 vs the corresponding control). D. A single intravenous injection of αATD-NR1 (160 µg, 4 hours post-clot onset) reduced ischemic damage in wild-type C57Bl6 (WT) but not in tPA knockout (tPA−/−) mice (n=10 per group; †P<0.05 vs the corresponding control; ‡P<0.05 vs untreated WT). rtPA indicates recombinant tissue-type plasminogen activator.

Figure 4. αATD-NR1 confer long-term benefits after stroke. A. Mice were treated with saline or αATD-NR1 (160 µg), 4 hours post-ischemia (no rtPA treatment). At the indicated times, they were placed in a 7-Tesla MRI for T2 brain imaging and determination of the apparent diffusion coefficient (ADC) at 24 hours (representative images, n=3 per group). B, Lesion volumes (illustrated in A) were measured by MRI 24 hours post-clot onset (n=3 per group; *P<0.05 vs corresponding control). C. Mice were treated with saline or αATD-NR1 (160 µg) 4 hours after stroke and their neurological deficits were evaluated 24 hours, 30 days, and 3 months post-ischemia (n=10 per group; *P<0.05 vs corresponding treated control). rtPA indicates recombinant tissue-type plasminogen activator; MRI, magnetic resonance imaging.
the patterns of thionine stainings (45% reduction of the lesion volume 24 hours post-ischemia; \( P < 0.05 \); Figure 4B), brain protection being maintained for up to 1 week. Like in the clinical setting, we then investigated whether brain protection translated into improvement of neurological deficits. Stroke-induced neurological deficits evaluated at 24 hours were potently reduced by the intravenous administration of \( \alpha \)-ATD-NR1 20 minutes (Supplemental Figure III) or 4 hours \( (P < 0.05) \) post-MCAO. Providing further support for the clinical relevance of this treatment modality, this beneficial effect was maintained for at least 3 months (Figure 4C).

\( \alpha \)-ATD-NR1 Prevents rtPA-Induced Alterations of the Neurovascular Unit After Stroke

Because ischemic injury and rtPA’s beneficial/deleterious effects are highly interrelated with alterations of the BBB integrity, Evans blue extravasation and MMP activation were evaluated. On late administration, rtPA produced a significant extravasation of Evans blue into the brain parenchyma (Figure 5A). Accordingly, rtPA induced MMP-9 activity in the ipsilateral cortex, effects that were more pronounced \( (P < 0.01) \) after late (4 hours) than early (20 minutes) administration (Figure 5B). Consistent with the effects on ischemic lesions, both early and late administration of \( \alpha \)-ATD-NR1 alone reduced the extent of stroke-induced Evans blue extravasation \( (60\% \text{ versus clotted animals}; \ P < 0.05 \text{; Figure 5A}) \). Moreover, immunotherapy efficiently reduced the damaging effect of late rtPA administration on Evans blue extravasation \( (41\% \text{ versus the corresponding rtPA-treated animals}; \ P < 0.05 \text{; Figure 5A}) \). Similar effects were observed in the MMP-9 assays (Figure 5B). Indeed, compared with MCAO-only animals, reductions of 44% and 33% were obtained after early (20 minutes) and late (4 hours)
antibody post-clot injection, respectively. Compared with rtPA-treated animals, the respective decreases in the αATD-NR1/rtPA group were 57% and 65% (P<0.05). Similar observations were performed regarding MMP-3 levels (Supplemental Figure IV).

We further investigated whether the global beneficial effect of the αATD-NR1 could also affect the pro-PDGF-C/PDGF-CC/PDGF-RRα cascade.10 Immunoblots from ischemic cortices confirmed that rtPA treatment leads to huge increases of PDGF-CC levels (almost 8-fold when injected 4 hours post-MCAO). This stimulation was significantly antagonized (~40%) by the αATD-NR1 (Figure 5C). Similarly, rtPA increased the activity of PDGF-RRα after ischemia (+74% when injected 4 hours post-MCAO; Figure 5D). This increase was abolished by the αATD-NR1 and even reverted into a significant decrease compared with animals exposed to MCAO with no treatment (approximately ~75% versus MCAO alone and ~90% versus MCAO with tPA; P<0.05).

**Discussion**

Despite the beneficial results of the National Institute of Neurological Disorders and Stroke trial,1 stroke has remained a challenging clinical problem. Indeed, thrombolysis can be applied to few patients only, and improved treatment modalities for stroke are mandatory. The limitations of the use of rtPA are mainly due to nonfibrinolytic effects.12 Controlling the prohemorrhagic and proneurototoxic effects of tPA (endogenous and exogenous) is one of the most urgent issues in the field of stroke. We demonstrate that an antibody preventing the interaction of tPA with the NMDAR could be an ideal therapeutic candidate in ischemic stroke, alone or in combination with tPA.

Our aim was to assess whether targeting the deleterious effects initiated by the tPA/NMDAR interaction can afford brain protection. Central to our strategy was a dedicated thromboembolic model of stroke, in which we previously demonstrated that early rtPA-induced thrombolysis reduces ischemic lesions and cognitive deficits.15 We have further validated this model by showing that like in the clinical setting, brain protection is better the sooner rtPA-based thrombolysis is performed. The noxious effects of tPA, whether released from parenchymal cells and/or administered late after stroke, can largely be attributed to its potentiating effect on NMDAR activity.5,7 Although the mechanism has been debated, tPA is undoubtedly a positive modulator of the NMDAR-mediated glutamatergic signaling pathways in neurons,12–14 and possibly in endothelial cells.19 Based on our proposed mechanism of action, in which tPA directly (ie, with no requirement of plasminogen) interacts with the NMDAR NR1 subunit,7 we produced polyclonal antibodies recognizing the N-terminal end of NR1 in both rodents and humans. Importantly, these antibodies do not alter NMDA-induced neurotransmission (toxicity, calcium influx) but specifically target the effect of tPA on these receptors. We show in vitro that these antibodies limit rtPA-induced NMDA-evoked neuronal calcium overload and, by this, are highly effective against rtPA-dependent aggravation of excitotoxicity. This ability finds its expression in our in vivo investigations, which demonstrate that a single intravenous injection of the αATD-NR1 alone leads to massive neuroprotection, irrespective of the timing (20 minutes or 4 hours post-clot onset), whereas coadministration averts the deleterious effects of late rtPA administration, making late thrombolysis as safe as early reperfusion. Perhaps most important, clinically relevant criteria (MRI analysis and neurological scores) support the contention that αATD-NR1 cannot only confer brain protection but also improve long-term neurological outcome.

A growing body of evidence indicates that tPA is a central mediator of ischemia-induced increase in neurovascular unit permeability (and consequently edema and hemorrhagic transformation) in experimental models12 and possibly in thrombolysed stroke patients.20 We demonstrate that the αATD-NR1 cause beneficial changes in events considered as hallmarks of BBB disruption, that is, BBB permeabilization, MMP-3 and MMP-9 activation, and PDGF-RRα activation by PDGF-CC, irrespective of being caused by MCAO alone (ie, mediated by endogenous tPA) or by late administration of rtPA. These observations strengthen the therapeutic interest of the αATD-NR1, because brain edema and bleeding are associated with a poor clinical outcome in patients with stroke.1 They also support the view that parenchymal damages are at the origin of (or at least contribute to) BBB leakage, tPA, and other mediators being released from stressed neurons to promote harmful events at the BBB.17

Targeting the brain with antibodies has been the subject of question of feasibility and major safety concerns. However, consistent with previous observations,21–25 we show that low levels of antibodies can enter the brain parenchyma in the absence of injury and that the passage of the BBB is increased under ischemic conditions. Moreover, our antibodies do not induce encephalopathy or cognitive, behavioral, or mnesic deficits (not shown). This last observation rules out the possibility that the protective neurological effect induced by the αATD-NR1 after stroke could be due to a downregulation of the inhibitory neurotransmission. Moreover, αATD-NR1 is highly specific of their target, because it does not mediate protection in tPA-deficient mice and does not alter the antiprotective effect of tPA (data not shown), which is independent on the interaction with NMDAR.26 The necessity for a more specific NMDAR blockage27 lends greater weight to our antibody-based strategy, because it only targets a neuromodulatory rather than a whole neurotransmission system.28

In line with ongoing experimental strategies targeting undesirable rtPA functions to optimize stroke treatment,5,29,30 we demonstrate that this immunotherapy targeting the ability of endogenous tPA to potentiate NMDAR signaling reduces excitotoxic damages, BBB leakage, and the attendant brain injury and neurological deficits. We also provide proof of concept for the use of these antibodies as an adjunct to rtPA-based thrombolysis with the potential to antagonize brain and neurovascular unit damage and, by this, to extend the therapeutic window.

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Disclosures
None.

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Supplemental methods

**Protein extraction:** Ice-cold TNT buffer (50mmol/L Tris-HCl pH 7.4; 150mmol/L NaCl; 0.5% Triton X-100)-dissociated tissues were centrifuged (10,000g, 4°C, 15 min), and protein content assessed by the BCA method (Pierce, France).

**Immunoblotting:** Proteins (20µg) were separated by 10% SDS-PAGE (15% for PDGF-C) and transferred onto a PVDF membrane. Membranes were blocked with TBS (10mmol/L Tris; 200mmol/L NaCl; pH 7.4) containing 0.05% Tween-20, 5% dry milk, and incubated overnight at 4°C with primary antibodies: our mouse αATD-NR1 or control Igs (1:2000), a goat anti-C-ter-NR1 (1:200; Santa Cruz, Germany), a goat anti-histidine (1:1000; Qiagen, France) or a goat anti-PDGF-C (1:800; R&D system, France). After incubation with the appropriate peroxydase-conjugated secondary antibodies (1:5000), proteins were visualized with an enhanced chemiluminescence ECL-Plus detection system (Perkin Elmer-NEN, France).

**Immunoprecipitation:** Supernatants from TNT-lysed tissues were incubated overnight at 4°C with the mouse monoclonal antibody anti-pTyr (1:200; Santa Cruz Biotechnology, Germany) or with a goat polyclonal antibody anti-MMP3 (1:200, Abcam, France). Samples were then coupled to Protein G-Sepharose beads as described by the manufacturer (GE-Healthcare, France). Proteins were separated by 6% SDS-PAGE (for PDGFRα experiment) or 10% SDS-PAGE (for MMP3 experiment), blots were exposed with a goat anti-mouse PDGFRα primary antibody (1:800; 4°C, overnight, R&D system, France) or mouse anti-goat MMP3 primary antibody (1:800, 4°C, overnight, Abcam, France) and revealed following the procedure described above.

**Immunohistology:** For immunohistochemistry studies of the passage of antibodies through the BBB, mice were injected i.v. with 160µg of Alexa<sup>555</sup>-αATD-NR1, 20min or 4h after thromboembolic ischemia. After 2h, mice were perfused with heparinised saline and fixed with PBS
0.1mol/L, pH 7.4, containing 2% paraformaldehyde and 0.2% picric acid. Brains were harvested and cut in 10μm-thick slices. Slices were incubated with a goat antibody raised against type IV collagen (1:800, Abcam, France) before an overnight incubation with the secondary antibodies, F(ab’)2 fragments of donkey anti-rabbit IgG linked to FITC (1:300, Jackson ImmunoResearch, PA, USA). Slices were then cover-slipped with antifade medium containing DAPI. All sections were examined with a Leica DM6000 microscope. Images were digitally captured using a coolsnap camera and visualized with Metavue software (n=3). The proportion of passage of the antibodies was calculated by transforming the fluorescence signal measured in the tissue into the corresponding dose of antibody (over a standard curve), and then normalizing to the total dose of antibody injected.

**Immunocytochemistry:** Neuronal cultures (on 24 wells plates or glass bottom Petri dishes; MatTek corporation, MA, USA) were fixed with 4% of paraformaldehyde in PBS (0.1M, pH 7.4) and incubated in the presence of mouse αATD-NR1 (1:2000), goat anti-NR1 (1:500; Santa Cruz Biotechnology, Germany), or chicken anti-MAP2 (1:2000, Abcam, France) polyclonal antibodies. For glass bottom Petri dishes, secondary antibody F(ab’)2 fragments of donkey anti-Goat IgG linked to TRITC (1:300, Jackson ImmunoResearch, PA, USA) were incubated overnight and images were digitally captured using a Nikon Eclipse (TE2000-E) inverted confocal microscope equipped with an oil immersion Nikon x60 objective. For 24 well plates, chicken peroxydase-conjugated secondary antibodies (1:5000) were incubated overnight and revealed with a chromogenic substrate (diamino-benzidine). Pictures were obtained with a bright field camera.

**Immunoblotting:** Proteins (50μg) were separated by 5-10% SDS-PAGE and transferred onto a PVDF membrane. Membranes were blocked with TBS containing, 5% dry milk, and incubated overnight at 4°C with primary antibodies: our mouse αATD-NR1 (1:200), a goat anti-C-ter-NR1 (1:200; Santa Cruz, Germany). After incubation with the corresponding
secondary peroxydase-conjugated streptavidine reagent antibodies (1:4000), proteins were visualized with an enhanced chemiluminescence ECL-Plus detection system (Perkin Elmer-NEN, France).

**Human tissues experiments:** Brain biopsies were harvested from a total of 5 deceased patients (3 women and 2 men) who had been admitted to the neurovascular unit of the Vall d’Hebron hospital (Barcelona) within the previous 2-4 days. All samples were obtained within the first 6h after death and snap frozen in liquid nitrogen and stored at -80°C for Western blot or fixed with 4% paraformaldehyde for immunohistochemistry techniques. This study was approved by the local Ethics Committee and conducted in accordance with the Declaration of Helsinki. All relatives gave written informed consent before the autopsy. Studies were performed on the non ischemic hemisphere. **Immunoblotting:** Proteins (50µg) were separated by 5-10% SDS-PAGE and transferred onto a PVDF membrane. Membranes were blocked with TBS containing, 5% dry milk, and incubated overnight at 4°C with primary antibodies: our mouse αATD-NR1 (1:200), a goat anti-C-ter-NR1 (1:200; Santa Cruz, Germany). After incubation with the corresponding secondary peroxydase-conjugated streptavidine reagent antibodies (1:4000), proteins were visualized with an enhanced chemiluminescence ECL-Plus detection system (Perkin Elmer-NEN, France). **Immunohistology:** For immunohistochemistry, brains were harvested and cut in 12µm-thick slices. Slices were incubated with the mouse αATD-NR1 antibody (1:50) and a chicken anti-MAP2 antibody (1:300) before an overnight incubation with the secondary antibodies, F(ab’2) fragments of donkey anti-mouse IgG linked to TRITC or anti-chicken IgG linked to FITC. Before a cover-slipped with antifade medium containing DAPI all slices were treated with Sudan Black B to avoid autofluorescence.

**MRI analyses:** MRI experiments were carried out on a Pharmascan 7 T/12 cm system with Paravision 4.0 software (Bruker, Germany) using a 72mm inner diameter birdcage for radio frequency transmission and a 25mm diameter surface coil for reception. During the MRI
experiments, anaesthesia was maintained using isoflurane (70%/30% mixture of NO₂/O₂). Mice were monitored for changes in their respiratory rate in order to adjust the anaesthetic concentration. T2-weighted images were acquired using a RARE sequence: TE/TR 41/3000 ms with 4 averages (Matrix 256x256, FOV 23.7x20mm). Diffusion-weighted images were acquired with a 6 directions EPI-DTI sequence: TE/TR 34/3750 ms using two b values (0 and 800 s/mm²) with 2 experiments for each direction (Matrix 128x128, FOV 20.5x19.2mm). A different sequence of DWI was used: High resolution diffusion-weighted images were obtained with a standard spin echo imaging modified with a Stejskal-Tanner gradient scheme. Parameters were set as follows: TR/TE 2500ms/33ms, matrix size of 256x256 giving an in-plane resolution of 78µm x 78µm, slice thickness of 0.5mm, one direction diffusion gradient (in the frequency encoding direction) with a b factor of 1000 s/mm². Apparent diffusion coefficients (ADCs) in mm²/s were calculated by pixel-by-pixel curve fitting using a monoexponential model. Angiographies were acquired with TE/TR 12/7 ms with 2 averages (Matrix 256x256, FOV 20x20mm).

**Behavioural analyses:** Behavioural assessments were performed in a chamber (67X53X55 cm, BIOSEB®, France) constructed from black methacrylate walls and a Plexiglas front door. The floor consisted of 22 stainless steel bars (3mm in diameter, spaced 1.1cm center-to-center). The signals generated by the mouse movements were recorded and analyzed through a high sensitivity weight transducer system. The analog signal was transmitted to the Freezing software module through the load cell unit for recording and posterior analysis in activity/immobility (Freezing).

**Euglobulin clot lysis time:** Human plasma was collected and the euglobulin fraction, containing β and γ-globulins, was separated by dilution of one volume of chilled anticoagulated plasma in 20 volumes of chilled acetic acid 2.9mM. After 15min at 4°C and centrifugation at 3000g for 10min, the euglobulin fraction was precipitated and resuspended
in HEPES buffer (10mmol/L HEPES pH=7.4, NaCl 150mmol/L). The clot was initiated by addition of 15mmol/L calcium chloride. Then, rtPA (60 ng/mL) alone or with increasing concentrations of αATD-NR1, control antibody, and vehicle were added to the reaction mix at 37°C (quantity ratio antibody:tPA spreading from 1:1 to 1000:1). Absorbance was read at 405nm. Tests were performed in triplicate. For each condition, the time to complete clot lysis was measured and normalized to the condition tPA alone.
Supplemental figure 3

Drug i.v., 20 minutes post-MCAO
Supplemental figure 4

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Supplemental figure legends

Figure S1: **αATD-NR1 interact with the Amino-Terminal Domain of the murine and human NMDA receptor NR1 subunit.**

(A) Postulate: by preventing the binding of (r)tPA to the ATD domain of the NMDA receptor NR1 subunit, the αATD-NR1 should prevent the potentiating effect of tPA on NMDA receptor signalling. (B) Representative immunoblots (IB, 3 independent experiments) showing the ability of αATD-NR1 to recognize the immunogenic peptide (recombinant polyhistidin-tagged N-terminal domain of NR1, rATD-His). Control IgGs gave no signal. Protein extracts from (C) murine cultured neurons, (D) mouse brain and (E) post-mortem human brain biopsies, were resolved by SDS-PAGE and immunoblots were performed with either the αATD-NR1 or a commercially available anti-human C-ter-NR1 antibody. Both antibodies recognize the same protein at 120kDa in mice and 150kDa in humans (representative IB, 3 independent experiments). Nota: double bands represent differentially glycosylated forms of NR1. (F) Immunofluorescence stainings of murine cultured neurons with a commercially available anti-human C-ter-NR1 (red) or with αATD-NR1 (red) antibodies. MAP-2 (green) was used as a neuronal marker. (G) Immunofluorescence staining of human brain tissue with the αATD-NR1 (red) confirms that this antibody can recognize human neurons (arrows; see also stainings around vessels in the box).

Figure S2: **αATD-NR1 do not influence clot lysis by rtPA in vitro.**

A time to clot lysis assay showed that even high concentrations of the αATD-NR1 do not affect rtPA-induced clot lysis (n=3 per group).

Figure S3: **αATD-NR1 confer neurological benefits after stroke.** Mice were treated with αATD-NR1 (160µg) 20 minutes after stroke and were placed in a fear-conditioning room 24 hours post-clot onset. The neurological outcome is expressed as the percentage of freezing time during a 5 min period (n=10 per group); *, significant difference between the MCAO
and treated groups by the Kruskall–Wallis’ test, followed by post hoc comparisons, with the Mann–Whitney’s test with p<0.05.

Figure S4: \textit{αATD-NR1 prevent rtPA-induced activation of MMP-3 after stroke.} MMP-3 activation after MCAO in Swiss mice 24h post clot onset, 20 hours after injection of \textit{αATD-NR1} (160µg per mouse). Brains were solubilised and lysed with detergent. The lysates were immunoprecipitated with antibodies specific for MMP-3, followed by immunoblotting with MMP-3 specific antibodies.