Protease-independent action of tissue plasminogen activator in brain plasticity and neurological recovery after ischemic stroke

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Emerging evidence suggests that tissue plasminogen activator (tPA), currently the only FDA-approved medication for ischemic stroke, exerts important biological actions on the CNS besides its well-known thrombolytic effect. In this study, we investigated the role of tPA on primary neurons in culture and on brain recovery and plasticity after ischemic stroke in mice. Treatment with recombinant tPA stimulated axonal growth in culture, an effect independent of its protease activity and achieved through epidermal growth factor receptor (EGFR) signaling. After permanent focal cerebral ischemia, tPA knockout mice developed more severe sensorimotor and cognitive deficits and greater axonal and myelin injury than wild-type mice, suggesting that endogenously expressed tPA promotes long-term neurological recovery after stroke. In tPA knockout mice, intranasal administration of recombinant tPA protein 6 hours poststroke and 7 more times at 2 d intervals mitigated white matter injury, improved axonal conduction, and enhanced neurological recovery. Consistent with the proaxonal growth effects observed in vitro, exogenous tPA delivery increased poststroke axonal sprouting of corticobulbar and corticospinal tracts, which might have contributed to restoration of neurological functions. Notably, recombinant mutant tPA-S478A lacking protease activity (but retaining the EGFR-like domain) was as effective as wild-type tPA in rescuing neurological functions in tPA knockout stroke mice. These findings demonstrate that tPA improves long-term functional outcomes in a clinically relevant stroke model, likely by promoting brain plasticity through EGFR signaling. Therefore, treatment with the protease-dead recombinant tPA-S478A holds particular promise as a neurorestorative therapy, as the risk for triggering intracranial hemorrhage is eliminated and tPA-S478A can be delivered intranasally hours after stroke.

Significance

Therapies that boost brain plasticity and repair processes without deleterious side effects have the potential to transform the clinical management of stroke. Here we report that tissue plasminogen activator (tPA), known as a clot buster for clinical treatment of ischemic stroke, exerts additional neurorestorative effects in a murine model of stroke by stimulating axonal regeneration and facilitating functional recovery. Importantly, functional restoration was independent of tPA’s proteolytic activity—a mutant, protease-inactive form of tPA, tPA-S478A, was as effective as regular tPA. Results of our preclinical studies support the view that tPA-S478A has the therapeutic potential to enhance brain regeneration and functional restoration in stroke victims. Unlike regular tPA, tPA-S478A does not bear the risk of inducing intracerebral hemorrhage.

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protective effects of tPA against excitotoxicity and ischemic injury in neurons and oligodendrocytes (12, 13). In recent years, the long-term influence of tPA on CNS repair and plasticity after injury has begun to be elucidated. For example, tPA stimulates axonal sprouting at chronic stages after ischemic stroke (14), traumatic brain injury (15), and spinal cord injury (16, 17), and these salutary effects correlate with improvements in neurological functions. However, whether tPA plays an indispensable role in poststroke functional recovery and the underlying mechanisms warrant further investigation. Furthermore, even if tPA does boost brain plasticity and repair, safety concerns regarding intracranial hemorrhage in stroke patients surely would hamper the use of tPA as a neuroprotectant beyond its FDA-approved therapeutic timeframe.

To address the aforementioned gaps in the field, we investigated the role of tPA in long-term neurological outcomes after ischemic stroke and the underlying mechanisms. Our results show that tPA enhances brain plasticity and functional recovery even when delivered at 6 h after stroke onset and 7 more times at 2 d intervals, in a clinically relevant mouse model of permanent focal cerebral ischemia. Importantly, such restorative effects are independent of tPA’s proteolytic functions. Protease-inactive mutant tPA demonstrates efficacy comparable to that of wild-type (WT) tPA, but without the risk of inducing intracranial hemorrhage.

**Results**

**tPA Stimulates Axonal Growth Independent of Its Protease Activity.**

The functional restoration of neural circuits after brain injury is dependent upon axonal sprouting and the plasticity of pyramidal tracts (18). To evaluate the effects of tPA, we quantified the length of axons immunolabeled by tau in cultured primary cortical neuron cultures (SI Appendix, Fig. S1). Recombinant tPA protein stimulated axonal outgrowth in a concentration-dependent manner, with median axonal length peaking at 10 ng/mL, as determined after 24 and 48 h of incubation (SI Appendix, Fig. S1 B and C). Next, we subjected the neurons to an ischemia-like insult, oxygen–glucose deprivation (OGD), which induced cell death and axonal shortening (SI Appendix, Fig. S1 D–G). After 60 min of OGD and 48 h of reperfusion, axonal length was 25% shorter than in non-OGD controls (median: 31.1 μm vs. 20.4 μm; P < 0.001). However, axons were longer after incubation with recombinant tPA for 48 h beginning at the onset of post-OGD reperfusion compared with post-OGD axons treated with vehicle (Fig. 1 A–C). At a concentration of 10 ng/mL, tPA resulted in axonal length 97.0% greater compared with vehicle treatment after OGD (median: 135.4 μm vs. 68.7 μm; P < 0.001). tPA protected axons after OGD without affecting cell

![Image of axonal growth](https://www.pnas.org/doi/10.1073/pnas.1821979116)
viability, according to two independent cell viability/death assays (SI Appendix, Fig. S1G).

The effects of tPA on axonal outgrowth in the CNS have been ascribed to proteolytic breakdown of extracellular matrix (ECM) molecules, which establishes a permissive microenvironment for axonal elongation and regeneration after injury (16, 19, 20). However, it is unknown whether tPA still retains such proaxonal growth effects in the absence of its proteolytic activities. To address this question, we created a mutant tPA protein bearing a single amino acid substitution (S478A) in the catalytic domain, which completely abolishes its serine protease activity (21).

Interestingly, tPA-S478A demonstrated positive effects on axonal protection and axonal growth in cultured neurons similar to those of WT tPA, under OGD and non-OGD conditions, respectively (Fig. 1 D–F), suggesting that the effect of tPA on axons is independent of its proteolytic activity. The impact of tPA and tPA-S478A on axonal length was further determined in a microfluidic device (Fig. 1G and SI Appendix, Fig. S2). In this two-chambered setup connected by microgrooves, tau-immunopositive axons of neurons placed in one chamber traverse microgrooves into a second chamber, whereas microtubule-associated protein 2 (MAP2)-immunopositive cell dendrites fail to reach the second chamber due to the length of the microgroove barricade (22). We found that 96 h of tPA treatment markedly increased the length of axons both under OGD and control non-OGD conditions (Fig. 1H). Furthermore, tPA-S478A exerted similar enhancing effects to WT tPA on axonal length in the microfluidic device. Collectively, these data demonstrate that tPA administered at nanomolar concentrations can promote axonal growth under physiological conditions and protect against deleterious effects of OGD on axonal length, respectively; such effects were independent of its proteolytic activity and without an impact on cell survival.

tPA Promotes Axonal Outgrowth Through EGF Receptor Signaling. We further investigated the mechanism underlying the proaxonal growth effects afforded by tPA. tPA is a multidomain protein and exerts cytokine and growth factor-like functions beyond its protease role (1). Specifically, the EGF-like domain of tPA is homologous to EGF, a potent stimulator of cell proliferation. Therefore, we tested the hypothesis that tPA stimulates axonal growth by activating EGF receptor (EGFR)-dependent signaling through its EGF-like domain. Indeed, robust phosphorylation of EGFR was detected in cultured neurons upon treatment with 10 ng/mL of tPA for 24 h (Fig. 2A), indicating receptor activation. Consistent with these findings, we observed activation (via increased phosphorylation) of several mitogen-activated protein kinases (MAPKs) in tPA-treated neurons, including ERK1/2, p38, and JNK (Fig. 2A), which are known downstream effectors of EGFR activation. The protease-inactive tPA-S478A showed similar magnitudes to WT tPA in activating EGFR–MAPK signaling in neurons (Fig. 2A). Furthermore, the phosphorylation of EGFR, ERK1/2, p38, and JNK was reduced in tPA or tPA-S478A–treated neurons by the EGFR inhibitor AG1478 (Fig. 2B and C), supporting the specific activation of EGFR-dependent signaling cascades. To test whether tPA-induced axonal elongation was achieved through EGFR signaling, we measured axonal length in neurons treated with 0–200 nM AG1478. The results showed that AG1478 did not alter axonal length under control conditions without tPA treatment (Fig. 2D and E).

Importantly, AG1478 inhibited tPA- or tPA-S478A–induced axonal outgrowth in a concentration-dependent manner; 200 nM of AG1478 reduced the median axonal length by 29.4% and 18.6% after tPA or tPA-S478A treatment, respectively (Fig. 2D and E; P < 0.001 vs. control). In summary, these data strongly support our hypothesis that tPA stimulates axonal growth through EGFR signaling in vitro.

Deletion of Endogenous tPA Worsens Long-Term Neurological Deficits After Ischemic Stroke. Given the potent stimulating effects of tPA on axonal growth under ischemic conditions, tPA may be involved in postischemic brain repair and long-term functional recovery. Therefore, we examined whether endogenous tPA plays an essential role in long-term neurological recovery after ischemic stroke by using tPA knockout (KO) mice. Permanent focal cerebral ischemia was induced in tPA KO mice and age- and weight-matched WT control mice by unilateral occlusion of the left distal middle cerebral artery (dMCAO) (SI Appendix, Fig. S3A), a model that reproducibly elicits cortical infarction and long-term neurological deficits (23). WT and tPA KO mice showed comparable reductions of cortical cerebral blood flow (CBF) as determined 30 min after dMCAO (SI Appendix, Fig. S3B) and semiquantitative data on phospho-EGFR and phospho-ERK1/2 levels. Shown are representative blots and densitometry data. Error bars represent the mean ± S.D. (n = 6–8 independent experiments). *P < 0.05, **P < 0.01, ***P < 0.001 vs. vehicle. **P < 0.05, ***P < 0.01 vs. non-AG1478 control. (D and E) Primary neurons were incubated with indicated concentrations of AG1478 for 1 h, followed by 10 ng/mL of tPA or tPA-S478A treatment for 48 h. Axon length was measured on tau-immunostained cells. (D) AG1478 reduced tPA and tPA-S478A–induced axonal outgrowth in a concentration-dependent manner. (E) Histograms show that 200 nM of AG1478 shifted the frequency distribution of axonal length to the left in tPA- and tPA-S478A–treated neurons, but not in vehicle-treated neurons. n = 99–104 neurons from three independent experiments. **P < 0.01, ***P < 0.001 vs. 0 nM.
dMCAO (SI Appendix, Fig. S3 D and E), *tPA KO mice showed significantly poorer motor recovery after dMCAO compared with WT mice (Fig. 3E). Furthermore, dMCAO-induced forelimb use asymmetry in the cylinder test was markedly exacerbated in tPA KO animals (Fig. 3F). In the hanging wire test, tPA KO mice demonstrated greater motor deficits than WT mice on days 1 and 3 after dMCAO (Fig. 3C); however, all groups of mice recovered to prestroke levels by 14 d after dMCAO.

We further assessed ischemia-induced long-term spatial learning and memory deficits using the Morris water maze test at 22–27 d after dMCAO (Fig. 3D). dMCAO did not induce statistically significant deficits in spatial learning or memory in WT mice. Notably, dMCAO tPA KO mice demonstrated prominent deficits in spatial learning (Fig. 3E). In the memory test dMCAO tPA KO mice were marginally worse than dMCAO sham mice (Fig. 3F), but that may be ascribed to their poorer learning. All mice displayed similar gross motor skills, as reflected by similar swimming speeds across all experimental groups (Fig. 3G).

Together, these results support a significant role of endogenous tPA in long-term recovery of sensorimotor functions and spatial learning capability after ischemic stroke; the recovery can occur despite insignificant protection against macroscopic gray matter loss.

tPA Knockout Exacerbates White Matter Injury After Ischemia. To determine the pathological basis underlying the exacerbation of neurological outcomes in tPA KO stroke mice, we assessed white matter integrity at 35 d after dMCAO using ex vivo diffusion tensor imaging (DTI) and immunohistochemistry. dMCAO reproducibly resulted in ischemic lesions in subcortical white matter, including the corpus callosum (CC) and external capsule (EC) (23), which were detected by both DTI and immunohistochemistry (Fig. 4A). On the directionally encoded color (DEC) maps, distortion of white matter tracts was observed in the ipsilesional EC in both WT and tPA KO mice (Fig. 4A). White matter integrity was further quantified by measuring fractional anisotropy (FA), axial diffusivity (AD), and radial diffusivity (RD) values, which reflect overall white matter integrity, axonal degeneration, and demyelination (24). Despite the DEC maps showing visible white matter fiber distortion in the ipsilesional EC, no significant differences were found in FA, AD, and RD values between ipsilesional and noninjured contralesional hemispheres for this region of interest in WT mice (Fig. 4B). However, this white matter degeneration was greater in tPA KO mice, as manifested in a robust decline of FA values and an increase of RD values in the ipsilesional CC and EC, compared with the noninjured contralesional side or WT mice (Fig. 4B). These results demonstrate that endogenous tPA deficiency exacerbates ischemia-induced long-term white matter degeneration, which likely encompases damage to the myelinated fibers.

In accordance with the DTI findings, immunofluorescent labeling of myelin basic protein (MBP) at 35 d after dMCAO revealed similar injury patterns in the ipsilesional CC and EC areas (Fig. 4C). The thickness of the ipsilesional CC and EC was measured using immunochemistry (Fig. S4 A and B), resulting in a markedly increased SMI-32/MBP ratio, an established indicator of white matter injury (Fig. 4D), suggesting that tPA KO mice lost more white matter adjacent to the cortical lesion. Furthermore, we assessed changes within the ipsilesional CC, EC, and cortex by dual immunostaining for MBP and SMI-32 (a marker for demyelinated axons; Fig. 4E) (25). dMCAO decreased MBP and increased SMI-32 immunofluorescence in the CC/EC and cortex (Fig. 4E and SI Appendix, Fig. S4 A and B), suggesting that tPA KO mice lost more white matter after ischemic stroke than WT mice.

Poststroke Intranasal tPA Administration Improves White Matter Integrity in tPA Knockout Mice. Our data thus far indicate that endogenous tPA contributes to the maintenance of white matter integrity after ischemia. We next investigated whether exogenously supplied tPA could rescue the white matter deterioration in tPA KO stroke mice. To this end, mice were subjected to dMCAO, and recombinant tPA protein was administered intranasally to anesthetized mice at a dose of 2 mg/kg. The first dose was given 6 h after stroke onset, followed by administrations once every 2 d for 14 d. This treatment regimen was designed according
At 35 d after dMCAO, assessment of MAP2-immunostained coronal brain sections showed no statistically significant difference in cortical neuronal tissue loss between tPA- and vehicle-treated tPA KO mice (SI Appendix, Fig. S5). However, white matter integrity was significantly improved in tPA KO mice by intranasal tPA treatment. In the ipsilesional CC, EC, and cortex, vehicle-treated tPA KO mice demonstrated worse white matter injury than vehicle-treated WT mice, as shown by reduced MBP but increased SMI-32 immunofluorescence (Fig. 5A and B and SI Appendix, Fig. S4C). Strikingly, intranasal tPA treatment substantially reduced SMI-32 expression and/or preserved MBP expression in tPA KO mice, indicating the improvement of white matter integrity (Fig. 5A and B). Consistent with these immunohistochemical findings, Western blotting on protein extracts from the ipsilesional CC and EC at 35 d after dMCAO or sham operation revealed that ischemia-induced loss of MBP was rescued by tPA treatment in tPA KO mice (Fig. 5C and D and SI Appendix, Fig. S6B). Immunoreactivity for neurofilament heavy chain (NF200), another marker for axonal integrity, was also restored in tPA KO mice by tPA treatment (Fig. 5C and D), suggesting that recombinant tPA ameliorated axonal injury. We counted the number of viable oligodendrocytes labeled by adenomatous polyposis coli (APC) in the ipsilesional CC and EC at 35 d after dMCAO (Fig. 5E and F and SI Appendix, Fig. S6C). There were significantly fewer viable oligodendrocytes in tPA KO mice than in WT mice, which was partially rescued in the EC by intranasal delivery of tPA.

In addition to the structural examinations of white matter, we determined white matter functional integrity by measuring the transmission of action potentials in the ipsilesional CC and EC (Fig. 5G). Evoked compound action potentials (CAPs) reflect axonal conduction in the white matter and typically yield a multiphasic waveform consisting of an initial segment (N1, represents faster conducting myelinated axons) followed by a second segment (N2, represents more slowly conducting axons) (26). In both vehicle-treated WT and tPA KO mice, dMCAO decreased the amplitude of the N1 segment but not the N2 segment compared with sham control mice (Fig. 5H and SI Appendix, Table S2), suggestive of damage to the white matter that mainly encompasses myelinated axons. Notably, intranasal tPA treatment after dMCAO elevated the N1 amplitudes in tPA KO mice to the levels in vehicle-treated WT mice, but tPA treatment had no statistically significant impact on the N2 amplitude (Fig. 5H).

Summary, these data indicate that poststroke administration of exogenous tPA not only promotes white matter structural integrity, but also improves nerve impulse transmission through white matter, especially by faster conducting fibers.

**Poststroke Intranasal tPA Administration Alleviates Long-Term Neurological Deficits in tPA Knockout Mice.** We determined whether exogenously delivered recombinant tPA protein was capable of improving long-term neurological recovery after dMCAO in tPA KO mice, using the same intranasal treatment paradigm as above. Indeed, intranasal tPA treatment significantly promoted sensorimotor recovery for at least 35 d after dMCAO compared with vehicle treatment in tPA KO mice, as shown by both the rotarod and cylinder tests (Fig. 6A and B). In the foot fault test, which assesses sensorimotor coordination during spontaneous locomotion, tPA KO stroke mice treated with tPA performed as well as vehicle-treated WT stroke mice, without any change to what can be readily manageable in clinical settings and taking into consideration the potential beneficial effects of tPA on brain repair at chronic stages after ischemic injury. Vehicle control mice received the same anesthesia and an equal volume of sterile PBS treatment. In a pilot experiment, we verified successful delivery of tPA into the brain after intranasal administration in tPA KO mice. In the absence of background endogenous tPA, intranasal delivery resulted in detectable concentrations of tPA or tPA-S478A within 0.5 h in various brain regions, including the cortex, striatum, and hippocampus (SI Appendix, Fig. S5).

Fig. 4. Ablation of endogenous tPA worsens white matter injury after ischemic stroke. WT and tPA KO mice were subjected to dMCAO or sham operation, and white matter structural integrity was assessed after 35 d by ex vivo DTI and immunofluorescent staining of MBP and abnormally dephosphorylated filaments (SMI-32). (A) Shown are representative DEC maps from DTI and MBP immunofluorescence in coronal brain sections captured 0.62 mm anterior to bregma. dMCAO induced distortion of white matter fiber tracts on DEC maps and reduced MBP immunosignals in the ipsilesional EC (arrowhead). The color coding for DEC maps, which report the orientation of the primary eigenvector is: red, left/right; green, anterior/posterior; and blue, dorsal/ventral. Dashed rectangles demarcate the area of enlargement of the ipsilesional (IL) and contralateral (CL) CC and EC in the second row. (B) The average values of FA, AD, RD, and RD were quantified in the EC from five axial brain slices encompassing the ischemic lesion to assess changes in white matter integrity. n = 6 mice per group. ***P < 0.01, ****P < 0.001. (C) Images of MBP immunofluorescence (green) illustrate the morphology of the ipsilesional CC and EC. Dashed lines show the boundaries of CC/EC, cg, cingulum; LV, lateral ventricle. (D) Measurements of the thickness of the ipsilesional CC and EC at 20 equally spaced intervals (0.15 mm each) from the midline reveal greater atrophy of white matter in the EC of tPA KO mice. n = 10 mice per group. *P < 0.05 KO vs. WT. (E) Double-label immunofluorescence of MBP and SMI-32 in the ipsilesional CC/EC and cortex. Dashed lines illustrate the border of the CC/EC. (F) The ratio of SMI-32 to MBP fluorescence intensities was calculated, and an increase in this ratio is an indicator of white matter injury. n = 6 mice per sham group. n = 10 mice per dMCAO group. ***P < 0.001. (Scale bars: 1 mm in A; 400 μm in C; 50 μm in E.)
in total steps (SI Appendix, Fig. S7 A and B). In the Morris water maze test, vehicle-treated tPA KO stroke mice performed significantly worse than vehicle-treated WT stroke mice in both spatial learning and memory phases (Fig. 6 C–F). Moreover, tPA treatment improved spatial learning 25–26 d after dMCAO and spatial memory 27 d after dMCAO in tPA KO mice (Fig. 6 C–F). Further analyses on the same cohort of mice undergoing neurobehavioral tests and immunohistochemical assessments revealed a robust inverse correlation between the white matter injury marker (SMI-32/MBP immunofluorescence ratio) in the ipsilesional EC and cortex and the neurological function parameter (latency to fall in the rotarod test) at 35 d after dMCAO (SI Appendix, Fig. S7 C).
matter functionality (represented by the CAP amplitudes) was also statistically correlated with performance in the rotarod test (SI Appendix, Fig. S7D). In summary, these data demonstrate that intranasal tPA treatment promotes long-term neurological recovery after ischemia in tPA KO mice, very likely contributed by improvements in white matter integrity.

Exogenous tPA Enhances CNS Plasticity After Stroke in tPA Knockout Mice. Axonal sprouting is an important mechanism underlying neural plasticity and functional restoration in poststroke brain (27, 28). In our in vitro models, we observed that tPA potently promoted axonal growth under both physiological and ischemia-mimicking conditions. To test whether tPA influences axonal sprouting after ischemia in vivo, we assessed the plasticity of intracortical connections and the corticobulbar and corticospinal tracts in WT and tPA KO mice after dMCAO. The tract-tracer biotinylated dextran amine (BDA) was injected into the contralateral (right) motor cortex 21 d after dMCAO or sham surgery and the mice were then allowed to survive for an additional 14 d (Fig. 7A). BDA is anterogradely transported along the pyramidal tracts. The sprouting of BDA+ axons across the midline into the denervated facial nucleus and spinal cord (Fig. 7A) reflects the plasticity of the corticobulbar tract and corticospinal tract, which are implicated in neck and limb movements (27).

Sham-operated WT and tPA KO mice exhibited similar intensities of BDA fluorescence signal and similar numbers of BDA+ fibers at all levels examined (Fig. 7B and C). The fluorescence signal of BDA in the ipsilesional, perifocal cortex was comparable between tPA KO mice and WT mice after dMCAO, suggesting unaltered intracortical plasticity by tPA KO. Intranasal tPA treatment after dMCAO resulted in a statistical trend toward increased cortical BDA fluorescence signal in tPA KO mice compared with vehicle treatment (Fig. 7B and C; P = 0.065). In the ipsilesional brainstem at the level of the facial nuclei and in the contrallesional C7 segment, the number of BDA+ midline-crossing fibers was significantly reduced in tPA KO mice after dMCAO compared with WT mice (Fig. 7B and C). Remarkably, poststroke treatment with recombinant tPA significantly increased the numbers of BDA+ fibers crossing the midline into the contralateral facial nucleus and spinal cord, respectively, in tPA KO mice, with the latter exhibiting comparable numbers of fibers as WT stroke mice (Fig. 7B and C). These results support (i) a critical role of endogenous tPA in axonal sprouting during the chronic/recovery stage after ischemic stroke and (ii) the axonal growth-promoting properties of exogenous tPA repletion.

Protease-Inactive Mutant tPA Rescues Neurological Deficits in tPA Knockout Mice After Ischemic Stroke. Our data so far suggest that recombinant tPA is a promising therapeutic agent that can enhance poststroke CNS plasticity and neurological recovery. However, safety concerns on intracerebral hemorrhage will limit the use of tPA on stroke patients even if it can enhance brain repair. Our data derived from cultured neurons suggest that the axonal protective/regenerative effects of tPA are possibly achieved through its EGF-like domain but not its protease activity (Figs. 1 and 2). Therefore, we determined if the protease-inactive mutant tPA was also efficacious in rescuing the tPA KO phenotype from ischemic injury. We administered tPA-S478A to tPA KO stroke mice, using the same intranasal treatment regimen described above, and the effects of tPA-S478A on sensorimotor and cognitive deficits were assessed up to 35 d after dMCAO. In the rotarod test, cylinder test, and foot fault test, intranasal delivery of tPA-S478A or wild-type tPA comparably mitigated dMCAO-induced sensorimotor deficits in tPA KO mice compared with vehicle treatment (Fig. 8A–C). Furthermore, tPA-S478A significantly improved spatial learning, but not time in goal quadrant, in tPA KO mice at 22–27 d after dMCAO, as revealed by the Morris water maze test (Fig. 8D–F).

Importantly, the use of tPA-S478A offered a safety advantage over wild-type tPA with respect to hemorrhage in a transient MCAO stroke model. i.e. administration of wild-type tPA (10 mg/kg) to mice after 2 h of transient MCAO elicited prominent intracerebral hemorrhage (SI Appendix, Fig. S8), consistent with previous reports (29). Strikingly, tPA-S478A did not induce any detectable hemorrhage when delivered at 10 mg/kg, a dosage five times that shown to enhance neurological recovery after stroke. These data are consistent with a higher therapeutic index of exogenous mutant tPA compared with wild-type tPA.

Discussion

This study investigated the role of tPA in poststroke brain plasticity and long-term functional recovery using a rodent model of permanent focal cerebral ischemia not involving clot formation. The results demonstrate that deletion of endogenous tPA worsens ischemic white matter injury, reduces axonal sprouting, and exacerbates long-term neurological deficits, all of which can be rescued by exogenously delivered recombinant tPA. Potentially, the most important finding is that a mutant form of tPA protein lacking proteolytic activity is equally effective in improving functional recovery in this stroke model, while eliminating the risk of triggering intracerebral hemorrhage.
tPA is best known as a thrombolytic enzyme with the zymogen plasminogen as its primary substrate (1). However, tPA holds additional potentially important roles in the CNS through specific domain interactions. For example, tPA can bind to the membrane receptor low-density lipoprotein receptor-related protein (LRP) through its finger domain, resulting in receptor phosphorylation, intracellular signal transduction, and altered gene expression (30). Besides this cytokine-like function, tPA can also exert growth factor-like functions through the binding and activation of the EGFR via its EGF-like domain (13). Although interactions between the Kringle 2 domain and the NMDA receptor are reported to potentiate neuronal cell death (11, 31), many studies favor a neuroprotective role for tPA under CNS injury conditions (32, 33). Consistent with the latter action, we observed increased neuronal cell death in the absence of endogenous tPA at 3 d after ischemic stroke (SI Appendix, Fig. S9A). However, this phenotype was not reversed by exogenous tPA treatment, and the difference in neuronal death between WT and tPA KO mice diminished during subsequent injury progression. At 35 d after ischemia, cortical tissue loss volumes were comparable among tPA and vehicle-treated mice, although the degree of loss varied widely (SI Appendix, Fig. S6A). We therefore speculate that the persistent improvement of neurological functions elicited by tPA treatment does not result from acute neuroprotection, but from a long-term effect on white matter restoration, as discussed below. As the white matter restoration by tPA was not accompanied by changes in the number of cortical somata, and callosal fibers are known to originate in cortical neurons, we further speculate that the NeuN and MAP2 histological markers do not distinguish between cortical neurons that are fully functional versus neurons that are dysfunctional and silent but are still present within the tissue. The active neurons might retain function due to the prolonged presence of tPA, whereas the quiescent, severely damaged cell bodies may be unable to recover, even in the presence of tPA. The phenomenon that tPA promotes axonal outgrowth and CNS plasticity is well documented (15, 16). Traditionally, such effects were attributed to the digestion of ECM components by tPA, thereby creating a permissive microenvironment for the elongation of axons (16, 19, 20). Our study, however, reveals additional protease-independent mechanisms, as the proaxonal growth effects were largely preserved in cultured neurons treated with the protease-inactive tPA-S478A mutant. The blockade of tPA-induced axonal elongation by the EGFR inhibitor AG1478

Fig. 7. Intranasal administration of recombinant tPA boosts poststroke axonal sprouting and CNS plasticity in tPA KO mice. WT and tPA KO mice were subjected to dMCAO or sham operation. Recombinant tPA was administered intranasally to tPA KO mice at 6 h after dMCAO and then once every 2 d for 14 d. (A) Schematic diagram of tract-tracing experiments. Mice received stereotaxic injections of BDA in the contralesional (Right) motor cortex 21 d after dMCAO. BDA fluorescent signal was examined at three levels in the brain and spinal cord at 35 d after dMCAO (depicted in green): (1) Projections from the right hemisphere into the left hemisphere; (ii) corticobulbar projections from the motor cortex (motor areas M1 and M2) into the left and right facial nuclei (FN); and (iii) corticospinal projections from the motor cortex into the cervical spinal cord (C7). (B) Representative photomicrographs of streptavidin-FITC BDA+ label (green) in the left periflactal cortex (Upper blue box in A), in the ventromedial medulla at the level of the FN (Lower blue box in A), and in C7 spinal cord. The midline is depicted in red, and dashed boxes indicate areas that are enlarged in the fourth row. White dots and arrows indicate sprouting axons at the level of FN and C7, respectively. (C) Quantification of BDA fluorescence intensity in the cortex and numbers of midline-crossing axons per section in the FN and C7 spinal cord. n = 4–8 mice per sham group. n = 5–14 mice per dMCAO group. **P < 0.01, ***P < 0.001 dMCAO KO + vehicle vs. dMCAO WT + vehicle. ###P < 0.001 dMCAO KO + tPA vs. dMCAO KO + vehicle. NS, no significant difference. (Scale bars: 100 μm.)
further substantiates the growth factor-like function of tPA through interactions with EGFR. These in vitro data were supported by in vivo experiments, which demonstrated compromised axonal sprouting after dMCAO in the absence of endogenous tPA and facilitation of sprouting by intranasal tPA treatment. Interestingly, deficiency of endogenous tPA impaired axonal sprouting only in poststoke mice but not in the noninjured sham mice, indicating that endogenous tPA is not essential in the growth and maintenance of axons under physiological conditions. After dMCAO, we observed a robust increase in BDA-labeled fibers at C7 levels in the corticospinal tract (arising from the contralesional cortex) compared with sham controls, suggesting that endogenous tPA activity might be increased in the spinal cord after cortical stroke. Despite previous reports that tPA activity is up-regulated in the spinal cord after contusion injury (16), future studies are warranted to obtain direct evidence that tPA, especially the protease-inactive tPA-S478A, promotes axonal sprouting in the poststroke brain and that the effect of tPA on corticospinal tract remodeling indeed contributes to enhanced neurological recovery in stroke animals.

Our data reveal a fundamental impact of both endogenous and exogenous tPA on white matter integrity after ischemic stroke. White matter tracts are vital for communication between widely separated neurons and contain both myelinated and nonmyelinated axons. We observed exacerbation of white matter injury encompassing both axons and myelin in tPA KO mice after ischemia, and this injury was mitigated at both the structural and functional levels by intranasal tPA treatment. Several parameters that differed between WT and tPA KO mice implicate a major contribution by myelinated fibers, such as the increase of radial diffusivity in DTI, loss of MBP protein, and the marked difference in the N1 component of CAPs. Consistent with these findings, the number of surviving myelin-producing oligodendrocytes in white matter was lower in tPA KO mice 35 d after stroke and partially restored by exogenous tPA. tPA has been reported to exert antiapoptotic functions on oligodendrocytes 24 h after ischemic insults (15). Although we observed comparable numbers of viable oligodendrocytes in the white matter of WT and tPA KO mice at 3 d after dMCAO (SI Appendix, Fig. S9B), we cannot rule out the possibility that tPA protects oligodendrocytes against cell death at even earlier time points. Nevertheless, we speculate that the increase in the overall number of viable oligodendrocytes with tPA treatment at 35 d after dMCAO arises from both early protection and oligodendrogenesis. Future studies will further delineate the potential effects of tPA on white matter repair mechanisms, such as oligodendrogenesis and remyelination.

Although tPA is an FDA-approved, gold standard treatment for ischemic strokes, the critical barrier that limits its use within 4.5 h after stroke onset is the safety concern about intracerebral hemorrhage. tPA-induced hemorrhage has been documented in previous reports (34, 35), but the underlying mechanisms are multifaceted and incompletely understood. tPA and matrix metalloproteinases (MMPs) are two major proteolytic systems regulating the ECM in adult brains, and these two systems are functionally connected (36). tPA-generated plasmin can activate MMPs, thereby amplifying their proteolytic actions (37). Plasmin also directly regulates the cytoskeleton of astrocytes and increases BBB permeability (38). Other substrates of tPA, such as platelet-derived growth factor (PDGF)-CC (39), also disrupt BBB integrity after stroke upon activation by tPA (40). Aside from the protease activity of tPA, there are other protease-independent mechanisms through which tPA contributes to BBB breakdown and hemorrhage. For example, the binding of tPA with LRP is involved in the induction of MMPs and BBB breakdown after stroke (41, 42). Our data show that wild-type tPA protein caused intracerebral hemorrhage 24 h after brain ischemia at a dose of 10 mg/kg (SI Appendix, Fig. S8). However, the protease-inactive tPA-S478A did not trigger any detectable hemorrhage compared with vehicle controls, even when delivered at 10 mg/kg—a dosage five times higher than that needed for neurorestorative effects (2 mg/kg). Our study suggests that the proteolytic activity of tPA is not required to induce intracerebral hemorrhage after stroke. More importantly, the elimination of protease function does not dampen the neurorestorative effects of tPA, as tPA-S478A demonstrated equal efficacy in promoting axonal sprouting in vitro (Fig. 1) and functional recovery in vivo (Fig. 8). With the risk for hemorrhage avoided, tPA-S478A is a promising restorative agent for future clinical development.

Recent years have witnessed exciting advances in reperfusion therapies against ischemic strokes, such as extension of the therapeutic time window to 24 h after stroke onset in some patient subpopulations (6). However, both thrombolytic and recanalization therapies are limited to small patient populations due to the risk of lethal intracerebral hemorrhage, the presence of contraindications (e.g., uncontrolled hypertension and abnormal blood glucose) (43), ineffective arterial recanalization, and suboptimal responses to adequate reperfusion (6). Therefore, there remains an urgent need for safe and effective neuroprotectants for use alone or in conjunction with reperfusion therapies in stroke patients. In this context, the present study has considerable implications for clinical translation. First, the noninvasive intranasal delivery route and 6-h poststroke efficacy for recombinant tPA treatment are practical in clinical settings. Second, we measured long-term neurological functions—the primary outcome assessed in stroke patients—as a major test of efficacy. Third, use of protease-inactive mutant tPA avoided the risk of triggering intracranial hemorrhage, a major obstacle for...
applying tPA as a neuroprotectant in stroke patients. However, a few limitations of the present study warrant further investigation to enhance translatability. For example, we used a permanent ischemia model that does not involve acute reperfusion. Further studies must also examine the neuroprotective or neurorestorative efficacy and therapeutic time window of mutant tPA in conjunction with re-canalization or thrombolysis in preclinical models with reperfusion. Furthermore, key biological variables and comorbid conditions such as age, gender, hypertension, and hyperglycemia must be considered.

In conclusion, the present study demonstrates profound neurorestorative effects of both endogenous and exogenous tPA after ischemic brain injury. The protease-inactive tPA-S478A should be explored further as a treatment for ischemic stroke.

Materials and Methods

Key resources that are essential to reproduce the results are in SI Appendix, Table S1. Focal cerebral ischemia was induced in adult male mice by permanent occlusion of the left distal middle cerebral artery (SI Appendix, Fig. S3A) and left common carotid artery. We refer to this ischemia model as dMCAO. All experimental procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee and performed in accordance with the Guide for the Care and Use of Laboratory Animals (44). Surgeries and all outcome assessments were performed by investigators blinded to mouse genotype and experimental group assignments. All statistics are summarized in SI Appendix, Table S2.

For further methodological details, please consult SI Appendix.

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