Tissue plasminogen activator promotes white matter integrity and functional recovery in a murine model of traumatic brain injury

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Recombinant tissue plasminogen activator (tPA) is a Food and Drug Administration-approved thrombolytic treatment for ischemic stroke. tPA is also naturally expressed in glial and neuronal cells of the brain, where it promotes axon outgrowth and synaptic plasticity. However, there are conflicting reports of harmful versus neuroprotective effects of tPA in acute brain injury models. Furthermore, its impact on white matter integrity preclinical traumatic brain injury (TBI) has not been thoroughly explored, although white matter disruption is a better predictor of long-term clinical outcomes than focal lesion volumes. Here we show that the absence of endogenous tPA in knockout mice impedes long-term recovery of white matter and neurological function after TBI. tPA-knockout mice exhibited greater asymmetries in forepaw use, poorer sensorimotor balance and coordination, and inferior spatial learning and memory up to 35 d after TBI. White matter damage was also more prominent in tPA knockouts, as shown by diffusion tensor imaging, histological criteria, and electrophysiological assessments of axon conduction properties. Replenishment of tPA through intranasal administration of the recombinant protein in tPA-knockout mice enhanced neurologic function, the structural and functional integrity of white matter, and postinjury compensatory sprouting in corticofugal projections. tPA also promoted neurite outgrowth in vitro, partly through the epidermal growth factor receptor. Both endogenous and exogenous tPA protected against white matter injury after TBI without increasing intracerebral hemorrhage volumes. These results unveil a previously unappreciated role for tPA in the protection and/or repair of white matter and long-term functional recovery after TBI.

Tissue plasminogen activator (tPA) is employed in stroke patients to lyse blood clots, but its therapeutic potential in brain trauma victims is far from settled, partly due to fears of internal bleeding. The long-term effects of tPA upon the white matter of the brain and neurological outcomes after head trauma are also uncertain. To address these gaps, we performed electrophysiological, anatomical, and behavioral studies in genetically modified or wild-type mice subjected to traumatic brain injury and infused tPA through the nose. We found that natural or synthetic forms of tPA protect or repair white matter tracts without magnifying internal bleeding. Thus, tPA treatment after brain injury promotes communication across neuronal networks through nerve fiber tracts and improves long-term adaptive behavior.


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The present study was designed to determine the effects of tPA deletion on long-term outcomes after controlled cortical impact (CCI), an established model of TBI. Measurement outcomes were focused on long-term sensorimotor and cognitive functions and white matter parameters, including axonal injury, demyelination, impulse conduction, and regeneration of nerve fibers in white matter tracts. The results demonstrate that tPA-KO mice exhibit lower white matter integrity and poorer recovery of neurological deficits than WT mice up to 35 d after TBI. Furthermore, intranasal delivery of recombinant tPA after TBI restores the long-term functional recovery in tPA-KO mice to WT levels. tPA evokes these protective and/or restorative effects without increasing TBI-induced intracerebral hemorrhage. Thus, both endogenously expressed and exogenously delivered tPA can play a significant role in promoting white matter integrity and long-term neurological recovery after TBI.

**Results**

**The Absence of Endogenous tPA Expression in tPA-KO Mice Exacerbates Long-Term Neurological Impairments After TBI.** We investigated if deletion of the tPA-encoding gene improves or exacerbates long-term neurological dysfunction up to 35 d after TBI or sham operation. Following sham operation, WT and tPA-KO mice exhibited no forelimb use asymmetry or sensorimotor coordination deficits throughout the entire study, as assessed with the cylinder (Fig. L1, Upper) and rotarod (Fig. 1B, Upper) tests, respectively. TBI elicited forelimb asymmetry between 5 and 35 d after TBI, and this motor deficit was significantly magnified in tPA-KO mice (Fig. 1A, Lower). Similarly, both WT and tPA-KO mice fell off the accelerating rotating rod sooner than sham-treated control mice between 3 and 35 d after surgery, and this motor deficit was exacerbated in tPA-KO mice (Fig. 1B, Lower).

Long-term spatial cognitive functions were assessed by the Morris water maze test from day 29 to day 34 post-TBI (Fig. 1C–H). All mice exhibited gradual decreases in escape latency over time (Fig. 1C–F). WT and tPA-KO mice displayed comparable learning and memory capacity after sham surgeries (Fig. 1E and G). After TBI, tPA-KO mice exhibited greater learning and memory deficits than WT mice, as manifested by significantly longer escape latencies (loss of spatial learning) (Fig. 1F) and decreased time in the target quadrant (loss of spatial memory) (Fig. 1G). Mice from all four groups exhibited similar swim speeds, suggesting that the memory tests were not confounded by major differences in swim abilities (Fig. 1H).

**Exacerbation of White Matter Damage in tPA-KO Mice May Impede Long-Term Neurological Recovery After TBI.** Ex vivo diffusion tensor imaging (DTI) was performed to assess white matter integrity in the CC and external capsule (EC) at 35 d after TBI or sham operation (Fig. 2A and B and SI Appendix, Fig. S1). Reductions in fractional anisotropy (FA) and increases in radial diffusivity (RD) with DTI indicate the loss of white matter integrity (19). Compared with sham-operated mice, both TBI groups exhibited significant decreases in FA and increases in RD in the CC and EC of the ipsilesional hemisphere (SI Appendix, Fig. S1). However, after TBI the tPA-KO mice exhibited significantly greater changes in both FA and RD values in the ipsilesional hemisphere than WT mice (Fig. 2B), suggesting that tPA KO exacerbates white matter damage induced by TBI.

We assessed the integrity of myelin with immunostaining against myelin basic protein (MBP) and the degree of demyelination with immunostaining against nonphosphorylated neurofilament H (Sternberger monoclonal clone 32; SMI-32) in the perilesional cortex (CTX), EC, and striatum (STR) at 35 d after TBI. In both sham-operated groups, the SMI-32 signal in the EC and STR was extremely low. However, the SMI-32 signal was readily detectable in the uninjured CTX, perhaps indicating relatively higher numbers of unmyelinated axons within this region (Fig. 2C). TBI elevated the SMI-32/MBP ratio in the CTX, EC, and STR to significantly higher levels in tPA-KO mice than in WT mice (Fig. 2D). We further observed that the ratios of SMI-32/MBP in the CTX and EC, but not in the STR, were positively correlated with asymmetry in the cylinder test at 35 d after TBI (Fig. 2E–G) and were inversely correlated with the latency to fall in the rotarod test (D). TBI increased asymmetry and decreased latency to fall; sham operations had no effect. (C–H) Long-term spatial learning (escape latency), spatial memory (target quadrant time), and swim speeds were assessed by the Morris water maze test. (C and D) Representative images illustrate swim paths on days 33 (learning) and 34 (memory) after TBI (C) or sham operation (D). (E–G) Escape latency and target quadrant time were not affected by sham operation but were increased after TBI, and even more in tPA-KO than in WT mice. (H) Swimming speed was unaffected by tPA-KO or TBI. Data are presented as mean ± SEM. n = 9–12 per group. *P < 0.05, **P < 0.01 for KO vs. WT mice by two-way repeated-measures ANOVA followed by Tukey post hoc correction in G and H, n.s., not significant. See SI Appendix, Table S1 for statistical details for all figures.
somatodendritic marker microtubule-associated protein 2 (MAP2) 35 d following TBI (SI Appendix, Fig. S3). WT and tPA-KO mice exhibited similar cortical lesion volumes, suggesting comparable damage to gray matter at the injury core. Thus, tPA KO has little effect on the cortical contusion size in this TBI model.

Collectively, these findings suggest that endogenous tPA mitigates TBI-induced white matter injury at extended survival times and that the extent of white matter injury within the perilesional zones in both the CTX and EC may partly dictate the evolution of long-term sensorimotor deficits after TBI.

TBI Elicits Greater Acute White Matter Damage in tPA-KO Mice than in WT Mice. The decline of motor function in tPA-KO mice within 1 wk after TBI (Fig. 1 A and B) suggested that tPA deficiency may also worsen early brain damage. Therefore, we assessed white matter injury at 3 d after TBI with immunofluorescent staining for the accumulation of β-amyloid precursor protein (β-APP), an established indicator of axonal damage (20), and the SMI-32/MBP protein ratio, an established measurement for demyelination (21).

The numbers of β-APP+ axonal varicosities were significantly increased along NF200+ neurofilaments in the perilesional CC, EC, and STR at 3 d after TBI in both WT and tPA-KO groups (Fig. 3 A–C). β-APP varicosity densities in the perilesional EC and STR, but not in CC, were significantly higher in tPA-KO mice than in WT mice (Fig. 3 A and B). Moreover, the SMI-32/MBP ratios in perilesional CTX, EC, and STR were significantly increased in tPA-KO mice compared with WT mice (Fig. 3 D and E).

To determine whether the histological results in white matter were associated with functional changes, we measured evoked compound action potentials (CAPs) in the perilesional CC and EC 3 d after TBI (Fig. 3 F–H). In this assay, the amplitude of the CAPs is a measure of axonal conduction (21, 22). There was no difference in amplitude between sham-injured WT and tPA-KO mice, suggesting that loss of tPA did not affect the baseline conduction properties of myelinated axons (Fig. 3 G and H). The amplitudes were significantly decreased after TBI in the WT group, reflecting the expected axonal injury (Fig. 3 G and H). In accordance with the histological changes in white matter, CAP amplitudes were further significantly decreased in tPA-KO mice compared with WT mice.

Taken together, these results strongly suggest that endogenous tPA mitigates both acute and chronic white matter damage after TBI at the histological and functional levels.

**Fig. 2.** Deletion of tPA exacerbates long-term white matter disruption after TBI. WT and tPA-KO mice were subjected to sham injury or unilateral TBI in the right hemisphere. (A) Representative DTI data of ex vivo brains [directionally encoded color (DEC), FA, and RD maps] and MBP staining 35 d after TBI or sham operation. In DEC axial maps centered on the level of the lesion, the color indicates the direction of water diffusion along white matter fibers (red = mediolateral, blue = dorsoventral, and green = anteroposterior). The contralateral (CL) and ipsilesional (IL) hemispheres are indicated. Arrowheads in the Sham column point to the uninjured CC and EC, and arrows in the TBI columns point to the injured CC and EC. (Scale bar, 1 mm.) (B) Quantification of average FA and RD values in the CC and EC area of the IL and CL hemispheres. (C) Representative images of dephosphorylated neurofilament protein (SMI-32, red) and MBP (green) in CTX, EC, and STR 35 d after TBI. (Scale bar, 50 μm.) (D) Illustration of regions of interest relative to lesion and quantification of the ratios of SMI-32 to MBP fluorescence intensity in the CTX, EC, and STR, illustrated as fold-change compared with the sham WT average value. (E–J) Correlation between the ratio of SMI-32 to MBP intensity in CTX, EC, and STR and forelimb use asymmetry in the cylinder test (E–G) or the latency to fall in the rotarod test (H–J) on day 35 after TBI or sham surgery. Data are presented as mean ± SEM. n = 4 per group in B; n = 8–11 per group in D; n = 5–6 per group in E–J. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 by two-way ANOVA followed by Tukey post hoc correction in B and D; P values stated in E–J were determined by two-tailed linear correlation analyses.
Intranasal Delivery of Recombinant tPA Rescues Neurological Functions After TBI. As the absence of endogenous tPA worsens neurological impairments and white matter injury at both acute and delayed time points after TBI, we hypothesized that intracerebral delivery of exogenous tPA to tPA-KO mice would ameliorate neurological deficits after TBI. To test this, we employed the intranasal approach for tPA delivery into the brain (23). In a pilot dose–response study we tested the effects of various intranasal tPA dosages (0, 0.5, 1, and 1.5 mg/kg) on behavioral performance up to 14 d after TBI using the rotarod test. The results showed that 0.5 mg/kg was the lowest dose among the tested doses to improve post-TBI performance in the rotarod test significantly. Higher doses did not exhibit further protection. Therefore, 0.5 mg/kg was selected as the optimal dose for all subsequent studies. tPA-KO and WT mice were subjected to TBI, and equal volumes of PBS or tPA (0.5 mg/kg, freshly dissolved in PBS at 1 μg/μL) were delivered into their nostrils 2 h after the injury and then once every other day for 2 wk (days 0, 2, 4, and so forth.). Sham-operated tPA-KO and WT mice treated with PBS exhibited no sensorimotor deficits in the cylinder or rotarod test and no differences across groups—an important control showing lack of effects on baseline sensorimotor functions (SI Appendix, Fig. S4 A and B). In contrast, after TBI the tPA-KO mice exhibited greater sensorimotor dysfunction than WT mice in both tests (Fig. 4 A and B) as well as greater cognitive dysfunction in the Morris water maze test (Fig. 4 C and D), consistent with the previous behavior data (Fig. 1). However, after post-TBI intranasal tPA administrations, the sensorimotor function of tPA-KO mice was equivalent to that of WT mice (Fig. 4 A and B). Although tPA treatment failed to improve spatial learning significantly in tPA-KO mice (Fig. 4 C), tPA rescued long-term spatial memory in tPA-KO mice, raising it to the levels of PBS-treated WT mice (Fig. 4 D). Swim speeds were unaffected (Fig. 4 E), as in Fig. 1. These results
we investigated whether tPA treatment increased axonal conduction at 35 d after TBI. Compared with sham controls (Fig. 3H), TBI markedly decreased CAP amplitude in WT mice, and this effect was even more profound in tPA-KO mice (Fig. 5 E and F). tPA treatment significantly enhanced conduction amplitude in tPA-KO mice to levels comparable to those in WT mice after TBI (Fig. 5 E and F). Taken together, these data reveal that intranasal delivery of tPA replaces the endogenous tPA missing from KO mice and prevents long-term demyelination and loss of axonal conduction after TBI. Furthermore, white matter loss was associated with deterioration of axonal conductivity, as shown by a negative correlation between the SMI-32/MBP ratio in the CTX and EC and the amplitude of evoked CAPs (Fig. 5 G–I). Strong correlations were also observed between evoked CAP amplitude and the asymmetry rate in the cylinder test (Fig. 5J) and the latency to fall in the rotarod test (Fig. 5K). These findings indicate that the electrophysiology measurements are an excellent predictor of functional performance after TBI.

Intranasal Delivery of tPA Reverses Axonal Injury Induced by TBI in tPA-KO Mice Without Magnifying the Intracranial Hemorrhage. Thus far, we have demonstrated that tPA administration improves long-term white matter integrity in tPA-KO mice and is associated with superior neurological outcomes. As acute axonal damage after TBI was greater in tPA-KO mice (Fig. 3), we examined whether exogenous tPA treatment might alleviate this detrimental effect by measuring β-APP⁺ varicosities 3 d after TBI. No immunoreactivity for β-APP was detected in either sham injury group. In accordance with our previous results, β-APP accumulated within injured axons in the perilesional EC and STR in PBS-treated WT mice after TBI, and this effect was greater in the tPA-KO mice, as expected (Fig. 6 A and B). However, tPA treatment significantly decreased β-APP staining in tPA-KO mice to levels similar to those in WT mice, suggesting that exogenous tPA administration reduced axonal injury in the acute phase after TBI.

The clinical use of recombinant tPA is viewed as contraindicated in head trauma victims due to the concern that tPA might increase the risk of cerebral hemorrhage. A recent paper reported that TBI induces less brain hemorrhage in tPA-KO mice than in WT mice (15), suggesting that endogenous tPA might augment cerebral bleeding after TBI. Therefore, we tested the safety of intranasal tPA administration and found that intranasal tPA treatment (0.5 mg/kg) failed to increase brain hemorrhage in WT mice (Fig. 6 C and D and SI Appendix, Fig. S7). Furthermore, there was no decrease in the amount of intracranial hemorrhage in tPA-KO mice compared with WT mice 24 h after TBI (Fig. 6 C and D and SI Appendix, Fig. S7B). These results do not support a major contributing role for tPA in TBI-induced brain hemorrhage.

Intranasal Delivery of tPA Compensates for the Detrimental Effects of tPA KO on White Matter After TBI. Macroscopic loss of brain neuronal tissue was assessed by staining for the neuron marker MAP2 at 3 and 35 d after TBI. No significant difference in cortical tissue volume loss was observed between the tPA and PBS treatment groups at either early or extended survival time points (SI Appendix, Figs. S5 and S6), suggesting that TPA administration does not rescue the focal cortical lesion after TBI, consistent with the tPA-KO data (SI Appendix, Fig. S3) and with the severity of the mechanical trauma in the focal injury zone.

We investigated whether intranasal tPA infusions reduce white matter disruption in tPA-KO mice subjected to TBI. SI Appendix, Figs. S5 and S6). Strong transverse tPA-treated injection into the cervical spinal cord segment 7 (C7). The sprouting of BDA⁺ axons across the midline into the derivated facial nucleus (ipsilesional) and spinal cord (contralateral) is a robust measure of neuroplasticity (27, 28). Thus, brains and spinal cords were harvested 2 wk after BDA infusions, and the numbers of BDA⁺ fibers in the ipsilesional brainstem at the level of the facial nuclei and the contralateral C7 segment were counted to assess axonal sprouting from motor neurons of the contralateral cortex (Fig. 7 B and C). Sham-operated WT and tPA-KO mice exhibited similar numbers of BDA⁺ fibers. The absence of endogenous tPA in tPA-KO mice dramatically reduced...
the number of midline-crossing fibers in C7 after TBI compared with WT mice (Fig. 7C). In the facial nuclei, TBI significantly reduced the number of midline-crossing axons in WT mice, and tPA KO decreased this measure further. Notably, tPA-KO mice infused with recombinant tPA exhibited similar numbers of midline-crossing fibers as WT mice in both areas (Fig. 7C), revealing an essential role of tPA in axonal sprouting during the recovery period after TBI.

To determine the strength of the relationship between increased sprouting and improvements in sensorimotor function by exogenously delivered recombinant tPA, Pearson product correlation analyses were performed. The number of midline-crossing BDA+ fibers at C7 was negatively correlated with the forelimb use asymmetry in the cylinder test (Fig. 7D). These results are consistent with the view that tPA promotes neurological outcomes after TBI partly through an increase in axon sprouting.

**Exogenous tPA Application Promotes Neurite Outgrowth in Vitro.** To explore the mechanisms by which tPA promotes axon sprouting under pathological conditions, an in vitro assay was designed to mimic the inhibition of neurite outgrowth after TBI by application of chondroitin sulfate proteoglycan (CSPG) (29). CSPG has been implicated in the inhibition of spontaneous axonal plasticity after TBI (30, 31). The neurotrophic effects of tPA are thought to be partly mediated by the epidermal growth factor receptor (EGFR) (32, 33). As expected, CSPG decreased the length of tau+ axons in neurons in a concentration-dependent manner (SI Appendix, Fig. S8 A and B), and axon length was significantly increased after tPA treatment (SI Appendix, Fig. S8 C and D). The increases in axon length by tPA were diminished by the EGFR inhibitor AG1478. These results suggest that the axon growth-promoting effects of tPA are mediated, at least in part, by EGFR.

**Discussion**

The present study demonstrates white matter-protective and neurorestorative roles for endogenous tPA after TBI, according to histological, MRI, electrophysiological, tract-tracing, and behavioral criteria. Despite the lack of a tissue-sparing effect at the cortical contusion site, endogenous tPA and intranasally delivered tPA robustly improved the structural and functional integrity of white matter tracts in perilesional brain regions in both acute and chronic injury phases. The salutary effects of tPA on white matter repair of white matter and long-term functional recovery after TBI.
Quantification of hemorrhage volumes can be viewed in production and release of TNF-α after stroke injury may be mediated by tPA-induced proliferation and maturation of oligodendrocyte progenitor cells, which promote neurovascular remodeling or regeneration (17, 18, 27, 36). The clinical recanalization rates of 10–30% (48). In a rat model of physiological thrombosis induced by crushing and stenosis of the common carotid artery, the i.v. "human dose" of 0.9 mg/kg was ineffective for rodent clot lysis, and the authors concluded that "rat doses" between 1.8 and 4.5 mg/kg were more likely to achieve the upper limits of the clinical recanalization rates of 10–30% (48). In a rat stroke model, intranasal delivery of 2.2–2.4 mg/kg iPA failed to increase intracerebral hemorrhage (49). Nevertheless, the neuroprotective iPA dose tested in the current study (0.5 mg/kg) is considerably lower than the reported doses that cause intracerebral hemorrhage in rodents (15, 46, 47). Furthermore, TBI-induced brain hemorrhage was not reduced in iPA-KO mice. These collective results do not support a major contributing role for endogenous or low-dose exogenous iPA in TBI-induced intracerebral hemorrhage.

The intranasal route is thought to be a superior method for quickly accessing the brain, as it minimizes systemic exposure and bypasses the blood–brain barrier, because mutant forms of iPA devoid of the protease activities, such as iPA-S481A, do not induce brain hemorrhage (12, 13, 16). The discrepancies in the literature on the beneficial versus toxic properties of iPA are likely attributed to variable doses and/or the timing of iPA treatment (10, 44). The dose of iPA for thrombolytic therapy in humans is 0.9 mg/kg, which is associated with an increased risk of intracerebral hemorrhage only if the delivery of iPA is delayed for >4.5 h after the onset of stroke (45). In rodent models of TBI and stroke, intracerebral hemorrhage is typically induced by i.v. delivery of iPA of 5 and 10 mg/kg, respectively (15, 46, 47). In a rat model of physiological thrombus induced by crushing and stenosis of the common carotid artery, the i.v. "human dose" of 0.9 mg/kg was ineffective for rodent clot lysis, and the authors concluded that "rat doses" between 1.8 and 4.5 mg/kg were more likely to achieve the upper limits of the clinical recanalization rates of 10–30% (48). In a rat stroke model, intranasal delivery of 2.2–2.4 mg/kg iPA failed to increase intracerebral hemorrhage (49). Nevertheless, the neuroprotective iPA dose tested in the current study (0.5 mg/kg) is considerably lower than the reported doses that cause intracerebral hemorrhage in rodents (15, 46, 47). Furthermore, TBI-induced brain hemorrhage was not reduced in iPA-KO mice. These collective results do not support a major contributing role for endogenous or low-dose exogenous iPA in TBI-induced intracerebral hemorrhage.
that intranasal delivery of 1.86 mg/kg tPA in rats leads to dramatically higher tPA protein and activity levels in the brain as late as 24 h postinfusion (55). However, brain bioavailability will depend upon formulation and other chemical properties of the intranasal infusate, as the intranasal routes were found to be similar to the i.v. route for some drugs (56, 57). Furthermore, we caution that generalizations of effective drug dosages from rodents to human patients are fraught with caveats, in-