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Subacute Intranasal Administration of Tissue Plasminogen Activator Increases Functional Recovery and Axonal Remodeling after Stroke in Rats

Zhongwu Liu[a], **Yi Li**[a], **Li Zhang**[a], **Hongqi Xin**[a], **Yisheng Cui**[a], **Leah R. Hanson**[b], **William H. Frey II**[b], and **Michael Chopp**[a,c,*]

[a]Department of Neurology, Henry Ford Hospital, Detroit, MI

[b]Alzheimer's Research Center at Regions Hospital, HealthPartners Research Foundation, St. Paul, MN

[c]Department of Physics, Oakland University, Rochester, MI

Abstract

As a thrombolytic agent, application of recombinant tissue plasminogen activator (tPA) to ischemic stroke is limited by the narrow time window and side effects on brain edema and hemorrhage. This study examined whether tPA, administered by intranasal delivery directly targeting the brain and spinal cord, provides therapeutic benefit during the subacute phase after stroke. Adult male Wistar rats were subjected to permanent right middle cerebral artery occlusion (MCAo). Animals were treated intranasally with saline, 60 μg or 600 μg recombinant human tPA at 7 and 14 days after MCAo (n=8/group), respectively. An adhesive-removal test and a foot-fault test were used to monitor functional recovery. Biotinylated dextran amine (BDA) was injected into the left motor cortex to anterogradely label the corticorubral tract (CRT) and the corticospinal tract (CST). Naive rats (n=6) were employed as normal control. Animals were euthanized 8 weeks after stroke. Compared with saline treated animals, significant functional improvements were evident in rats treated with 600 μg tPA ($p<0.05$), but not in 60 μg tPA treated rats. Furthermore, 600 μg tPA treatment significantly enhanced both CRT and CST sprouting originating from the contralesional cortex sprouting into the denervated side of the red nucleus and cervical gray matter compared with control group ($p<0.01$), respectively. The behavioral outcomes were highly correlated with CRT and CST axonal remodeling. Our data suggest that delayed tPA intranasal treatment provides therapeutic benefits for neurological recovery after stroke by, at least in part, promoting neuronal remodeling in the brain and spinal cord.

Keywords

functional recovery; middle cerebral artery occlusion; neuronal remodeling; tissue plasminogen activator

[*]Correspondence to Michael Chopp, PhD, Neurology Research, E&R Bldg., Room 3056, Henry Ford Hospital, 2799 West Grand Boulevard, Detroit, MI 48202, USA, Tel: (313)916-3936 Fax: (313)916-1318 chopp@neuro.hfh.edu.

Introduction

Recombinant tissue plasminogen activator (tPA) is the only FDA approved thrombolytic agent for acute treatment of ischemic stroke; however, the efficacy and safety of its therapeutic application are limited by the narrow 4.5 hour treatment time window and adverse side effects on brain edema and hemorrhagic transformation (Cronin, 2010). Presently, fewer than 5% of stroke patients in the United States receive tPA. For decades, the primary stratagem for stroke treatment has been focused on neuroprotection to reduce the cerebral infarction. Unfortunately, all neuroprotective agents developed in the laboratory have failed in clinical trials (Rother, 2008). There is therefore a compelling need to develop neurorestorative therapies to enhance neurological recovery, by primarily treating the intact cerebral hemisphere and the compromised cerebral tissue to promote neuronal plasticity to compensate for the damaged tissue during the subacute and chronic phases after stroke.

tPA has pleiotropic actions in the brain besides its well established fibrinolytic action. It induces neural injury in the setting of acute stroke (Benchenane et al., 2004). However, it is also involved in synaptic plasticity (Samson and Medcalf, 2006), dendritic remodeling (Mataga et al., 2004) and axonal outgrowth (Minor et al., 2009). We have demonstrated that endogenous tPA mediates bone marrow stromal cell-induced neurite outgrowth and functional recovery after stroke (Shen et al., 2011). Thus, we hypothesized that exogenous administration of tPA during the subacute phase may also provide beneficial effects on stroke recovery by promoting axonal remodeling.

The catalytic activity of tPA is rapidly inactivated through binding of protein inhibitors, primarily plasminogen activator inhibitor-I (PAI-1). The tPA/PAI-1 complex is cleared from the circulation by the liver. Therefore, tPA has a short half-life of 5 to 10 min in the bloodstream (Gravanis and Tsirka, 2008). Intranasal delivery method has been demonstrated to directly target the brain and spinal cord along olfactory and trigeminal nerves innervating the nasal passages to bypass the blood-brain barrier (Dhuria et al., 2010). To avoid the rapid inactivation and clearance of tPA from the circulation system, we for the first time examined the effect of tPA administered by intranasal delivery on sensorimotor functional recovery in adult rats during the subacute phase after ischemic stroke. In addition, to investigate the neuronal substrate of the behavioral recovery, we examined axonal remodeling of the corticorubral tract (CRT) and corticospinal tract (CST) originating from the intact cortex into the denervated side of the red nucleus and spinal cord.

Materials and Methods

Adult male Wistar rats (n=30, 2 month-old, weighing 250–275 g) were used throughout this study in a blinded fashion for treatment, behavioral measurements and tissue anlysis. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Henry Ford Hospital.

Animal model

Permanent right side middle cerebral artery occlusion (MCAo) was induced using a method of intraluminal vascular occlusion, modified in our laboratory (Chen et al., 1992). Briefly, a length of 4-0 monofilament nylon suture (18.5–19.5 mm) with a heating rounded tip was advanced from the external carotid artery into the lumen of the internal carotid artery to block the origin of the MCA.

Functional measurements

A series of behavioral tests were performed before MCAo, 3 days after MCAo and weekly thereafter to evaluate the sensorimotor disability and recovery with an adhesive-removal test

(Schallert and Whishaw, 1984), which measures the sensory and motor deficits by recording the time required to remove an adhesive tab from the stroke-impaired left forepaw, and a foot-fault test (Hernandez and Schallert, 1988), which measures the accuracy of forepaw placement on a non-equidistant grid as the percentage of foot-faults of the left forepaw to total steps.

Intranasal administration of tPA

The animals were randomly selected to receive saline (n=8), low dose (60 μg, n=8) or high dose (600 μg, n=8) recombinant human tPA (Genentech Inc, San Francisco, CA) intranasally at 7 and 14 days after MCAo, respectively. The delivery method described by Thorne et al (Thorne et al., 2004) was modified for intranasal treatment. Briefly, under Forane anesthesia, the rats were placed in a supine position with a rolled 2 × 2 inch gauze under the neck to maintain a horizontal head position. Ten 6-μL drops for a total volume of 60 μL of saline or tPA solution in saline were placed alternately onto each nostril with a 3-min interval between drops and naturally sniffed in by the rat. The animals were kept in supine position for an additional 10 min. An additional animal group without MCAo and treatment (n=6) was employed as normal control. To validate the efficiency of intranasal delivery, a test performed in tPA−/− mice showed that the tPA was successfully delivered into the brain (Supplementary Material).

Anterograde axonal tracing

Fourteen days before euthanasia, the rats were restricted in a Kopf stereotaxic apparatus and a craniotomy was performed over the left frontal sensorimotor cortex with a high speed drill under ketamine anesthesia. Ten % solution of biotinylated dextran amine (BDA, 10000 MW; Invitrogen, Carlsbad, CA) in saline was injected through a finely drawn glass capillary into 4 points (stereotaxic coordinates: 0.5 and 1.5 mm rostral to the bregma, 2 and 3 mm lateral to the midline, 1.5 mm deep from the cortical surface; 100 nl per injection) in the left forepaw motor cortex to anterogradely label the axons of CRT and CST originating from these areas.

Tissue preparation and data analysis

Animals were sacrificed under deep Ketamine anesthesia at 8 weeks after MCAo. Rats were perfused transcardialy with saline, followed by 4% paraformaldehyde. The entire brain and spinal cord were immersed in 4% paraformaldehyde overnight. The brain was cut into 7 equally spaced (2 mm) coronal blocks. The brain blocks and tissue from the medulla and cervical cord were cut into 100 μm-thick coronal sections using a vibratome. The lesion volume was measured on sections from each brain block as percentage of the lesion area compared with the contralateral hemisphere, as previously described (Swanson et al., 1990). The remaining sections were incubated with avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA) at 4°C for 48 hr, and the BDA-labeling was visualized with 3,3′-diaminobenzidine-nickel (Vector).

The number of BDA-positive fibers in the pyramidal tract at the medulla level ipsilateral to the injection site was counted and averaged on 3 consecutive coronal sections for each animal. The NIH image software (Image J) was employed to measure the BDA-positive CRT density in the bilateral red nucleus on 5 continuous midbrain sections, and the BDA-positive CST length in the stroke-impaired side of the ventral gray matter on 40 consecutive cervical cord sections (C5–7). To avoid inter-animal variation in tracing efficiency, axonal remodeling of the CRT was estimated by the ratio of axonal density in the ipsilesional denervated side to the contralateral intact red nucleus measured on same section analyzed with, and the CST remodeling was estimated by the total BDA-labeled axonal length

normalized with a quotient of individual BDA-labeled CST number in the pyramidal tract to the mean number calculated in all animal groups.

Statistics

All data are presented as mean ± SD. The experimental groups were compared statistically using the ANOVA test. A value of $p<0.05$ was taken as significant. Pearson's correlation coefficients were calculated between functional recovery and anatomical reorganization.

Results

Intranasal tPA administration enhances functional recovery

In the permanent suture MCAo model, the ischemic infarct included the forelimb area of the sensorimotor cortex, striatum, and the supraoptic area in the right cerebral hemisphere. As shown in the Table, there was no significant difference on lesion volume among the control and different treatment groups. In addition, intranasal tPA administration did not induce animal death or brain hemorrhage (data not shown).

The motor performance of the stroke-impaired left forelimb was assessed with the adhesive-removal test and foot-fault test. All animals showed a remarkable functional deficit on postoperative day 3, followed by gradual improvement with time over the 8 week experimental course. Compared with the saline treated group, animals that received high dose tPA began to exhibit behavioral improvement in the adhesive removal task at 1 week after treatment (ie, 2 weeks after stroke, $p<0.05$, Figure 1A), and showed significant functional enhancement in the foot-fault test starting 2 weeks after treatment ($P<0.05$, B). However, there was no significant therapeutic benefit on both functional tests in the low dose tPA treated animals.

Intranasal tPA administration promotes CRT and CST remodeling

To verify the neuroanatomical substrate of functional recovery after stroke with tPA treatment, we injected the anterograde neuronal tracer BDA into laminae V of the contralesional sensorimotor cortex (Figure 2A), to label the major descending axonal tracts of the CRT, which innervates the red nucleus for coordinating sensations and movements of the whole upper body (Cooper et al., 2000), and the CST, the most important neural pathway controlling precise voluntary movements (Heffner and Masterton, 1983). The CRT in normal rats is primarily an ipsilateral projection (B). In animals subjected to MCAo followed by saline (C) and low dose tPA (D) treatment, few BDA-labeled CRT fibers cross the midline and terminate within the contralateral de-afferentated red nucleus area. However, rats with high dose tPA treatment showed marked increased number of BDA-labeled fibers in the de-afferented red nucleus (E). To assess the CRT remodeling after stroke among different treatment groups, the ratio of BDA-labeled CRT axonal density in the denervated red nucleus to the intact side was calculated. Quantitative data showed a significant increase of midline crossing fibers in the high dose tPA treated animals by 2-fold more than the control group (F, $p<0.01$), while no significant difference was evident in the low dose tPA treated group.

At the cervical enlargement level, the descending CST motor fibers extend into the gray matter to form neural circuits with spinal motoneurons, which control forelimb muscle movements. By using BDA as an anterograde tracer injected into the unilateral cerebral cortex (Figure 3A), the BDA-positive CST projections in the spinal cord contralateral to the BDA injection side in normal animals (B) were labeled. In MCAo animals treated with saline (C) or low dose tPA (D) groups, few BDA-labeled CST fibers grow toward the denervated spinal gray matter. In contrast, the animals that underwent MCAo and treatment

with the high dose tPA showed increased BDA-positive fibers that originated from the contralesional cortical hemisphere and re-crossing the midline into the denervated side of the gray matter in the spinal cord (E). tPA treatment-induced CST axonal remodeling in ischemic rats were evaluated by measurement of the total length of the BDA-positive fibers in the denervated gray matter obtained from 40 adjacent cross sections at the cervical enlargement (C5–7). Our data indicate that high dose tPA intranasal administration significantly promoted axonal reorganization in the denervated spinal cord after experimental stroke (F, $p<0.01$).

CRT and CST remodeling highly correlate with behavioral outcome after stroke

To test the hypothesis that contralesional neuronal remodeling functionally contributes to neurological outcome after stroke, we examined the relationship of behavioral performance with the neuronal status 8 weeks after MCAo. Our data show that the forelimb skilled motor tasks assessed with the adhesive-removal test and foot-fault test were highly correlated with the cortical innervation originating from the contralesional cortex at both red nucleus and cervical spinal cord levels (Figure 4A–D, $p<0.05$).

Discussion

A recent clinical study reported that tPA-treated patients continue to improve faster and to a larger extent during the rehabilitation period beyond the acute phase compared with an equivalent cohort of non-tPA treated patients (Meiner et al., 2010). Desmoteplase, a recombinant form of the plasminogen activator from saliva of the vampire bat, is designed to dissolve the blood clot without the side effects of tPA (Armstead et al., 2006). However, a phase III clinical trial of desmoteplase has failed to demonstrate any beneficial effects on neurological improvements and survival (Hacke et al., 2009), suggesting that the therapeutic benefits of tPA for acute ischemic stroke may not be only attributed to the reperfusion of the occluded vessel by thrombolysis. tPA has both intravascular and extravascular effects in the CNS. A wide distribution of tPA biosynthesis in the brain is associated with different actions of tPA, such as facilitating synaptic plasticity (Samson and Medcalf, 2006) and axonal regeneration (Armstead et al., 2006), which may contribute to neural repair. Therefore, in the present study, we examined the use of tPA to treat ischemic stroke beyond the acute stage of lesion onset by promoting neuronal remodeling. Our data demonstrated that intranasal tPA administration at the subacute phase significantly improved motor outcome and induced compensatory axonal re-innervation in both denervated red nucleus and spinal cord in rodent MCAo model. Although further pharmacological studies are needed to optimize the administration dose and frequency to achieve the best outcome, the present study provides a robust proof-of-principle that subacute treatment (7d after stroke) of stroke with intranasal administered tPA is neurorestorative and enhances neurological recovery.

The CRT and CST projections primarily show a unilateral topographical innervation pattern to the ipsilateral red nucleus and contralateral spinal cord. After MCAo and treatment with tPA, sprouted CRT and CST axons originating from the contralesional cortex cross the midline to arborize into the contralateral denervated red nucleus and gray matter of the spinal cord, respectively. Although it is technically difficult to substantiate a direct causative relationship for the axonal sprouting to behavioral outcome, the present study demonstrated that the axonal remodeling is highly correlated with sensorimotor functional improvement of the forelimb after stroke treated with tPA intranasally. Our prior studies have demonstrated that activation of endogenous tPA induced by bone marrow stromal cells promotes neurite outgrowth in mice after stroke (Shen et al., 2011; Xin et al., 2011; Xin et al., 2010). tPA has been shown to activate matrix metalloproteinases-9 (MMP-9) through plasmin-dependent and -independent mechanisms (Candelario-Jalil et al., 2009), that may promote stroke recovery by modulating neurovascular remodeling, since inhibition of MMP-9 during the

subacute phase (7 to 14 days) after stroke impairs functional recovery (Zhao et al., 2006). The beneficial effects of tPA may also be mediated through plasmin, which converts a precursor of brain-derived neurotrophic factor (BDNF) into mature BDNF (Pang et al., 2004). Binding of tPA to low-density lipoprotein receptor-related protein 1 can also activate extracellular signal-regulated kinase 1/2 (Hu et al., 2006), that mediates corticospinal motor axon regeneration (Hollis et al., 2009). In addition, tPA also promotes degradation of the inhibitory proteoglycans in the extracellular matrix, which is important for synaptic remodeling and formation of new axonal varicosities (Benarroch, 2007). Previous studies have demonstrated that tPA knockout mice show delayed functional recovery after sciatic nerve crush (Siconolfi and Seeds, 2001), and local application of tPA promotes axonal regeneration and functional recovery after sciatic nerve injury (Zou et al., 2006), suggesting that enhanced sensorimotor behavioral recovery after stroke in rats treated with tPA may be attributed to the increased CRT and CST sprouting to rewire the denervated red nucleus and the spinal cord. However, further studies to elucidate underlying mechanisms for tPA induced axonal remodeling, and other potential mechanisms such as vascular remodeling, angiogenesis, and neurogenesis promoted by tPA, are warranted.

Intranasal delivery circumvents the blood–brain barrier, and directly targets the brain and spinal cord (Dhuria et al., 2010), with a significantly lower elimination rate in the brain than intravenous administration (Bagger and Bechgaard, 2004). A previous study shows the tPA content in the intact rat brain is 7 ng/ml measured 2 hours after tPA (10 mg/kg) intravenous injection (Harada et al., 2005). In contrast, the content of tPA in the brain 2 hours after the beginning of intranasal administration was 228 ng/ml, while a higher content of 307 ng/ml was found at 30 minutes (Supplemental Material), suggesting the intranasal administration is an efficacious method to deliver drugs with molecular weight as high as tPA (72 KDa) to target the brain and spinal cord. Seven to 14 days after the stroke is the initiation and maintenance phase of axonal sprouting response (Carmichael, 2006). Because of the potential adverse side effects of tPA that may aggravate stroke injury and offset the therapeutic benefits when tPA is applied intravascularly and early after stroke, we delivered tPA at 7 and 14 days after stroke into the nasal cavity targeting the brain and spinal cord directly. To our knowledge, no one has investigated the potential therapeutic effects of tPA as a neurorestorative agent to improve motor functional recovery by promoting axonal remodeling to rewire the denervated peripheral tissue during the subacute phase after ischemic stroke. Recently, a pilot clinical trial has demonstrated that administration of intranasal insulin stabilized or improved cognition, function, and cerebral glucose metabolism for adults with amnestic mild cognitive impairment or Alzheimer disease (Craft et al., 2011). The results from this study provide an impetus for further pharmacokinetic and mechanistic studies, even future clinical trials, which may significantly impact the clinical needs of subacute and chronic stroke treatment.

Conclusions

In conclusion, this study demonstrated, for the first time, that intranasal administration of tPA at the subacute phase after ischemic stroke significantly improved sensorimotor functional outcome and enhanced compensatory axonal remodeling, suggesting that tPA may also be used as a neurorestorative agent for neurological disability by promoting neuronal remodeling in the brain and spinal cord.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

tPA	tissue plasminogen activator
MCAo	middle cerebral artery occlusion
BDA	biotinylated dextran amine
CRT	corticorubral tract
CST	corticospinal tract
PAI-1	plasminogen activator inhibitor-I
MMP-9	matrix metalloproteinases-9

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Highlights

- We examined the effect of subacute intranasal tPA treatment after stroke in rats.
- High dose tPA improved functional recovery and enhanced both CRT and CST remodeling.
- tPA may be used as a neurorestorative agent for neurological disability.

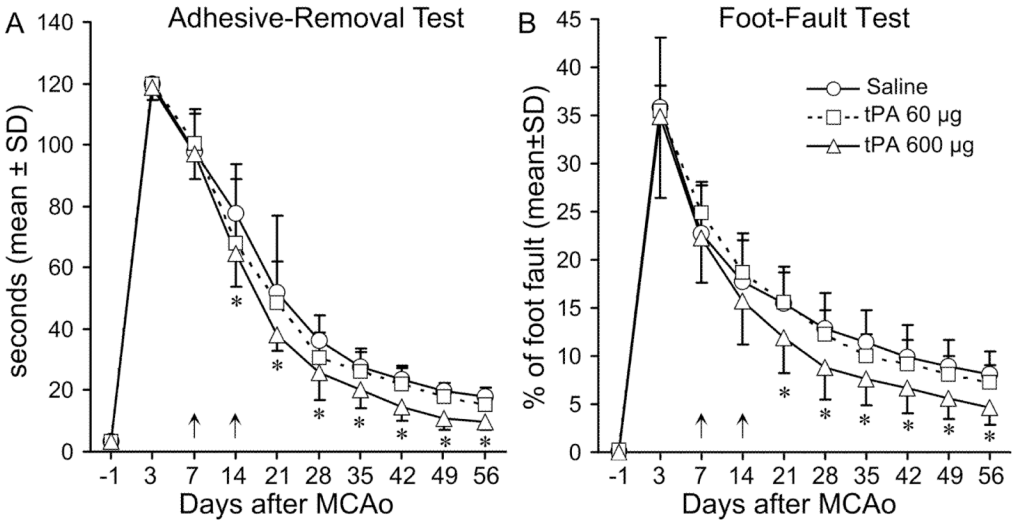

Figure 1.

Line graphs show the temporal profile of left forepaw behavioral deficit and recovery after right MCAo measured by the adhesive-removal test (A) and the foot-fault test (B). All rats exhibited severe neurological deficits after stroke, followed by progressive improvement with time. Intranasal tPA administration with high dose (600 µg, n=8), however, not low dose (60 µg, n=8), significantly enhanced functional recovery on the adhesive removing task starting 14 days and in the foot-fault test starting 21 days, respectively, compared with saline treated animals (n=8, $p<0.05$). Arrows in figures denote treatment times.

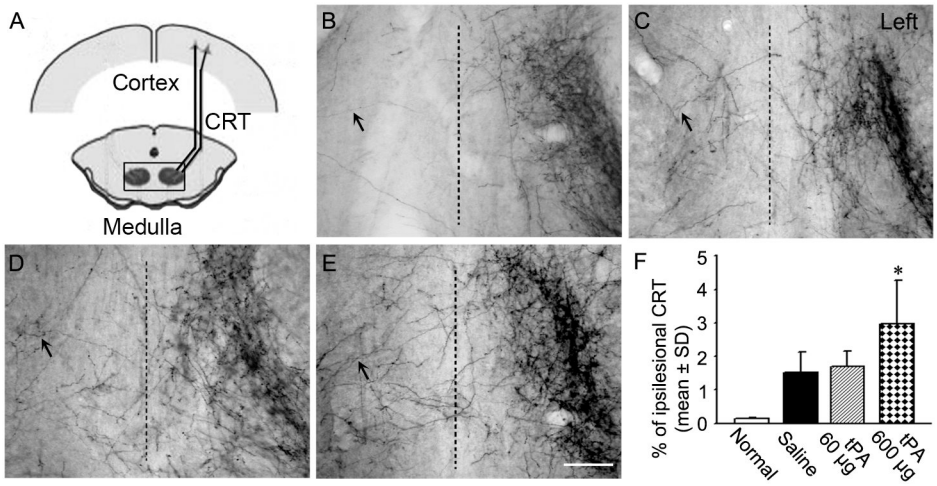

Figure 2.

High dose tPA intranasal treatment enhances corticorubral plasticity after stroke. The CRT arising from the left intact hemisphere were labeled by BDA cortical injections as shown in the schematic diagram (A). Corticorubral projection in a normal rat shows a unilateral innervation pattern (B). In MCAo rats treated with saline (C) or low dose tPA (60 μg, D), few BDA positive CRT fibers crossing the midline, while high dose tPA (600 μg, E) treated animals exhibit increased CRT fibers in the area of denervated red nucleus. Quantitative analysis of the percentage of BDA-labeled CRT axons in the denervated side of the red nucleus to the contralateral side demonstrated that high dose tPA significantly increased CRT axonal remodeling in comparison with control animals (F, $p<0.01$). Broken lines in A to D indicate the midline of the midbrain. Arrows indicate the right denervated red nucleus area. Scale bar=50 μm.

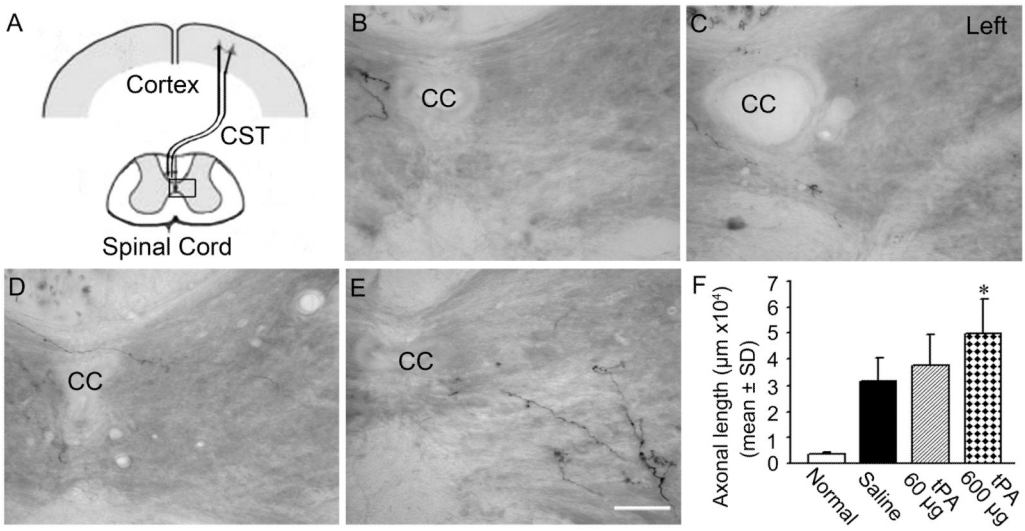

Figure 3.

Intranasal administration of tPA after stroke enhances CST axonal plasticity originating from the contralesional cortex at the cervical enlargement level. A schematic diagram shows the CST labeled with BDA injected into the left cortex (A). The normal rats showed a unilateral CST innervation manner (B). Scarce BDA-labeled midline-crossing CST axons extended toward to the ventral horn of the spinal gray matter in the denervated side of the cervical cord in rats subjected to MCAo treated with saline (C), low dose tPA (60 μg, D). Significantly increased BDA-positive CST axons were found in the denervated side of the spinal cord in high dose tPA (600 μg, E) treated animals. Quantitative data show that high dose tPA significantly promoted axonal sprouting in the denervated spinal cord, compared with control animals (F, $p<0.01$), while there was no significant difference between saline or low dose treated groups. A rectangle field in the spinal schematic drawing (A) indicates the position of the photomicrographs appearing in B to E. CC stands for central canal. Scale Bar=50 μm.

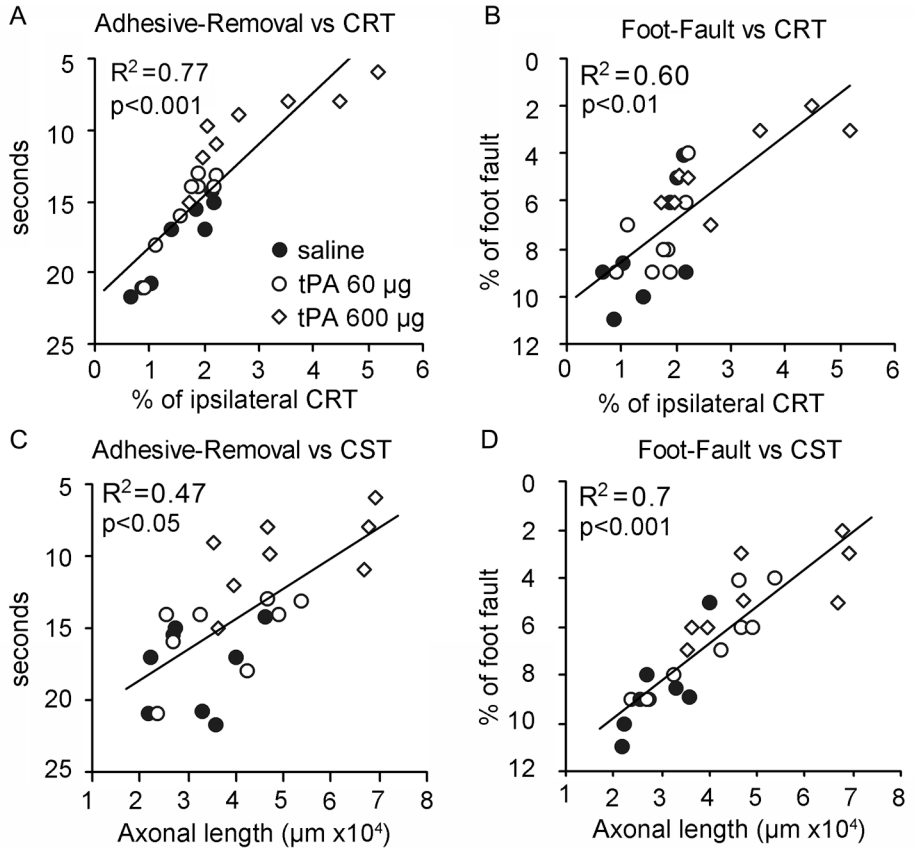

Figure 4.

Data point graphs of correlations between neural remodeling and behavioral recovery. The behavioral outcome assessed by both adhesive-removal test and foot-fault test were highly correlated ($p<0.05$) with the midline-crossing CRT axons in the denervated red nucleus and CST axons in the ventral horn of the denervated side of cervical spinal cord.

Table

Lesion volume among experimental groups

Groups	Infarct Volume (% of Contralesional Hemisphere)
Saline	38.2±7.2
tPA 60 μg	38.6±9.6
tPA 600 μg	37.4±6.3