

# Vampire Bat Salivary Plasminogen Activator (Desmoteplase) A Unique Fibrinolytic Enzyme That Does Not Promote Neurodegeneration

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**Background and Purpose**—Tissue-type plasminogen activator (tPA) promotes excitotoxic and ischemic injury within the brain. These findings have implications for the use of tPA in the treatment of acute ischemic stroke. The plasminogen activator from vampire bat (*Desmodus rotundus*) saliva (*D rotundus* salivary plasminogen activator [DSPA]; desmoteplase) is an effective plasminogen activator but, in contrast to tPA, is nearly inactive in the absence of a fibrin cofactor. The purpose of this study was to compare the ability of DSPA and tPA to promote kainate- and *N*-methyl-D-aspartate (NMDA)-induced neurodegeneration in tPA<sup>-/-</sup> mice and wild-type mice, respectively.

**Methods**—tPA<sup>-/-</sup> mice were infused intracerebrally with either tPA or DSPA. The degree of neuronal survival after hippocampal injection of kainate was assessed histochemically. Wild-type mice were used to assess the extent of neuronal damage after intrastratial injection of NMDA in the presence of tPA or DSPA. Immunohistochemistry and fibrin zymography were used to evaluate DSPA and tPA antigen or activity.

**Results**—Infusion of tPA into tPA<sup>-/-</sup> mice restored sensitivity to kainate-mediated neurotoxicity and activation of microglia. DSPA was incapable of conferring sensitivity to kainate treatment, even when infused at 10-fold higher molar concentration than tPA. The presence of tPA also increased the lesion volume induced by NMDA injection into the striatum of wild-type mice, whereas DSPA had no effect.

**Conclusions**—DSPA does not promote kainate- or NMDA-mediated neurotoxicity in vivo. These results provide significant impetus to evaluate DSPA in patients with ischemic stroke. (*Stroke*. 2003;34:537-543.)

**Key Words:** excitotoxins ■ neuronal death ■ stroke ■ tissue plasminogen activator

The synthesis and release of tissue-type plasminogen activator (tPA) from cells within the vascular system and the subsequent generation of plasmin from plasminogen are considered the primary means to degrade intravascular blood clots and fibrin deposits. The relative fibrin dependence of tPA led to its development as a therapeutic modality for acute myocardial infarction and ischemic stroke. Despite this, tPA still causes systemic plasminogen consumption and fibrinogenolysis when given to patients. Nonetheless, the clear benefits of tPA in reperfusion of vessels outweigh these negative features, and tPA is widely used in the treatment of myocardial infarction. The use of tPA in the treatment of ischemic stroke, however, is restricted. Although effective if administered within 3 hours of onset,<sup>1</sup> later use is contraindicated because of an increase in the risk of cerebral hemorrhage.

The biology of tPA extends beyond the vascular system because it is now evident that tPA performs critical functions within the central nervous system. tPA is expressed by neurons and microglia, participates in neuronal plasticity and

memory formation,<sup>2</sup> and contributes to the late phase of long-term potentiation.<sup>3</sup> In pathological states, tPA has been shown to enhance *N*-methyl-D-aspartate (NMDA)-mediated neurodegeneration<sup>4</sup> and to mediate kainate-induced neuronal death. Indeed, animals deficient in either tPA or plasminogen are resistant to kainate-mediated neurodegeneration,<sup>5-7</sup> while intrahippocampal infusion of tPA restores sensitivity to kainate injury.<sup>8</sup>

Numerous animal studies have been undertaken to assess the role of tPA in ischemic stroke. tPA administration has displayed differential effects on the extent of neuronal injury when different stroke models are used. The most compelling findings have stemmed from studies using tPA<sup>-/-</sup> mice subjected to transient<sup>9</sup> or permanent<sup>10</sup> ischemic injury by mechanical occlusion of the middle cerebral artery (MCA). The absence of endogenous tPA was correlated with smaller infarct volumes, while tPA administration into tPA<sup>-/-</sup> mice increased infarct volume,<sup>9,10</sup> indicating that tPA had a negative impact on outcome. In contrast, other mechanical MCA stroke studies using wild-type rats showed that tPA did not

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exacerbate neuronal injury.<sup>11,12</sup> The reason for this inconsistency is unclear. On the other hand, a number of animal stroke studies that have assessed tPA on the basis of its thrombolytic action have reported a protective effect of tPA.<sup>13,14</sup> The protective effect of tPA under these conditions clearly relates to its ability to promote reperfusion.

Curiously, one prominent *in vitro* study yielded an unexpected protective effect of tPA with respect to excitotoxic injury.<sup>15</sup> In this study tPA inhibited zinc-mediated neurotoxicity of rat neuronal cultures<sup>15</sup> in a manner independent of its proteolytic action, suggesting that its effect was independent of plasminogen activation. However, other *in vitro* studies using cultured cortical neurons from tPA<sup>-/-</sup> mice subjected to oxygen/glucose deprivation showed that the absence of tPA reduced neuronal death, while addition of exogenous tPA exacerbated neuronal loss.<sup>16</sup> Additionally, tPA has been shown to significantly amplify hemoglobin-induced neurotoxicity of rat neuronal cultures,<sup>17</sup> raising the possibility that tPA may have negative effects in hemorrhagic ischemic stroke.

With respect to ischemic injury, it appears that tPA has both positive and negative effects depending on the stroke model used.<sup>18</sup> While controversy still remains, a pattern is emerging that implicates tPA as a potential neurotoxic agent if permitted access to brain tissue after injury.<sup>19</sup> This has led to the notion that prolonged or delayed use of tPA in ischemic stroke patients could exacerbate neurodegeneration and destruction of the blood-brain barrier and thereby contribute to the increased risk of hemorrhage seen in these patients.

The limited fibrin specificity of tPA and its possible neurotoxic effects have fueled the search for other plasminogen activators displaying greater fibrin dependence and selectivity but that lack detrimental effects within the central nervous system. One candidate enzyme has been found in the saliva of the blood-feeding vampire bat, *Desmodus rotundus*. Four distinct proteases have been characterized and are referred to as *D rotundus* salivary plasminogen activators (DSPAs). Full-length vampire bat plasminogen activator (DSPA $\alpha$ 1) is the variant most intensively studied and exhibits >72% amino acid sequence identity with human tPA.<sup>20</sup> However, 2 important functional differences are apparent. First, unlike tPA, the DSPAs exist as single-chain molecules and are not cleaved into 2 chain forms.<sup>20</sup> Second, the catalytic activity of the DSPAs is critically dependent on a fibrin cofactor.<sup>21,22</sup> This is particularly conspicuous for DSPA $\alpha$ 1 because its catalytic efficiency increases 102 000-fold in the presence of fibrin yet only 8-fold by fibrinogen.<sup>21</sup> When the effect of fibrinogen is taken into account, the catalytic efficiency of DSPA $\alpha$ 1 is specifically increased 13 000-fold by fibrin. Since similar analyses have shown that the catalytic efficiency of tPA is specifically enhanced only 72-fold by fibrin,<sup>21</sup> it is clear that DSPA $\alpha$ 1 is substantially more fibrin dependent and fibrin specific than tPA. Furthermore, DSPA $\alpha$ 1 as well as DSPA $\alpha$ 2 has demonstrated faster and more sustained reperfusion than human tPA in animal models of arterial thrombosis.<sup>23</sup> Moreover, the DSPAs have been shown to cause less fibrinogenolysis<sup>22</sup> and antiplasmin consumption than tPA.

The aim of this study was to determine whether the exquisite fibrin dependence and specificity of DSPA $\alpha$ 1 would render it less likely to confer the neurotoxic effects described for tPA. To address this, we compared the ability of DSPA $\alpha$ 1 and tPA to promote kainate- and NMDA-mediated neurodegeneration in tPA<sup>-/-</sup> and wild-type mice, respectively.

## Materials and Methods

### Animals

Wild-type (c57/Black 6) and tPA<sup>-/-</sup> mice (c57/Black 6 background)<sup>24</sup> were used for this study. These experiments were performed in accordance with the guidelines of the National Health and Medical Research Council of Australia for live animal use. The experiments were approved by the Animal Ethics Committee, the Austin and Repatriation Medical Center Animal Welfare Committee, and the Physiology Animal Ethics Committee of Monash University.

### Protein Extraction From Brain Tissue

Mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (70 mg/kg) and then transcardially perfused with 0.1 mol/L PBS, and the brains were removed. The hippocampal region was dissected, weighed, and incubated in an equal volume (wt/vol) (approximately 30 to 50  $\mu$ L) of NP-40 lysis buffer (0.5% NP-40, 10 mmol/L Tris-HCl [pH 7.4], 10 mmol/L NaCl, 3 mmol/L MgCl<sub>2</sub>, 1 mmol/L EDTA) containing no protease inhibitors. Brain extracts were homogenized with the use of a hand-held glass homogenizer and left on ice for 30 minutes. Samples were centrifuged, and the pellet was discarded. Protein concentration was then determined (Bio-Rad reagent).

### Fibrin Zymographic Analysis of Proteases

The proteolytic activity in samples or brain tissue extracts was determined by fibrin zymography<sup>25</sup> with the use of either recombinant protein (up to 100 pmol) or brain tissue extracts (20  $\mu$ g). Gels were incubated in a 37°C humidified chamber until proteolysed zones appeared.

### Kainate-Induced Excitotoxicity

The kainate injury model was based on the approach previously described.<sup>5</sup> Animals were injected intraperitoneally with atropine (4 mg/kg), then anesthetized with pentobarbital. Mice were then placed into a stereotaxic frame, and a micro-osmotic pump (Alzet model 1007D) containing 100  $\mu$ L of 0.1 mol/L PBS, recombinant human tPA (alteplase, 0.12 mg/mL; 1.85  $\mu$ mol/L), or DSPA $\alpha$ 1 (1.85  $\mu$ mol/L; supplied by Berlex) was implanted subcutaneously between the shoulder blades. The pumps were connected via sterile tubing to a brain cannula and inserted through a burr hole made through the skull at coordinates bregma -2.5 mm, mediolateral 0.5 mm, and dorsoventral 1.6 mm to deliver the compound near the midline. The cannula was glued into position, and the pumps were allowed to infuse the respective solutions at rate of 0.5  $\mu$ L/h for a total of 7 days.

Two days after infusion of the proteases, mice were reanesthetized, and 1.5 nmol of kainate in 0.3  $\mu$ L of PBS was then injected unilaterally into the hippocampus. The coordinates were as follows: bregma -2.5 mm, mediolateral 1.7 mm, and dorsoventral 1.6 mm. The excitotoxin was delivered over 1 to 2 minutes. After kainate treatment, the injection needle remained at these coordinates for an additional 2 minutes to prevent reflux.

### Brain Processing

Five days after kainate injection, animals were transcardially perfused with 30 mL of PBS and then with 70 mL of a 4% paraformaldehyde solution; they were postfixed in the same fixative for 24 hours, followed by 24-hour incubation in 30% sucrose. Coronal sections (40  $\mu$ m) were cut on a freezing microtome and either stained

with thionin (BDH) to detect neurons or used for immunohistochemistry.

### Quantification of Neuronal Loss

Quantification of neuronal loss in the CA1 to CA3 hippocampal subfields was performed as described.<sup>5,6</sup> Five consecutive sections from the dorsal hippocampus from all treatment groups were prepared, and care was taken to ensure that the sections spanned the injection site and lesioned area. The hippocampal subfields (CA1 to CA3) on these sections were traced from camera lucida drawings of the hippocampus. The entire length of the subfields was measured by comparison to 1-mm standards traced under the same magnification. The length of tissue with viable pyramidal neurons (normal morphology) and length of tissue devoid of neurons (no cells present, no thionin staining) were determined. The lengths representing intact neurons and neuronal loss over each hippocampal subfield were averaged across sections.

### NMDA-Induced Excitotoxicity

This method was performed as previously described<sup>4</sup> with slight modifications. Anesthetized wild-type c57/Black 6 mice were placed in a stereotaxic frame. The left striatum was injected with a 1- $\mu$ L solution containing 50 mmol/L NMDA either alone or in combination with 46  $\mu$ mol/L (3  $\mu$ g) tPA or 46  $\mu$ mol/L DSPA $\alpha$ 1. tPA and DSPA $\alpha$ 1 (1  $\mu$ L; both at 46  $\mu$ mol/L) were also injected alone as controls. The injection coordinates were as follows: bregma -0.4 mm, mediolateral 2.0 mm, and dorsoventral 2.5 mm. The 1- $\mu$ L solutions were delivered over a 5-minute period at 0.2  $\mu$ L/min, and the needle remained for an additional 2 minutes after injection to minimize reflux of fluid. After 24 hours mice were transcardially fixed, and their brains were removed, postfixed for 24 hours, then incubated in 30% sucrose (see above). Then 40- $\mu$ m coronal sections were cut with the use of a freezing microtome and mounted onto gelatin-coated glass slides.

### Quantification of NMDA-Induced Lesion Area

The lesion area induced by NMDA in the striatal region was determined by the method described by Callaway et al.<sup>26</sup> This method does not require staining of sections and can be used to clearly distinguish between necrotic (appearing as a darkened area) and normal (appearing translucent) tissue. Ten consecutive coronal sections were prepared that spanned the lesion. The lesion area for each section was quantified with the use of a Micro Computer Imaging Device (MCID, Imaging Research Inc, Brock University), and the results were averaged.

### Immunohistochemistry

Sections were immersed in 3% H<sub>2</sub>O<sub>2</sub>/10% methanol for 5 minutes and then incubated with 5% normal goat serum for 60 minutes. Sections were incubated overnight with either an anti-MAC-1 antibody (1:1000; Serotec) to detect microglia or polyclonal anti-DSPA antibodies (provided by Schering AG) and then incubated in the appropriate biotinylated secondary antibodies (Vector Laboratories). This was followed by a final incubation in avidin/biotin complex (Vector Laboratories) for 60 minutes before visualization with 3,3'-diaminobenzidine/0.03% H<sub>2</sub>O<sub>2</sub>. Sections were then mounted on gelatin-coated slides.

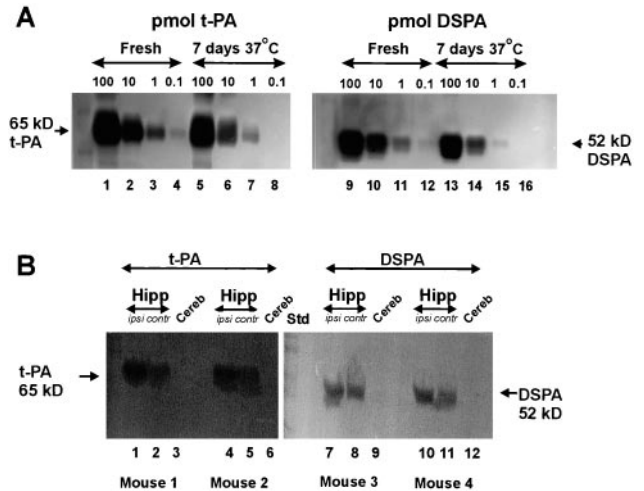
### Statistical Analyses

All values are expressed as mean  $\pm$  SEM. Statistical analyses were performed by 2-sample Student's *t* test. The null hypothesis was rejected at *P* < 0.05.

## Results

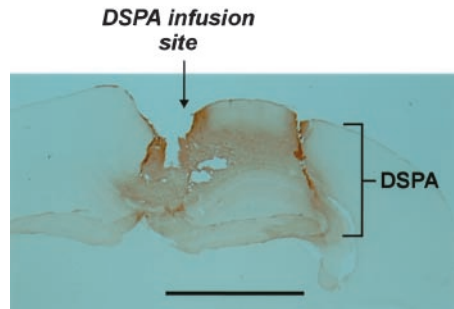
### tPA Activity and DSPA Activity Remain Stable After 7-Day Incubation at 37°C

To confirm that both DSPA and tPA retained proteolytic activity during the 7-day infusion period, aliquots of tPA and



**Figure 1.** A, tPA activity and DSPA proteolytic activity remain stable after 7-day incubation at 37°C. Samples of both tPA and DSPA were incubated at 37°C for 7 days. Serial 10-fold dilutions from 100 pmol were then performed, and proteolytic activity was assessed by fibrin zymography. Similar dilutions of freshly prepared tPA and DSPA were assessed in parallel as controls. The degree of proteolytic activity present in freshly prepared samples of either tPA or DSPA (lanes 1 to 4 and lanes 9 to 12, respectively) was compared with that seen in samples of tPA or DSPA incubated at 37°C for 7 days (lanes 5 to 8 and lanes 13 to 16, respectively). B, Recovery of tPA and DSPA proteolytic activity after infusion into the hippocampal region (Hipp) of tPA<sup>-/-</sup> mice. tPA<sup>-/-</sup> mice were infused with either tPA (mouse 1 and 2) or DSPA (mouse 3 and 4) for 7 days. Protein extracts prepared from both the ipsilateral (ipsi) and contralateral (contr) hippocampus were assessed for proteolytic activity by fibrin zymography. Extracts were also prepared from the cerebellum (cereb) as a negative control. As shown, DSPA and tPA proteolytic activity was recovered from the ipsilateral (lanes 1, 4, 7, 10) and contralateral (lanes 2, 5, 8, 11) regions of the hippocampus but was absent in the cerebellum (lanes 3, 6, 9, 12).

DSPA were incubated at 37°C for 7 days. Serial 10-fold dilutions of the samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis under nonreducing conditions, and proteolytic activity was assessed by zymographic analyses. Fresh aliquots of tPA and DSPA were used as positive controls. As shown in Figure 1A, only minor differences were seen in the degree of proteolytic activity of freshly prepared tPA or DSPA and the same concentrations of tPA and DSPA incubated for 7 days at 37°C (compare lanes 1 to 4 and lanes 9 to 12 with lanes 5 to 8 and 13 to 16, respectively). These results indicate that the proteolytic activity of both tPA and DSPA is relatively stable. Zymographic analysis verified that the proteases retained proteolytic activity after infusion into the brain. Brains of tPA<sup>-/-</sup> mice infused for 7 days with either DSPA or tPA were removed, and the ipsilateral and contralateral hippocampal regions were isolated. As shown in Figure 1B, tPA proteolytic activity of the predicted molecular weight (65 kDa) was recovered from both the ipsilateral and contralateral sides (lanes 1 to 2 [mouse 1] and lanes 4 to 5 [mouse 2]). Similarly, proteolytic activity of 52 kDa was recovered within the ipsilateral and contralateral regions of the hippocampus of mouse 3 and 4 infused with DSPA (lanes 7, 8, 10, and 11). This indicates that the infused proteases not only retained



**Figure 2.** Detection of DSPA in the hippocampal region of tPA<sup>-/-</sup> mice. Brains were removed from tPA<sup>-/-</sup> mice after the 7-day infusion of DSPA. DSPA antigen was detected in coronal sections with the use of a polyclonal anti-DSPA antibody. Bar=2 mm.

their activity in the brain but also had diffused bilaterally within the hippocampal region. No activity was detected in the extract prepared from the cerebellum from any tPA<sup>-/-</sup> mouse (lanes 3, 6, 9, and 12).

### Immunohistochemical Assessment of DSPA

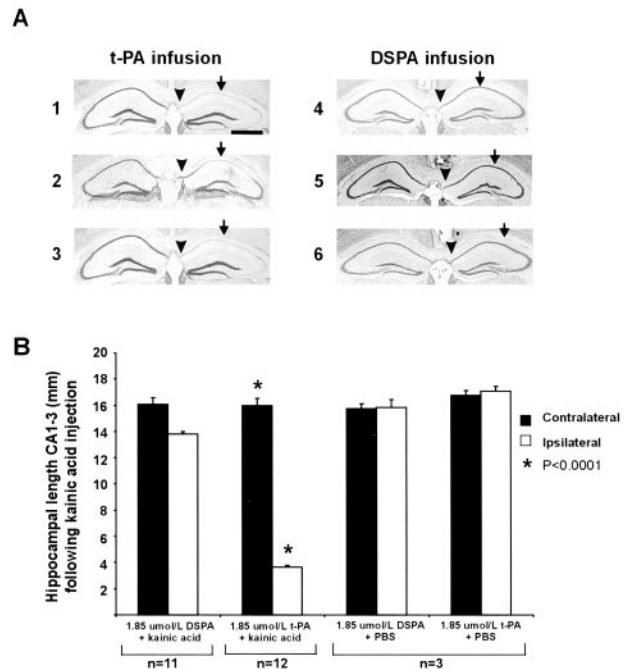
Coronal sections of brains of tPA<sup>-/-</sup> mice that had been infused with DSPA were assessed by immunohistochemistry. DSPA antigen was detected within the hippocampal and cortical regions, with the most pronounced staining apparent around the infusion site (Figure 2). This result confirms that the infused DSPA is soluble and had diffused within the mouse brain.

### DSPA Infusion Does Not Restore Kainate-Mediated Neurodegeneration In Vivo

tPA<sup>-/-</sup> mice were infused intrahippocampally with either 1.85  $\mu\text{mol/L}$  tPA or DSPA. Two days later animals were injected with kainate and then killed on day 7. The degree of neuronal staining was assessed in at least 4 consecutive coronal sections by thionin staining. Infusion of recombinant tPA clearly promoted kainate-mediated neurodegeneration within the ipsilateral hippocampal region. In contrast, infusion of the same concentration of DSPA into the hippocampal region of tPA<sup>-/-</sup> mice did not alter the sensitivity of these animals to kainate. Figure 3A shows representative thionin-stained sections of 3 mice infused with either protease. Of the 11 mice infused with DSPA, some minor neurodegeneration was seen in 2 of these animals, whereas marked neurodegeneration was seen in all 12 mice infused with tPA. Quantification of the extent of neuronal survival within the ipsilateral and contralateral sides of the hippocampus for all mice is shown in Figure 3B. Also as shown in Figure 3B, infusion of either protease alone followed by intrahippocampal injection of PBS did not promote neurodegeneration (n=3). On the basis of these findings, it is apparent that when tPA and DSPA $\alpha$ 1 are infused at equimolar concentrations, only tPA is capable of restoring sensitivity to kainate-induced neurodegeneration.

### Titration of DSPA and tPA into Mouse Hippocampus

The kainate-injury experiments were repeated with one tenth the concentration of tPA (0.185  $\mu\text{mol/L}$ ) and a 10-fold higher

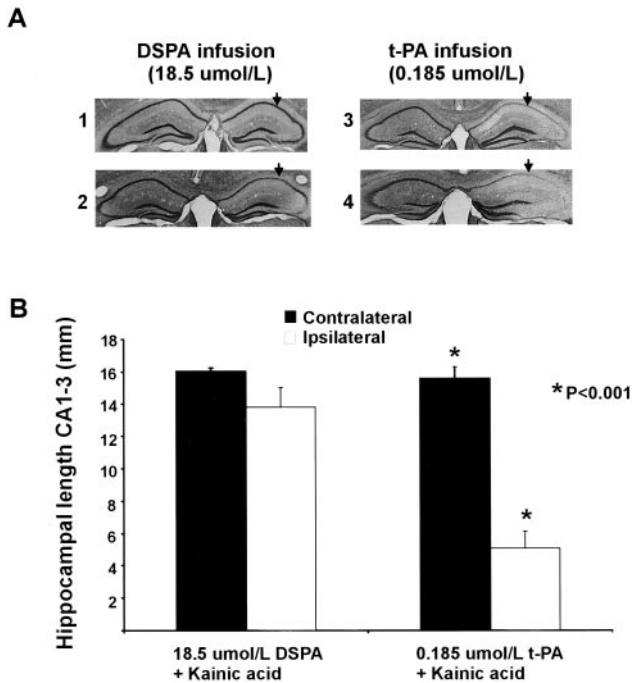


**Figure 3.** A, DSPA does not restore sensitivity to kainate-mediated neurodegeneration in tPA<sup>-/-</sup> mice. tPA<sup>-/-</sup> mice infused with either tPA or DSPA (1.85  $\mu\text{mol/L}$ ; arrowhead) were injected with kainate unilaterally into the hippocampus (arrow). Representative sections from 3 mice from each group are shown. The degree of neuronal loss in coronal sections was assessed by thionin staining. The degree of cell death is evident by the unilateral loss of neuronal staining within the hippocampal subfields. Bar=1 mm. B, Quantification of the degree of neuronal cell survival in the ipsilateral and contralateral regions of the hippocampus of tPA<sup>-/-</sup> mice infused with either tPA (n=12) or DSPA (n=11) followed by a unilateral injection of kainate. Results obtained from mice infused with either protease but injected with PBS are also shown (n=3). Error bars indicate SEM.

amount of DSPA (18.5  $\mu\text{mol/L}$ ). As shown in Figure 4, infusion of 18.5  $\mu\text{mol/L}$  DSPA caused only minor neuronal loss after kainate treatment, whereas the lower concentration of tPA (0.185  $\mu\text{mol/L}$ ) still restored sensitivity to kainate treatment (Figure 4A). This experiment was performed with the use of 3 mice in each group, and the combined quantified data are presented in Figure 4B. The minor degree of neuronal loss seen with the higher concentration of DSPA is virtually identical to that seen when the lower concentration of DSPA is used, suggesting that this minor effect is not due to DSPA. Indeed, tPA<sup>-/-</sup> mice have been shown to display a minimal response to kainate injury<sup>5</sup>. Taken together, these findings further highlight the relative inability of DSPA to promote sensitivity to kainate in tPA<sup>-/-</sup> mice.

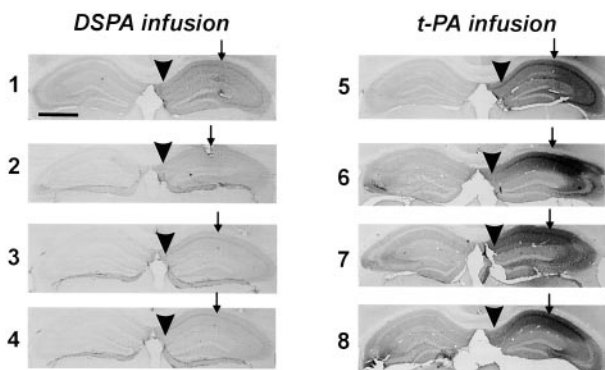
### Activation of Microglia

The restoration of kainate sensitivity in tPA<sup>-/-</sup> mice after tPA infusion has been shown to result in microglial activation.<sup>8</sup> To assess the degree of microglial activation after tPA or DSPA infusion and subsequent kainate treatment, coronal sections of mice were subjected to immunohistochemistry for activated microglia with the use of the anti-Mac-1 antibody. As shown in Figure 5, 3 of the 4 tPA<sup>-/-</sup> mice infused with

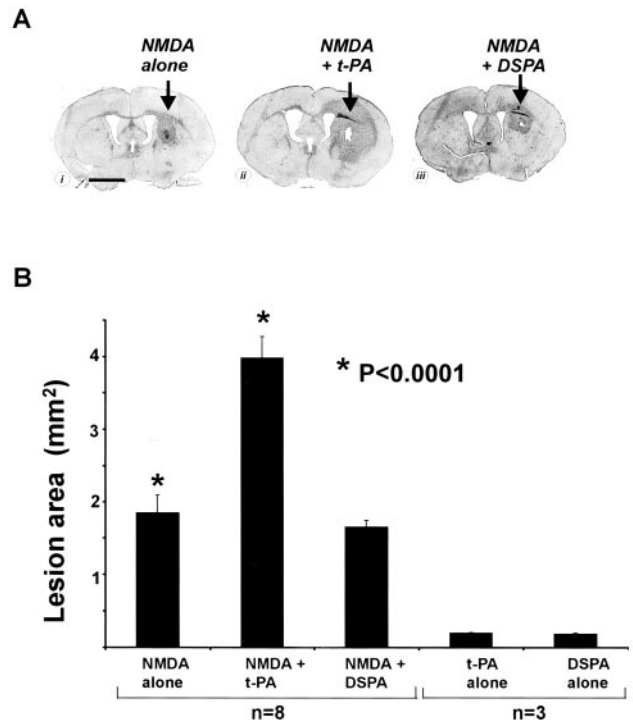


**Figure 4.** A, A 10-fold increase in the concentration of DSPA does not restore sensitivity of  $tPA^{-/-}$  mice to kainate treatment.  $tPA^{-/-}$  mice were infused with either 18.5  $\mu\text{mol/L}$  DSPA (sections 1 and 2) or 0.185  $\mu\text{mol/L}$  tPA (sections 3 and 4) (arrowhead). Mice were injected with kainate (arrow), and the animals were killed on day 7. A representative section from 2 mice of each group is presented. Bar=1 mm in section 4. B, The hippocampal length (contralateral and ipsilateral) after kainate injection of  $tPA^{-/-}$  mice infused for 7 days with either 18.5  $\mu\text{mol/L}$  DSPA or 0.185  $\mu\text{mol/L}$  tPA is presented. The data were derived from 3 mice infused with either protease and include the 2 mice presented in A. Error bars indicate SEM ( $n=3$ ).

DSPA before kainate treatment displayed minimal increase in Mac-1 staining (Figure 5). In contrast, all 4 mice infused with tPA before kainate injection produced a marked increase in microglial activation after 7 days (Figure 5). Infusion of either DSPA or tPA alone did not result in activation of microglia (data not shown). Higher magnification confirmed the specificity of staining for microglia (not shown).



**Figure 5.** Infusion of tPA activates microglia after kainate injection.  $tPA^{-/-}$  mice were infused with either DSPA or tPA and subsequently injected with kainate. Brain coronal sections were prepared, and the degree of microglial activation was assessed by staining for Mac-1. Bar=1 mm in first section.



**Figure 6.** A, DSPA does not enhance NMDA-mediated neurodegeneration in wild-type mice. The striatal region of wild-type mice was injected with kainate either alone (i) or in the presence of 46  $\mu\text{mol/L}$  tPA (ii) or DSPA (iii). Lesioned areas in consecutive unstained sections were photographed following previously described methods.<sup>24</sup> A representative section from each animal is presented. The lesioned area appears as a darkened zone. Bar=2 mm in i. B, Quantification of the striatal lesion. The lesioned area was determined as previously described.<sup>24</sup> Wild-type mice were used in each group (NMDA alone, NMDA+tPA, NMDA+DSPA;  $n=8$ ). Control mice were injected into the striatum with either tPA or DSPA alone ( $n=3$ ).

### Effect of tPA and DSPA on NMDA-Mediated Neurodegeneration in Wild-Type Mice

Injection of tPA into the striatum of normal mice enhances the neurodegenerative effects of the glutamate analogue NMDA.<sup>4</sup> This approach was used to compare the effects of tPA and DSPA (46  $\mu\text{mol/L}$ ) at promoting NMDA-induced neuronal cell loss in wild-type mice. As shown in Figure 6A, injection of tPA with NMDA markedly increased the lesion area compared with that produced by NMDA alone. In contrast, coinjection of the same concentration of DSPA did not cause any further increase in lesion size compared with NMDA alone. Injection of either tPA or DSPA alone did not result in any detectable neurodegeneration (not shown). These experiments were performed with the use of 8 mice in each treatment group, and composite quantitative results are presented in Figure 6B. These results illustrate that the presence of tPA results in 2.15-fold increase in the NMDA-induced lesion size ( $P<0.0001$ ). In contrast, DSPA produced no further increase in lesion size after NMDA treatment.

To verify that the injection of DSPA had indeed infused into the hippocampal region, immunohistochemistry was performed on coronal sections with the DSPA antibody. As shown in Figure 7, DSPA had indeed perfused into the striatal region.



**Figure 7.** DSPA antigen is detected after injection into the mouse striatum. The striatal region of wild-type mice was injected with NMDA+DSPA. After 24 hours, brains were removed, and DSPA antigen was detected by immunohistochemistry. Bar=2 mm.

### Discussion

We have compared the effects of tPA and DSPA using 2 different animal models of neurodegeneration: (1) using the kainate model of neurodegeneration in the hippocampus of tPA<sup>-/-</sup> mice and (2) using NMDA-induced lesion of the striatum of wild-type mice. Data obtained with the kainate injury model confirmed initial reports that highlight the critical need for tPA to promote kainate neurotoxicity and to activate microglia. However, under the same experimental conditions, infusion of an equimolar concentration of DSPA did not restore sensitivity to kainate, nor did it promote microglial activation. Indeed, infusion of a 10-fold higher concentration of DSPA (18.5  $\mu\text{mol/L}$ ) into tPA<sup>-/-</sup> mice still did not restore sensitivity to kainate treatment, whereas a 10-fold lower concentration of tPA (0.185  $\mu\text{mol/L}$ ) was still effective. Hence, tPA is at least 100-fold more potent than DSPA at promoting neurodegeneration after kainate treatment.

Our second model of neurodegeneration was established to assess the potential effects of both tPA and DSPA at promoting NMDA-mediated neurodegeneration in wild-type mice. The lesion area after injection of NMDA into the mouse striatum was increased 2.15-fold in the presence of tPA. In contrast, coinjection of DSPA $\alpha$ 1 did not exacerbate the neurotoxic effect of NMDA. Although our immunohistochemistry confirmed that DSPA was indeed present within the striatal region, similar experiments performed to visualize tPA after injection into the striatal region were unsuccessful because of excessive background staining of the antibodies. It could be argued that the greater lesion area produced in response to tPA+NMDA injection compared with DSPA+NMDA is a consequence of differential diffusion of the 2 proteases within the striatal region. However, tPA and DSPA are both highly soluble proteins with similar molecular weights and are unlikely to display differences in diffusion rates. Nonetheless, immunohistochemical data for tPA are required to formally rule out this possibility.

Our results have shown that DSPA does not enhance neurodegeneration even in the presence of plasma proteins that have diffused into the lesioned area as a consequence of NMDA-mediated neurodegeneration.<sup>27</sup> Taken together, our findings establish that DSPA $\alpha$ 1 is essentially an inert pro-

tease when present within the murine central nervous system and is incapable of promoting neurotoxicity induced by either kainate or NMDA.

The lack of effect of DSPA in this environment is most likely a consequence of the different biochemical properties of this vampire bat–derived protease. The catalytic activity of DSPA is exquisitely dependent on the presence of fibrin but not fibrinogen as a cofactor. Although not investigated directly in this study, it seems likely that fibrin formation does not occur to any significant extent after diffusion of fibrinogen into the brain following the breakdown of the blood-brain barrier. It also remains to be determined whether fibrin-activated DSPA would indeed act like tPA after excitotoxic injury. There has been much speculation that the neurotoxic effects of tPA are, at least in part, unrelated to its ability to activate plasminogen.<sup>4</sup> tPA has been shown to cleave the NR1 subunit of the NMDA receptor in a plasmin-independent manner, and it is likely that other substrates are direct targets for tPA. Whether fibrin-activated DSPA also recognizes these different substrates remains to be determined.

This study defines DSPA $\alpha$ 1 as a plasminogen activator that does not exhibit the inherent neurotoxic properties associated with tPA. It would be interesting to determine and compare the effects of DSPA $\alpha$ 1 and tPA at promoting neuronal loss after excitotoxic or ischemic injury when these proteases are administered intravenously.

The inability of DSPA to promote neurodegeneration provides substantial impetus to assess the efficacy of this protease in stroke patients. In this context, it is of interest that a clinical trial using DSPA (desmoteplase) in acute stroke (DIAS trial) has recently commenced in Europe (sponsored by PAION GmbH, P.I. Werner Hacke).

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### References

1. National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group. Tissue plasminogen activator for acute ischemic stroke. *N Engl J Med.* 1995;333:1581–1587.
2. Seeds NW, Williams BL, Bickford PC. Tissue plasminogen activator induction in Purkinje neurons after cerebellar motor learning. *Science.* 1995;270:1992–1994.
3. Baranes D, Lederfein D, Huang YY, Chen M, Bailey CH, Kandel ER. Tissue plasminogen activator contributes to the late phase of LTP and to synaptic growth in the hippocampal mossy fiber pathway. *Neuron.* 1998; 21:813–825.
4. Nicole O, Docagne F, Ali C, Margaill I, Carmeliet P, MacKenzie ET, Vivien D, Buisson A. The proteolytic activity of tissue-plasminogen activator enhances NMDA receptor-mediated signaling. *Nat Med.* 2001; 7:59–64.
5. Tsirka SE, Gualandris A, Amaral DG, Strickland S. Excitotoxin-induced neuronal degeneration and seizure are mediated by tissue plasminogen activator. *Nature.* 1995;377:340–344.

6. Tsirka SE, Rogove AD, Bugge TH, Degen JL, Strickland S. An extracellular proteolytic cascade promotes neuronal degeneration in the mouse hippocampus. *J Neurosci.* 1997;17:543–552.
7. Tsirka S, Rogove AD, Strickland S. Neuronal cell death and tPA. *Nature.* 1996;384:123–124.
8. Rogove AD, Siao C, Keyt B, Strickland S, Tsirka SE. Activation of microglia reveals a non-proteolytic cytokine function for tissue plasminogen activator in the central nervous system. *J Cell Sci.* 1999;112:4007–4016.
9. Wang YF, Tsirka SE, Strickland S, Stieg PE, Soriano SG, Lipton SA. Tissue plasminogen activator (tPA) increases neuronal damage after focal cerebral ischemia in wild-type and tPA-deficient mice. *Nat Med.* 1998;4:228–231.
10. Nagai N, Mol MD, Lijnen HR, Carmeliet P, Collen D. Role of plasminogen system components in focal cerebral ischaemic infarction. *Circulation.* 1999;99:2440–2444.
11. Klein GM, Li H, Sun P, Buchan AM. Tissue plasminogen activator does not increase neuronal damage in rat models of global and focal ischaemia. *Neurology.* 1999;52:1381–1384.
12. Meng W, Wang X, Asahi M, Kano T, Asahi K, Ackerman RH, Lo EH. Effects of tissue type plasminogen activator in embolic versus mechanical models of focal cerebral ischaemia in rats. *J Cereb Blood Flow Metab.* 1999;19:1316–1321.
13. Kilic E, Hermann DM, Hossmann KA. Recombinant tissue plasminogen activator reduces infarct size after reversible thread occlusion of middle cerebral artery in mice. *Neuroreport.* 1999;10:107–111.
14. Tabrizi P, Wang L, Seeds N, McComb JG, Yamada S, Griffin JH, Carmeliet P, Weiss MH, Zlokovic BV. Tissue plasminogen activator (tPA) deficiency exacerbates cerebrovascular fibrin deposition and brain injury in a murine stroke model. *Arterioscler Thromb Vasc Biol.* 1999;19:2801–2806.
15. Kim Y-H, Park J-H, Hong SH, Koh J-H. Nonproteolytic neuroprotection by human recombinant tissue plasminogen activator. *Science.* 1999;284:647–650.
16. Nagai N, Yamamoto S, Tsuboi T, Ihara H, Urano T, Takada Y, Terakawa S, Takada A. Tissue-type plasminogen activator is involved in the process of neuronal death by oxygen-glucose deprivation in culture. *J Cereb Blood Flow Metab.* 2001;21:631–634.
17. Wang X, Asahi M, Lo EH. Tissue-type plasminogen activator amplifies hemoglobin-induced neurotoxicity in rat neuronal cultures. *Neurosci Lett.* 1999;274:79–82.
18. Strickland S. Tissue plasminogen activator in nervous system function and dysfunction. *Thromb Haemost.* 2001;86:138–143.
19. Traynelis SF, Lipton SA. Is tissue plasminogen activator a threat to neurons? *Nat Med.* 2001;7:17–18.
20. Kratzschmar J, Haendler B, Langer G, Boidol W, Bringmann P, Alagon A, Donner P, Schleuning WD. The plasminogen activator family from the salivary gland of the vampire bat *Desmodus rotundus*: cloning and expression. *Gene.* 1991;105:229–237.
21. Bringmann P, Gruber D, Liese A, Toschi L, Kratzschmar J, Schleuning WD, Donner P. Structural features mediating fibrin selectivity of vampire bat plasminogen activators. *J Biol Chem.* 1995;270:25596–25603.
22. Toschi L, Bringmann P, Petri T, Donner P, Schleuning WD. Fibrin selectivity of the isolated protease domains of tissue-type and vampire bat salivary gland plasminogen activators. *Eur J Biochem.* 1998;252:108–112.
23. Mellott MJ, Stabilito II, Holahan MA, Cuca GC, Wang S, Li P, Barrett JS, Lynch JJ, Gardell SJ. Vampire bat salivary plasminogen activator promotes rapid and sustained reperfusion without concomitant systemic plasminogen activation in a canine model of arterial thrombosis. *Arterioscler Thromb.* 1992;12:212–221.
24. Carmeliet P, Schoonjans L, Kieckens L, Ream B, Degen J, Bronson R, De Vos R, van den Oord JJ, Collen D, Mulligan RC. Physiological consequences of loss of plasminogen activator gene function in mice. *Nature.* 1994;368:419–424.
25. Granelli-Piperno A, Reich E. A study of proteases and protease inhibitor complexes in biological fluids. *J Exp Med.* 1974;148:223–234.
26. Callaway JK, Knight MJ, Watkins DJ, Beart PM, Jarrott B, Delaney PM. A novel, rapid, computerized method for quantitation of neuronal damage in a rat model of stroke. *J Neurosci Methods.* 2000;102:53–60.
27. Chen Z-L, Indyk JA, Bugge TH, Kombrinck KW, Strickland S. Neuronal death and blood-brain barrier breakdown after excitotoxic injury are independent processes. *J Neurosci.* 1999;19:9813–9820.