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Award Number: DAMD17-99-1-9502

TITLE: Tissue Plasminogen Activator (tPA) Mediates Neurotoxin-Induced Cell Death and Microglial Activation

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REPORT DATE: July 2001

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2001	Final (1 Jul 99 - 30 Jun 01)

4. TITLE AND SUBTITLE
Tissue Plasminogen Activator (tPA) Mediates Neurotoxin-Induced Cell Death and Microglial Activation

5. FUNDING NUMBERS
DAMD17-99-1-9502

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Neuronal death occurs in the brain during development and in pathological conditions, like Alzheimer's disease and stroke. Tissue plasminogen activator (tPA), a protease converting plasminogen to plasmin, is necessary for neurodegeneration. In mice lacking tPA ($tPA^{-/-}$), neurons are resistant to neurotoxic death. Delivery of tPA into $tPA^{-/-}$ mice restores susceptibility to neuronal death, indicating that tPA is neurotoxic in the context of excitotoxic injury. Although tPA is synthesized by neurons, the increase in tPA upon injury derives primarily from activated microglia, the immune cells of the brain. Microglia in $tPA^{-/-}$ mice demonstrate reduced activation. Using tPA as tool, we are determining whether microglia are neuroprotective or neurotoxic, and what are the cell types involved in the sequence of events that lead from injury to neuronal death. We have established primary cultures and obtained information from them on the source of tPA that initiates neurotoxin-induced cell death (Spec. aim 2), and have narrowed the region of tPA that promotes microglial activation (Spec. aim 1). Since exaggerated neurodegeneration is evident in pathological conditions, understanding the underlying mechanisms could prove beneficial for interfering with the pathologies.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Neurotoxin	62
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Styliani-Anna E. Tsirka

PI - Signature Date

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Introduction

The application entitled "Tissue plasminogen activator (tPA) mediates neurotoxin-induced cell death and microglial activation" proposed to address 2 questions / objectives.

1. **How does tPA mediate microglial activation? Does the activation involve classical signal transduction pathways, namely tyrosine phosphorylation of a specific receptor and a cascade of activation of kinases, leading ultimately to transcriptional upregulation of critical immediately early or early genes? How does modulation of tPA / microglial activation affect neurotoxicity?**

We are in the process of defining the mechanism by which tPA mediates microglial activation using deletion mutants and eventually single point mutants, first in a cell culture system and then in vivo, in mice. We have now confirmed that tPA binds on to annexin II on the surface of microglia via its finger domain. We are doing a more detailed biochemical characterization of the interaction. We have determined that there is a co-receptor present on microglial cells that aids the signal transduction cascade, and our evidence points towards LRP for that role. Furthermore we are characterizing the signaling events using RNA interference techniques we knock-down annexin II in microglia and look for differences in activation after LPS or glutamate stimulation.

2. **What is the source(s) of tPA that mediates microglial activation and neuronal degeneration in response to excitotoxin-stimulation in culture?**

We have proposed to mix-and-match neuronal and microglial cultures from wild-type and tPA-deficient genotypes in the presence of excitotoxins and investigate the progress and mechanism by which neuronal death proceeds. We have established the mixing-and-matching protocols, and we have found that both paracrine and autocrine mechanisms are involved in microglial activation, with autocrine being the biologically more relevant.

Body

Objective 1) Which domain of tPA is required for microglial activation?
Objective 2) What is the source(s) of tPA that mediates microglial activation and neuronal degeneration in response to excitotoxin-stimulation in culture?

Microglia are the resident immune cells of the brain, migrating into it from the bone marrow during development. After completing the migration and early functional roles, they revert to a resting state. Upon injury, microglia become activated. The activation process consists of several steps, including migration to the site of injury, proliferation in the vicinity of neuronal injury, changes in gene expression, and phagocytosis. Both neurotoxic and neuroprotective roles have been attributed to microglia in the activated state. However, despite the recent explosion of literature concerning microglia, most research has been focused on the effects that microglia have on *neuronal* survival or the progression of specific diseases. Very little is known about microglial activation and physiology. We proposed to investigate these areas and related mechanistic questions using a variety of approaches and tools that we have in hand. The questions that were addressed are as follows:

- **Does microglial activation occur via an autocrine or paracrine pathway?** Microglial activation is mediated by tissue plasminogen activator (tPA), a serine protease that catalyzes the proteolytic conversion of plasminogen to the active protease plasmin. tPA is made both by microglia and neurons and plays important roles both in microglial activation and in neuronal

degeneration. In principle, each cell type could mediate its own response to tPA through an autocrine pathway. However, although gradients of chemokines function over a long distance to recruit microglia, tPA released locally by damaged neurons could act in a paracrine manner to limit full microglial activation to a locally restricted region. Additionally, control of neurodegeneration might be restricted in scope by having the microglia mediate it in a paracrine manner through release of additional and larger amounts of tPA at the site of injury. We have evidence that these events occur through a paracrine pathway. Microglial activation and neurodegeneration can be modeled *in vitro* using primary mixed cortical or hippocampal cultures, or reconstituted cultures consisting of purified neurons combined with purified microglia. To characterize the source of tPA that is required for microglial activation along with addition injury signals, homogeneous primary neurons and homogeneous microglia prepared from wild-type mice, $tPA^{-/-}$ mice, and transgenic $tPA^{-/-}$ mice expressing tPA under the control of a microglial or a neural promoter will be combined and assayed for microglial activation and neuronal degeneration. Finally, microglial activation and neuronal degeneration will be assessed *in vivo* in the transgenic mice after excitotoxic injury. Depending on the outcome of these experiments, methods to modify and interfere with the relevant tPA source could be devised to affect the progression of neuronal death in disease or injury.

- **What are the signaling events following the interaction of tissue plasminogen activator with its microglial receptor, annexin II**? Since the contribution of activated microglia to neurotoxicity is crucial and tPA activates microglia through a non-catalytic pathway which involves binding to a receptor, now identified as annexin II, we will now precisely define the sites of interaction between tPA and its receptor and examine the ensuing downstream signaling events that lead to microglial activation. Pharmacological blockade of the receptor or other steps along the pathway could offer novel approaches to inhibit microglial activation.
  Microglial activation follows stimulation / injury or infection of the CNS. We have been monitoring as outputs of microglial activation the changes in a cell surface marker (the glycoprotein F4/80), a secreted cytokine (TNFα), and the production of nitric oxide (NO, and its conversion to nitrites, NO_2^-). Neither the extracellular cues nor the intracellular signaling cascade that lead to microglial activation are well established. To analyze the pathways that confer microglial activation, we have been using adult *ex vivo* and neonatal primary microglial cultures, as well as *in vivo* experimental paradigms that result in activation.

Generation of an immortalized tPA-deficient microglial cell line:

For the culture experiments described below, we have been using primary microglia derived from wild-type and tPA- and plasminogen-deficient mice, prepared as described in the following section. While these primary cultures provide a very useful and relevant tool for studying microglial activation, they are also relatively labor-intensive, slow, and expensive since they depend on timed pregnancies of the animals, it takes two weeks to establish a microglial culture, and the microglial cell numbers recovered depend on the number of pups or embryos used. For some of the studies described below, it is imperative to have a large number of microglial cells that would be impractical to prepare as primary cultures. We also use the immortalized N9 mouse microglial cell line for initial experiments, and realized some time ago that it would be quite valuable to have a correspondingly immortalized tPA-deficient microglial cell line. Using tissue culture supernatants from N9 cells (which contain the secreted 3RV viral complex capable of transforming uninfected cells) and a protocol provided to us by

Dr. Righi (CNR Milan; a co-author on the original N9 cell paper), we have now established a $tPA^{-/-}$ immortalized microglial cell line (A. Kokkosis, C.-J. Siao and S.E. Tsirka, manuscript in preparation).

The cells are currently being characterized. We have confirmed that they are devoid of tPA activity, that they express several microglial markers as expected (see Figure 1), and that they respond to LPS stimulation with attenuated activation, comparable to primary $tPA^{-/-}$ microglial cells.

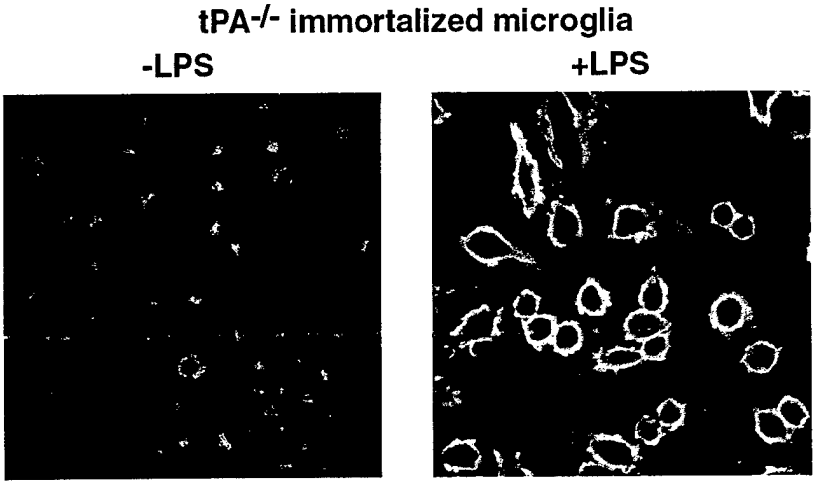


Figure 1: Expression of an established microglial marker in $tPA^{-/-}$ immortalized microglial cells. F4/80 was detected by immunohisto-chemistry in $tPA^{-/-}$ immortalized microglial cells. Note the upregulation of F4/80 expression after stimulation of microglial with LPS, very comparable to that seen *in vivo* in hippocampal sections from $tPA^{-/-}$ mice or in primary $tPA^{-/-}$ microglial cultures (but about 5.5-fold less than would be observed for wild-type microglia or the N9 cell line).

The addition of tPA promotes full microglial activation upon LPS stimulation to the level observed for wild-type N9 cells, as assessed by quantitative western blot analysis (using the FluorImager) and by measuring the concentration of NO produced using our standard fluorescent DAN assay. We will use these cells in some of our exploratory experiments for the intracellular events that lead to microglial activation.

Microglial activation – Autocrine versus paracrine; establishment of a primary co-culture system:

When injury occurs in the mammalian brain, the microglia become activated. The timing and sequence of events that lead to microglial activation remain unclear. It is possible that activation is initiated via an autocrine mechanism, namely that some endogenous microglial sensor detects the injury and sets the signaling cascade in motion. Alternatively, a paracrine mechanism may exist wherein the injured neuronal cells communicate with the microglia by secreting diffusible factors and the microglia locally present respond by becoming activated.

To evaluate whether signaling between neurons and microglia is necessary for full microglial activation and the subsequent neurodegeneration, we have established a co-culture system starting with the purified cell types (Zhang and Fedoroff, 1996).

The neonatal primary culture system that we use to examine these types of questions was established in our lab several years ago. It involves the preparation and use of purified microglia or neurons (or mixed or reconstituted cultures) (see Rogove et al., 1999; Rogove and Tsirka, 1998). Briefly, to prepare microglia, cortices are dissected out of one-day old pups. The meninges are removed and the tissue is subjected to limited trypsinization; then it is triturated and plated in DMEM with 10% FBS and 40 mg/l gentamycin onto poly-L-lysine coated 75mm^2 dishes at a density of two cortices per dish. These mixed cultures are maintained at 37°C for two weeks. At that point they are incubated with 12mM lidocaine for 10 min and finally rocked gently by hand. The microglial cells detach and are collected, washed, and plated onto poly-L-lysine coated flasks. Microglia attach very

rapidly to the plate. Other contaminating cells are removed by rinsing the plates with Hanks buffered salt solution (HBSS) 10 minutes after the cell seeding. Microglia are then put back into fresh medium. Using this protocol and judging by immunocytochemistry with specific microglial markers [Mac1 (marker for resting and activated microglia), F4/80 (marker for activated macrophages / microglia) and 5-D-4 (marker for activated microglia)], the microglial cultures are greater than 95% pure.

Neuronal cultures are prepared as follows: Hippocampi are dissected out from E17.5 mouse embryos as previously described (Rogove and Tsirka, 1998). In brief, pregnant C57/Bl6 mice carrying E17.5 day embryos are sacrificed, and the embryos are removed into chilled HBSS. The hippocampi are dissected and trypsinized gently (0.25% trypsin in HBSS) at 37°C for 15 minutes. Single cell suspensions are made by trituration and plated onto poly-L-lysine-coated coverslips at a density of 100,000 cells/ cm^2 in Neurobasal medium with B27 supplements, 25 μM glutamate, 0.5 mM L-glutamine and 10 g/L gentamycin sulfate (all from Life Technologies). The medium is changed 4 hours after the initial plating.

Since tPA is produced by both cell types and affects both processes, we first set out to address the consequence of mixing and matching neuronal and microglial cultures from primary cells isolated reciprocally from wild-type mice versus mice lacking the gene for tPA ($tPA^{-/-}$). Neuronal cells are plated on poly-L-lysine coated 6-well plates, whereas microglia are plated on inserts with 8μm pores. Our initial experiments indicate that full microglial activation is observed only when neuronal tPA (in addition to other factors released in the excitotoxic setting) is present to stimulate the microglial cells, thus supporting a model consisting of a paracrine mechanism for microglial activation (Siao and Tsirka (2002); see Figures 2 and 3). Moreover, neurodegeneration is not observed unless the microglia can also make tPA, suggesting that the signaling is paracrine in nature in both directions (not shown).

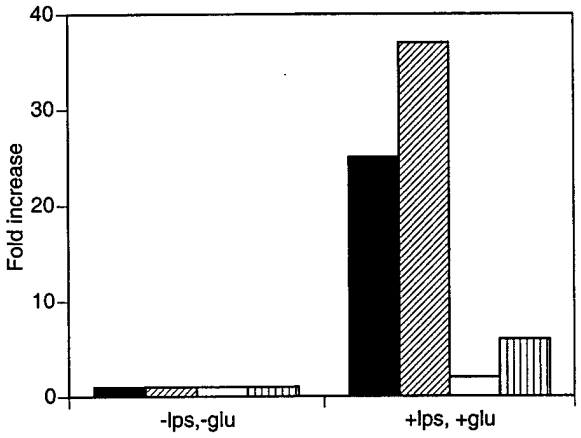


Figure 2: (Above) Quantification of the increase in F4/80 expression (a marker for microglial activation) using western blot analysis of a 'mixing-and-matching' experiment. Neurons were exposed to 25 μM glutamate prior to exposure to microglia, and microglia were stimulated with 100 ng/ml LPS prior to the addition to the neurons, ■, wild-type neurons and microglia (both sources of tPA); □, wild-type neurons, tPA-deficient microglia (only neuronal tPA present); □, tPA-deficient neurons, wild-type microglia (only microglial tPA present);□, tPA-deficient neurons and microglia (no tPA present).

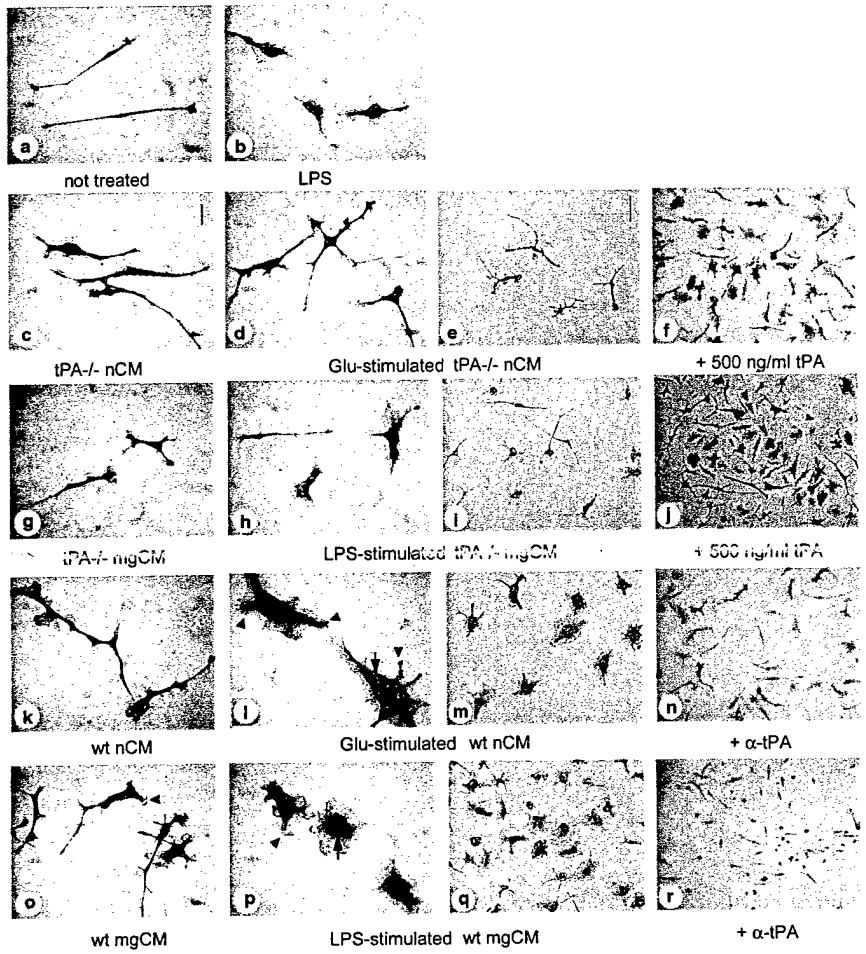


Figure 3: (Left) $tPA^{-/-}$ microglia in culture respond to signals released from injured neurons and microglia by becoming activated and changing morphology. Cells were treated as described below, and the expression of F4/80 was examined. a. Control, untreated $tPA^{-/-}$ microglia (400x); b. 100 ng/ml LPS (400x); c. Conditioned medium prepared from quiescent $tPA^{-/-}$ neurons (400x); d. Conditioned medium prepared from glutamate-stimulated $tPA^{-/-}$ neurons (400x); e. Same as d (100x); f. Conditioned medium prepared from glutamate-stimulated $tPA^{-/-}$ neurons additionally containing tPA at 500 ng/ml (100x); g. Conditioned medium prepared from quiescent $tPA^{-/-}$ microglia (400x); h. Conditioned medium prepared from LPS-stimulated $tPA^{-/-}$ microglia. The LPS was partially removed using a polymyxin B column prior to addition of the CM to the responding $tPA^{-/-}$ microglia (400x); i. Same as h (100x); j. Conditioned medium prepared from LPS-stimulated $tPA^{-/-}$ microglia additionally containing tPA at 500 ng/ml. The LPS was partially removed using a polymyxin B column prior to addition of the CM to the responding $tPA^{-/-}$ microglia (100x); k. Conditioned medium prepared from quiescent wild-type neurons (400x); l. Conditioned medium prepared from glutamate-stimulated wild-type neurons (400x); m. Same as l (100x); n. Conditioned medium prepared from glutamate-stimulated wild-type neurons additionally containing a-tPA antibody (100x); o. Conditioned medium prepared from quiescent wild-type microglia (400x); p. Conditioned medium prepared from LPS-stimulated wild-type microglia. The LPS was partially removed using a polymyxin B column prior to addition of the CM to the responding $tPA^{-/-}$ microglia (400x); q. Same as p (100x); q. Conditioned medium prepared from LPS-stimulated wild-type microglia additionally containing a-tPAantibody. The LPS was partially removed using a polymyxin B column prior to addition of the CM to the responding $tPA^{-/-}$ microglia (100x). Arrowheads, membrane ruffles or pseudopods. Arrows, phagocytic vacuoles. (Appendix).

Taken together, these results show that wild-type but not $tPA^{-/-}$ microglia and neurons, once primed / injured, release a factor(s) in the medium that activates $tPA^{-/-}$ microglia. Since this factor can be replaced by purified tPA and can be blocked by anti-tPA antiserum, it is most likely tPA. The results demonstrate that both neurons and microglia are capable of mediating microglial activation through the release of tPA. tPA alone does not suffice to activate microglia; instead, a second signal is required, which can be provided by LPS priming or by factors other than tPA released by stimulated microglia and neurons.

We also performed tPA add-back experiments. We detected an increase in F4/80 immunoreactivity after culturing $tPA^{-/-}$ microglia with LPS and increasing concentrations of tPA. From baseline unstimulated levels, we found a 0.8-fold increase with the lowest dose of tPA added (5 ng/ml), which increased to 2-fold with 50 ng/ml tPA, and to 2.4-fold with 500 ng/ml tPA, indicating that the level of activation is dependent on the concentration of tPA (data not shown). These tPA add-back experiments

also suggest that there is a threshold for microglial reactivity to injury (Figure 2 in Siao and Tsirka, in ; in the Appendix).

It is widely accepted that microglia are the sensors of injury in the brain (Kreutzberg, 1996), and we propose that tPA acts as a microglial sensor molecule. Normally, the level of tPA released into the extracellular space is tightly regulated. tPA is stored in intracellular vesicles and is only released by Ca^{2+} influx during neuronal depolarization (Gualandris et al.,1996). Another level of control is afforded by the presence of several serine protease inhibitors in many regions of the brain (e.g., plasminogen activator inhibitors-1, –2 and neuroserpin). We and others have shown that tPA plays an important role in physiological processes such as neurite outgrowth (Seeds et al., 1999; Wu et al., 2000). This highly localized secretion of tPA, and hence localized activation of plasmin, is probably necessary for neurites to degrade ECM structures so that they can reach their targets. Therefore, neurons must be able to tolerate significant local increases in tPA activity surrounding them without sending out injury / death and inflammatory signals. Like neurons, microglia must also be able to differentiate between high levels of tPA (alone, which may be physiological) and high levels of tPA and some other factor, the combination of which is now an injury signal for microglia to become activated. In most of our experimental conditions, that other factor is bacterial LPS, whose presence is uncommon in the normal CNS. Physiological candidates would include pro-inflammatory molecules such as TNF-α and IL-1β that play important roles in activating microglia in culture (Meda et al., 1996).

Despite the utility of this model system, there are respects in which it may not predict accurately the *in vivo* signaling requirements. For example, the setting of contact of the two cell types is artificial, and the relative numbers of neurons and microglia in contact are arbitrary; moreover, other possibly relevant cells are absent (i.e., astrocytes and oligodendrocytes). To validate these results, we have generated transgenic mice carrying tPA under the control of a neuronal or a microglial promoter to examine the signaling pathways in live animals while isolating the cellular source of tPA.

***Generation of transgenic mice*:** The first transgenic line expresses the tPA cDNA under the control of the1.7 kb neurofilament L (NF-L) neuronal promoter (Ivanov and Brown, 1992). The second line expresses the tPA cDNA under the control of the 5.3 kb CSF-1 receptor (CSFR) microglial promoter (Miyazaki et al., 1993). The transgenic mice were made in the tPA-deficient background; therefore tPA is expressed only in the intended cell type (neurons or microglia).

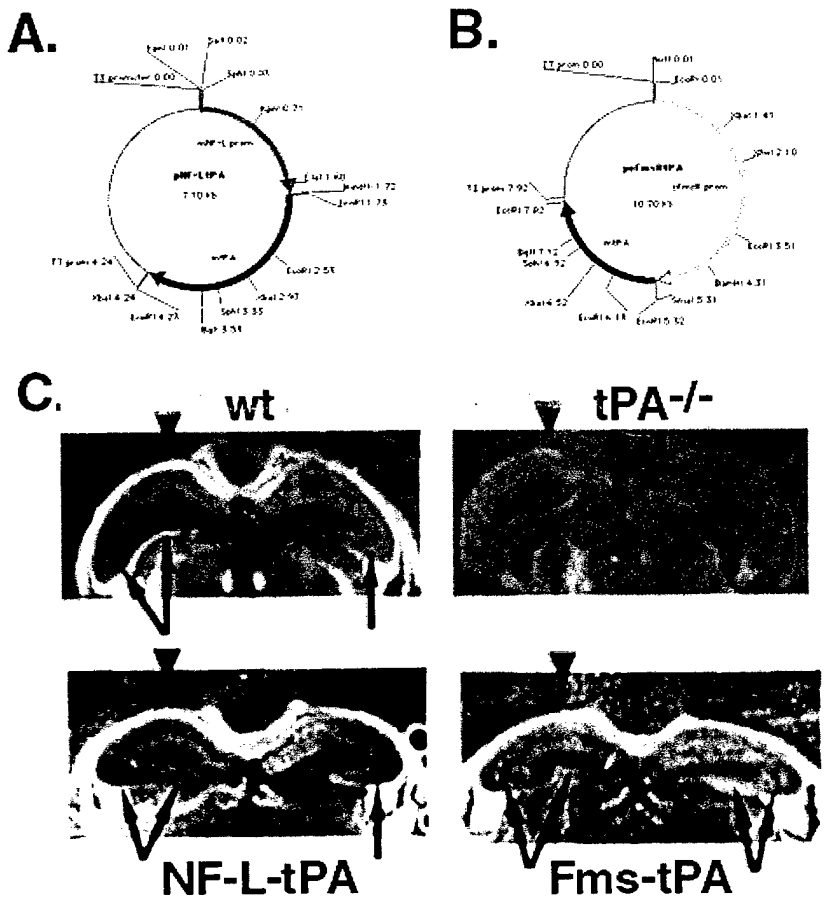


Figure 4: Constructs used to generate the NF-L-tPA (A) and cFMS-tPA (B) transgenic mice. In panel C, *in situ* zymographic assays show the presence of active tPA in both transgenic lines (arrows), as compared to lack of activity in $tPA^{-/-}$ mice. A table is also provided with the tPA activity measured in the brains of the different lines. See in the Appendix.

Genotype	tPA spec. activity (pg/sec/mg protein)
wt	9.63
Neuro-tPA line A	1.92
Neuro-tPA line B	12.53
Neuro-tPA line C	8.67
tPA-/-	0
Microglial-tPA line E	8.72
Microglial-tPA line J	10.92
Microglial-tPA line K	1.53
Microglial-tPA line L	not determined yet

We have characterized many transgenic founders and have established three separate lines for the neuronal transgene and four for the microglial one that express tPA at various levels compared to wild-type animals, thus providing a 'dose-response' curve to examine the amount of tPA required to be present in the CNS parenchyma to observe microglial activation and neurodegeneration. Experiments presently in progress involve subjecting the mice to our unilateral intrahippocampal kainate injection protocol to determine whether neuronal cell death proceeds normally through our excitotoxic paradigm, as is observed in wild-type but not $tPA^{-/-}$ mice (Siao and Tsirka, manuscript in preparation). As seen in Figure 4 above, the transgenic mice express active tPA in the brain. These mice will serve as a valuable tool to address multiple questions concerning the role of tPA in these processes.

The NF-L-tPA (neuronal tPA expression) and the Fms-tPA (microglial tPA expression) transgenic mice exhibit cell type-specific expression (Figs. 5 and 6).

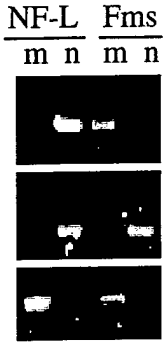


Figure 5: RT-PCR analysis of mRNAs isolated from neuronal and microglial cultures prepared from NF-L-tPA (A line) and Fms-tPA (E line) mice. The PCR used tPA (tPA), neuron-specific (APP; amyloid precursor protein), and microglia-specific (F4/80) primers. m: microglia; n: neurons.

Figure 6:

Expression of tPA mRNA in NF-L-tPA and Fms-tPA transgenic mice. Coronal hippocampal sections were hybridized with a Dig-UTP labeled tPA RNA probe. Neuronal tPA expression was evident in the CA1 region in NF-L-tPA animals, whereas punctate-microglial like expression was observed in the CA3 region and corpus callosum of Fms-tPA mice. Wild-type and $tPA^{-/-}$ mice were used for positive and negative controls, respectively.

Role of cell-type specific tPA release in kainate-elicited microglial activation and neurodegeneration. Each line was subjected to unilateral intrahippocampal injection of kainate following which neuronal death was assessed. The Fms-tPA mice exhibited earlier onset of cell death than wild-type mice. The NF-L-tPA mice exhibited a delayed onset. Neither line exhibited the extent of neuronal death observed in wild-type animals, suggesting a requirement for tPA expression by each cell type. We are presently crossing the transgenic lines together to generate 'wild-type-like' mice to confirm that full wild-type-like susceptibility is recovered (Fig. 7).

Genotype	Days post inj. (# of mice)	% neurons lost ± SEM
Wild-type	0.25 (2)	9.5 ± 6.7
	0.5 (2)	63.1 ± 7.5
	1 (4)	62.7 ± 4.2
	2 (1)	78.5 ± 0
	3 (2)	86.4 ± 4.3
	5 (3)	86.4 ± 1.3
NF-L-tPA	0.25 (2)	0
	0.5 (4)	18.6 ± 4.2
	1 (5)	29.3 ± 4.9
	2 (2)	53.9 ± 9.7
	3 (3)	59.2 ± 10.7
	5 (3)	69.9 ± 6.9
Fms-tPA	0.25 (3)	0
	0.5 (3)	39.9 ± 12
	1 (3)	62.2 ± 2.2
	2 (2)	60.8 ± 4.9
	3 (3)	50.1 ± 6.6
	5 (2)	60.7 ± 7.8

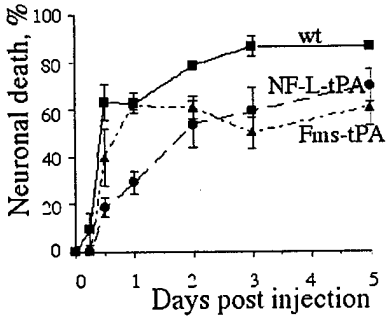


Figure 7: At different time-points after injection the animals were analyzed and the extent of cell death quantitated.

Microglial activation was followed in parallel using F4/80, 5D4, and Iba1 antibodies. Despite the lesser degree of neurodegeneration, the NF-L-tPA mice exhibited larger numbers of activated

microglia beginning at12 h after KA injection in comparison with the microglial tPA-expressing mice. Taken together, these findings suggest that microglia are most effectively activated by pathways triggered by the neuronal release of tPA, whereas neuronal degeneration is most effectively promoted by microglial release of tPA, and that both pathways are important for the full neurodegenerative response. This work has been submitted for publication.

***Microglial receptor for tPA*:** Catalytically-inactive tPA mediates microglial activation, implying that non-proteolytic domains in tPA act to trigger a microglial signaling pathway (Rogove et al., 1999). The tPA protein, aside from its catalytic serine-protease domain at the carboxy-terminus, also contains a finger domain, an EGF-like domain, and two kringle motifs, all of which are thought to potentially participate in protein-protein interactions. To investigate the mechanism by which tPA mediates microglial activation, the four non-protease structural domains of tPA (finger, growth-factor-like, kringle 1, and kringle 2) were expressed and added individually to primary mixed and pure microglial cultures. In addition, we obtained (from Genentech, Inc) and engineered deletion mutants each lacking one of the domains. Mutant 1 (ΔFinger) was made by PCR by directly introducing an ATG at the 5' end of the growth factor domain. Mutants 2 (ΔGrowth Factor),3 (ΔKringle1), and 4 (ΔKringle 2) were generated using PCR: the relevant domain(s) were deleted (looped out). The deletion mutants were confirmed by sequencing. All of the mutants were cloned into the pCGN vector (tPA expression is controlled by the CMV promoter) and co-transfected into HeLa cells along with pMC1neo, following which stable transformants were selected.

Mutant tPA proteins are being purified from the supernatants of the stable cell lines using affinity chromatography (on **E**rythrina **T**rypsin **I**nhibitor Sepharose column (Qiu et al., 1998), and the purity and concentrations of the recombinant proteins are assessed using SDS-PAGE.

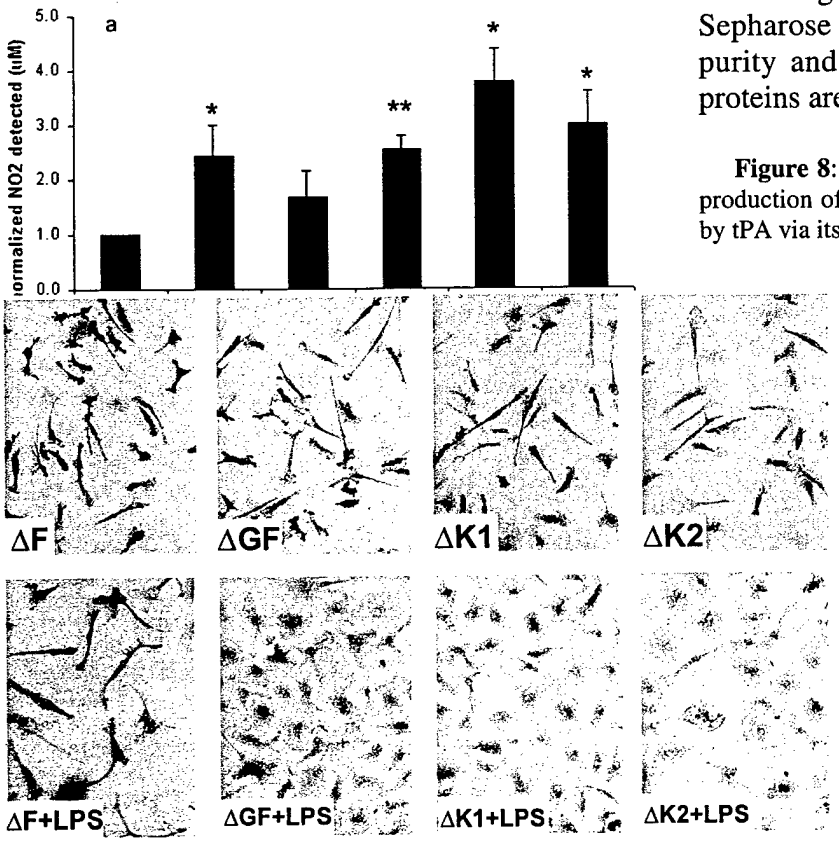


Figure 8: Microglial activation (measured here by the production of NO in tPA-deficient microglia) is mediated by tPA via its finger domain.

The purified deletion mutants are added individually to $tPA^{-/-}$-microglia and are tested for their ability to restore LPS-induced microglial activation. The level of microglial activation is assessed by morphology, by quantitation of the levels of F4/80 by western blot analysis, and by measurement of production of NO. Wild-type and $tPA^{-/-}$ microglia serve as controls for setting the upper and lower limits in the NO_2^- measurements.

Figure 9: $tPA^{-/-}$ microglia were isolated

as described above. Recombinant mutant tPA proteins (500 ng) were added for 24 hr prior to (or in the absence of) LPS stimulation (as in Rogove et al., 1999). Microglial activation is assessed here by morphology, i.e., the ability of activated microglia to acquire a rounded / macrophage-like shape. Note the attenuated microglial activation evident when ΔF and LPS are added to $tPA^{-/-}$ microglia (paper in Appendix).

Our data using the deletion mutants obtained from Genentech (Siao and Tsirka, 2002, see Figure 8) suggest that it is the Finger domain that binds to the microglial cell surface and promotes microglial activation: When the ΔF mutant is added to LPS-stimulated $tPA^{-/-}$ microglia, the levels of F4/80 and NO remain almost baseline. Furthermore, addition of the ΔK1 mutant resulted in even stronger stimulation of the LPS-treated $tPA^{-/-}$ microglia, suggesting that this domain may normally act to regulate the binding and microglial activation conferred by the Finger domain (see Figures 8 and 9).

Since the finger domain is involved in protein-protein interactions, we looked for binding partners / receptors for tPA that would mediate microglial activation. Two candidate receptors for tPA have been already identified and characterized in other tissues and cell types:

Annexin II: Annexin II, a Ca^{2+} and phospholipid binding protein, belongs to a family of proteins characterized by a highly conserved set of α-helical repeats in the C-terminus that mediate membrane binding (Mollenhauer et al., 1997). It has been shown to be the receptor for tPA on cultured endothelial cells (Hajjar and Hamel, 1990; Hajjar et al., 1994). It was demonstrated that annexin II is expressed by non-neuronal cells in the human hippocampus and that its expression is upregulated in pathological conditions, such as Alzheimer's disease, hypoxia, ischemia, or chronic seizure-related injury (Eberhard et al., 1994). Annexin II is regulated by Ca^{2+}, as is the LPS-induced microglial activation, and it is also involved in signal transduction and could thus participate in the pathway that leads to all of the changes associated with microglial activation. Using RT-PCR, we have determined that annexin II is present in microglial cells (Fig. 10; see also Siao and Tsirka (2002) in Appendix). Furthermore, using immunohistochemistry and confocal microscopy with an antibody provided by Dr. Blake Pepinsky (Biogen), annexin II appears to co-localize with tPA on the surface of microglial cells (Rogove and Tsirka, unpublished data). The binding between annexin II and tPA has been characterized extensively in endothelial cells (Hajjar et al., 1994). It involves the fibronectin-like 'finger' domain of tPA and is sensitive to reducing agents, possibly via disulphide bridges. Annexin II has also been shown to enhance the tPA-mediated conversion of plasminogen to plasmin in a fibrin-independent manner (Hajjar et al., 1994), a particularly interesting finding, given that we recently showed the absence of fibrin(ogen) in the mouse brain under normal circumstances and that plasmin mediates neuronal death in a non-fibrin-dependent manner (Tsirka et al., 1997).

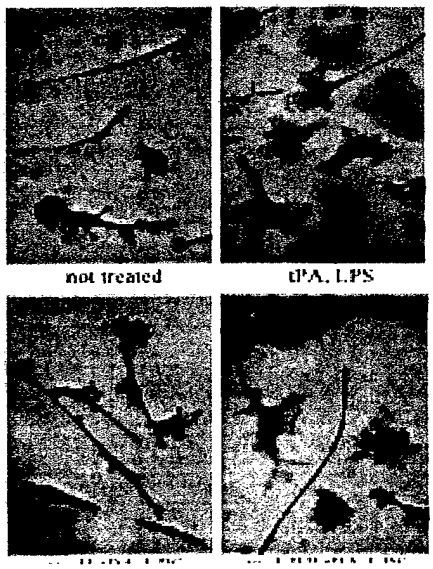


Figure 10: A. Expression of annexin II by primary mouse microglia but not neurons using RT-PCR. *Lane 1*: ladder; *lane 2*: mouse annexin II amplified from microglial cDNA; *lane 3*: mouse annexin II fails to be amplified from neuronal cDNA; *lane 4*: amyloid precursor protein (APP) amplified from neuronal cDNA (positive control). **B.** Western blot analysis for annexin II in protein extracts from primary microglial and neuronal extracts. **C.** $tPA^{-/-}$ microglia were cultured alone or with α-annexin II or α-LRP antibodies for 1 h, then 500 ng/ml tPA was added for another hour, then LPS was added and the culture continued overnight. Activation was examined using F4/80 immunocytochemistry on fixed cells.

A manuscript describing these findings was published in the Journal of Neuroscience (Siao and Tsirka, "Tissue plasminogen activator activates microglia via its finger domain through annexin II", Appendix).

We have recently generated anti-tPA polyclonal antibodies (A.D. Rogove, G. Spentzouris and S.E. Tsirka, unpublished data) by immunizing $tPA^{-/-}$ mice with recombinant tPA protein. We are characterizing currently these antibodies; they detect tPA in brain sections (immunohistochemistry) readily and efficiently immunoprecipitate tPA from primary cultures.

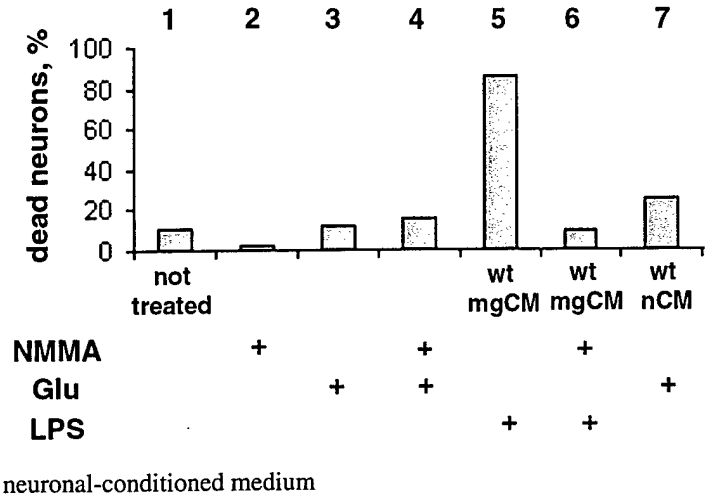
Low density lipoprotein receptor-related protein (LRP): tPA could trigger microglial activation through interaction with a cell surface receptor, such as LRP. LRP binds with high affinity and endocytoses different ligands including apoE, α_2-macroglobulin, and tPA. LRP is expressed in the brain (Bu et al., 1992a; Bu et al., 1992b), but its exact cellular site of synthesis has not been identified. Cell culture experiments and *in situ* mRNA hybridization analysis of human brain sections suggest possible localization on neurons or astrocytes (Bu et al., 1992b; Nykjær et al., 1992). LRP was shown recently to be expressed by neuronal cells (Zhuo et al., 2000), and thus it is possible that LRP is a mediator of cell-cell communication between neurons and microglia, and in particular between neuronal LRP and microglial tPA. Our preliminary experiments suggest both neuronal and microglial localization of LRP (see Fig. 11, and data not shown), using an anti-LRP antibody that was kindly provided by Dr. Bu, Washington University).

Figure 11: Immunolocalization of LRP on primary microglial cells in culture. Primary microglial cultures were fixed and incubated with anti-LRP polyclonal antibody.

Finally, there are scavenger receptors (LRP-like) on macrophage membranes that bind lipoproteins and become up-regulated by LPS. Note that one of the mRNAs we have found through microarray analysis to be increased after kainate injection is α_2-macroglobulin, one of the ligands for the LRP receptor.

We began addressing this question by assessing activation as a function of co-culturing neurons and microglia purified from wild-type and / or $tPA^{-/-}$ mice. We have now generated evidence that microglia can be activated in a paracrine manner by either injured neuronal cells or by previously stimulated neighboring microglia. This evidence was obtained by assessing activation after adding conditioned medium from the stimulated cell types to $tPA^{-/-}$ microglia (Siao and Tsirka, 2002).

Furthermore, the activated microglia can then mediate the death of neighboring neurons, as shown in Fig. 12. In brief, purified $tPA^{-/-}$ neurons (column 1) do not exhibit glutamate-induced neurotoxicity (column 3) under our culture conditions, nor do they exhibit much response to conditioned medium (CM) prepared from glutamate-injured wild-type neurons (column 7), even though there is sufficient tPA in the CM to activate $tPA^{-/-}$ microglia. The $tPA^{-/-}$ neurons do undergo apoptosis, however, in response to conditioned medium prepared from LPS-stimulated wild-type microglia (column 5), but

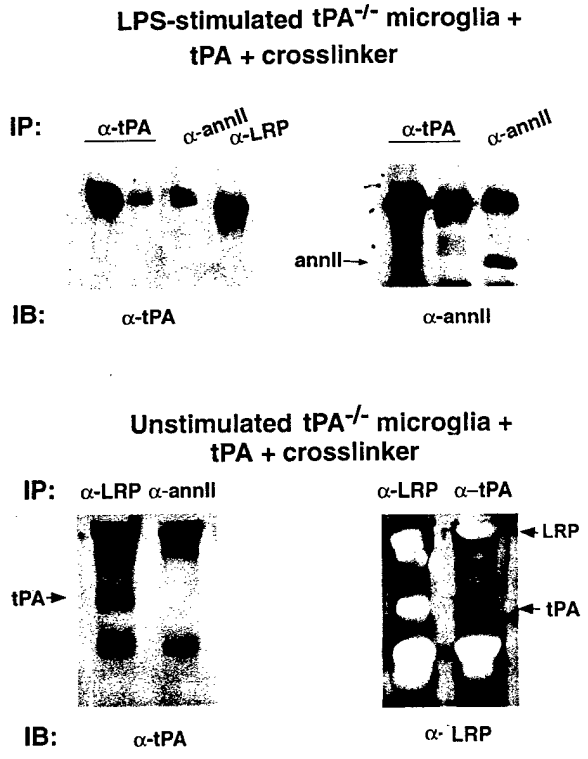


not from LPS-stimulated tPA$^{-/-}$ microglia (not shown). The neuronal cell death response requires tPA, but also clearly involves factors released by microglia in addition to tPA, since the Nitric Oxide Synthase inhibitor NMMA protected the neurons (column 6).

Figure 12: Quantitation of death of tPA$^{-/-}$ neurons in the presence of conditioned media from neurons or microglia. mgCM, (LPS-stimulated) microglial-conditioned medium; nCM, (Glutamate-stimulated)

We will look for these additional injury signals produced by activated microglia. Our existing evidence indicates that tPA alone is not sufficient to activate microglia, or to kill neurons. In both cases there is an additional factor(s) necessary. It is critical to have tPA of course, since in the absence of tPA neither phenomenon occurs like in wild-type animals.

We are further exploring the pathways of cell death by combining results from biochemical approaches as described above, microarray data, and the proposed *in vivo* experiments using the transgenic mice we have already generated.

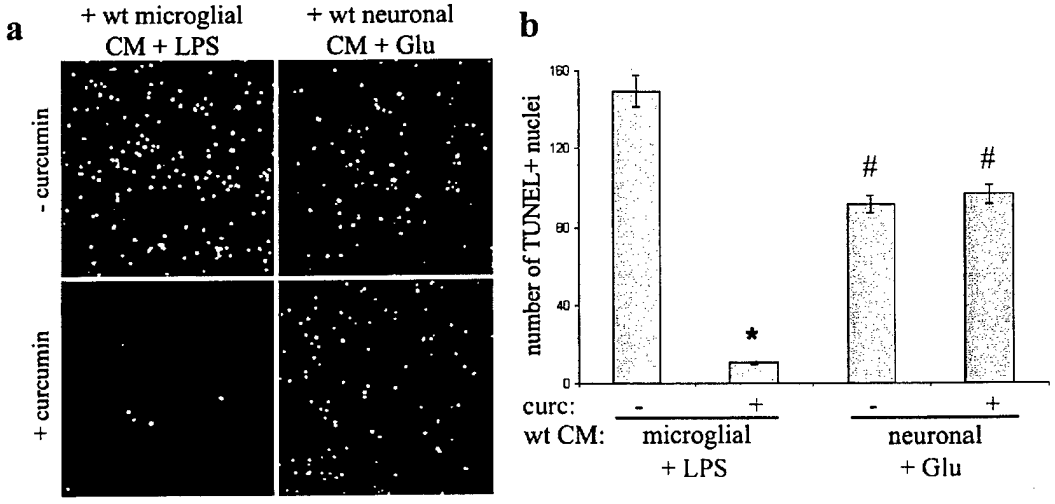


Our data suggest that the relevant receptor for tPA that mediates microglial activation is annexin II. However, we have detected LRP expressed on microglia. Since LRP is a known receptor for tPA, we decide to investigate its function in microglia further. We performed co-immunoprecipitation and western blot experiments on resident (non LPS-primed) and LPS-primed tPA$^{-/-}$ microglial cultures after exposure of the cells to exogenously added tPA in the presence of the crosslinking reagent BS^3 (Pierce Inc). As shown in Figure 13, tPA can be immunoprecipitated from resting microglia in complex with LRP, but not in complex with annexin II (lower left panel). This result changes, however, when the microglial cultures are stimulated with LPS prior to crosslinking: tPA now can be detected as a complex both with LRP and with annexin II (upper left panel). This result, in combination, with our previous data further suggests that annexin II is an excellent candidate to be the receptor with which tPA interacts to activate microglia. LRP may be a constitutive endocytosis and clearance receptor for tPA.

Figure 13: Co-immunoprecipitation and western blotting analysis experiments using anti-tPA, anti-annexinII and anti-LRP antibodies in LPS-unstimulated and stimulated $tPA^{-/-}$ microglia in the presence of exogenously added tPA and the crosslinking agent BS^3. Note the absence of tPA-annexin II interaction in unstimulated cells.

If LRP is a recycling / endocytosis receptor for tPA, it is possible that its function on microglia is to bind and remove secreted, extracellular tPA during the normal function of the brain. However, we would propose that when injury occurs, resulting in microglial "priming" and secretion from neurons of 'pathological' amounts of tPA, the microglia don't merely recycle tPA through LRP, but in addition also interact with tPA through the newly upregulated annexin II, which then triggers microglial activation.

NO is a co-injury signal released by microglia. Our data indicate that tPA promotes neuronal death in collaboration with other signaling pathways. NO is one such potential signal. We have examined the effect of using curcumin to inhibit nitric oxide synthase (NOS) from LPS-primed microglia or from Glu-stimulated neurons on neuronal survival (Fig. 14). Wild-type microglia were incubated with curcumin (final conc. 10 μM) for 2 h and then stimulated with LPS. The microglia were cultured for 16 h to allow stimulating factors to be secreted into the medium, which was then harvested and added to $tPA^{-/-}$ neurons. Wild-type neurons were incubated with the same concentration of curcumin for 2 h, glutamate (25 μM) for 2 h, then half of the medium was replaced by fresh complete medium, and the cells incubated for an additional 16 h prior to collection of conditioned media. $tPA^{-/-}$ neurons were cultured with these media for 16 h and then scored using TUNEL analysis. Panel a shows that significantly fewer $TUNEL^+$ neurons were observed when curcumin had been added to the microglia during generation of the CM. In contrast, no decrease was seen whether or not curcumin had been added during generation of the neuronal CM. The data are quantified in panel b. These results suggest that activated microglia secrete NO, which in combination with tPA, mediates neuronal degeneration.



Since the toxic counter-part of NO is peroxynitrite, we are currently evaluating the levels of this compound in neurons using immunohistochemistry for nitro-tyrosine, a cellular indicator for the presence of peroxynitrite.

Figure 14: NO and synergy with tPA. See text above for details. Statistical analysis was done using Student's t-test. *, $p < 0.05$ compared with –curcumin sample. #, $p < 0.05$ compared with microglial CM –curcumin.

The finger domain of tPA mediates microglial activation in vivo. The finger domain protein fragment was purified over polymixin B-agarose to remove bacterial LPS and was added with or without LPS priming to tPA$^{-/-}$ microglia, followed by F4/80 immunocytochemistry. The finger domain alone did not activate the resting microglia above the levels achieved with LPS alone. However, with LPS priming, synergistic activation was observed (Fig. 15).

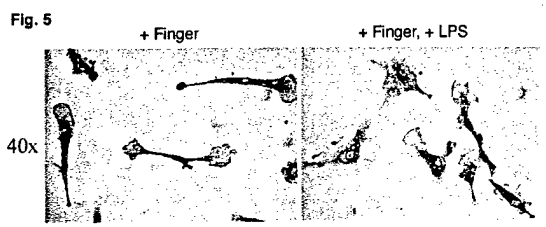
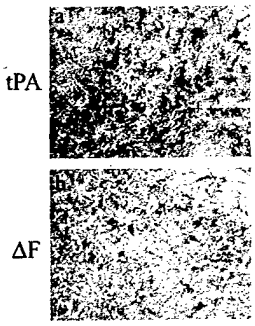


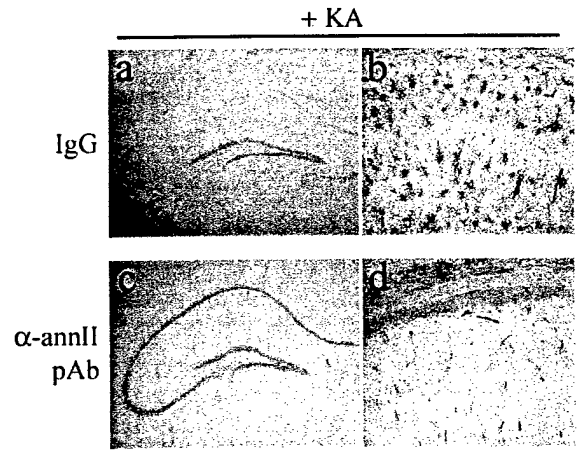
Figure 15: The finger domain mediates microglial activation



Infusion of the ΔF mutant prevents microglial activation. We infused either full-length tPA or the ΔF deletion mutant protein into the midline of tPA$^{-/-}$ mice for two days prior to initiating KA excitotoxicity. Five days later, the mice were sacrificed and microglial activation assessed. We found that microglia in the ΔF-infused mouse showed more limited microglial activation than the mice infused with the full-length tPA (Fig. 16). This result also suggests that other factors may be involved in co-mediating microglial reaction.

Figure 16: Infusion of ΔF tPA prevents microglial activation in vivo

In vivo assessment of annexin II-mediated microglial activation. Finally, we described in the proposal using our primary culture system that annexin II functions as a binding partner for tPA on the surface of microglial cells.



We have now shown that blocking annexin II *in vivo* similarly attenuates microglial activation and neuronal death. We infused a polyclonal α-annexin II antibody, or IgG control, into wild-type mice for two days, and then subjected them to KA excitotoxicity. Fig. 17a shows that the IgG control did not protect wild-type neurons from KA-induced death, but the polyclonal α-annexin II does (Fig.17c). Fig. 17d similarly shows that the α-annexin II pAb attenuated microglial activation in comparison with the control (Fig. 17b).

Figure 17: Blocking annexin II in vivo attenuates microglial activation.

A manuscript describing these findings is in preparation for publication.

Key Research Accomplishments

- Narrowed down the site of interaction between tPA and microglial cells. It is the finger domain
- Defined that the growth factor-like domain regulates the interaction, possibly acting as a dominant negative

- Determined that neuronal tPA is not sufficient to mediate neurotoxin-induced cell death, but needs the presence of higher concentrations of microglial tPA
- Determined that lipopolysaccharide-induced microglial activation and excitotoxin-induced microglial activation proceed via different signaling pathways
- Characterized initially the binding activity on microglial cells as annexin II
- Generated tPA-deficient immortalized microglial cell line
- Investigated and suggesting the presence of a co-receptor for tPA on microglial cells

Reportable Outcomes

A. Manuscripts:

- Chia-Jen Siao, and Stella E. Tsirka. (2002) 'Tissue plasminogen activator mediates microglial activation via its finger domain', *J Neurosci* 22, 3352-3358.
- C-J Siao, S.E. Tsirka (2001) Extracellular proteases and neuronal cell death. *Mol. Cell. Biol.*, 48: 151-161.
- Andrew D. Rogove, Weiquan Lu, and Stella E. Tsirka. (2002) "Microglial activation and recruitment, but not proliferation, suffice to mediate neurodegeneration' *Cell Death Differ* 9:801-806
- Chia-Jen Siao, and Stella E. Tsirka (2002) "Different roles for neuronal or microglial tissue plasminogen activator after excitotoxic injury", submitted for publication

B. Abstracts:

- CJ Siao, A. D. Rogove and SE Tsirka. "Neuronal- and microglial-specific tissue plasminogen activator play different but complementary roles in excitotoxicity-induced neurodegeneration in the mouse", 30th Annual SFN Meeting, New Orleans.
- CJ Siao, J. Watkins and SE Tsirka. 'Tissue plasminogen activator mediates microglial activation via a cell surface receptor', 31st Annual SFN Meeting, San Diego.

C. Funding applied for based on work supported by this award:

I have received fundable score (15.4%) for a R01 NIH grant on microglial activation based on some of the results described here.

Conclusions

The major findings of our funded research up to now point to the finger domain of tPA as the domain responsible for binding and thus mediating microglial activation. It appears that the interaction is mediated via binding on to annexin II on the surface of microglia cells. Knowing the binding domain and interacting molecule/receptor will allow us to generate a reagent very useful for turning on and off microglial activation. Such an accomplishment will allow us to modulate the neurotoxic effects of microglial activation, which is involved in promoting neuronal death in various neurodegenerative diseases.

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Introduction

The application entitled "Tissue plasminogen activator (tPA) mediates neurotoxin-induced cell death and microglial activation" proposes to address 2 questions / objectives.

1. **How does tPA mediate microglial activation? Does the activation involve classical signal transduction pathways, namely tyrosine phosphorylation of a specific receptor and a cascade of activation of kinases, leading ultimately to transcriptional upregulation of critical immediately early or early genes? How does modulation of tPA / microglial activation affect neurotoxicity?**

We are in the process of defining the mechanism by which tPA mediates microglial activation using deletion mutants and eventually single point mutants, first in a cell culture system and then in vivo, in mice. We have now confirmed that tPA binds on to annexin II on the surface of microglia via its finger domain. We are doing a more detailed biochemical characterization of the interaction. Furthermore, several classical pathways of signal transduction are evaluated, starting with the MAP-kinases pathway.

2. **What is the source(s) of tPA that mediates microglial activation and neuronal degeneration in response to excitotoxin-stimulation in culture?**

We have proposed to mix-and-match neuronal and microglial cultures from wild-type and tPA-deficient genotypes in the presence of excitotoxins and investigate the progress and mechanism by which neuronal death proceeds. We have established the mixing-and-matching protocols, and it appears that both paracrine and autocrine mechanisms are involved in microglial activation, with autocrine being the biologically more relevant.

Body

Objective 1) Which domain of tPA is required for microglial activation?

Objective 2) What is the source(s) of tPA that mediates microglial activation and neuronal degeneration in response to excitotoxin-stimulation in culture?

Tissue plasminogen activator (tPA) is a serine protease whose proteolytic activation of plasminogen into plasmin leads to neuronal death during excitotoxicity. tPA also mediates activation of microglial cells, but this role appears not to require its proteolytic function. Using cultured cells, we have further characterized the mechanism and pathway through which tPA functions to activate microglia. We show that the activation pathway can be either paracrine (via tPA released by glutamate-injured neurons) or autocrine (via tPA released by bacterial liposaccharide-primed microglia), supporting the role of microglia in the central nervous system as sensors of injury and infection. Furthermore, tPA activates microglia in a dose-dependent manner and blocking a potential cell surface microglial tPA receptor, annexin II, prevents microglial activation. This finding suggests that tPA acts as a neural cytokine with respect to microglia. Moreover, these findings raise the possibility that it may be feasible to selectively interfere with tPA-mediated microglial activation by blocking tPA's binding to its microglial receptor, which would provide a way to modulate inflammation in the brain without restricting tPA's other important physiological roles.

<u>tPA induces a dosage-dependent increase in microglial activation</u>.

Under basal conditions, $tPA^{-/-}$ microglial cells exhibited a resting, ramified morphology characterized by a small cell body and long, thin processes (see Fig 1, and Rogove et al., 1999). When

microglia were treated with rtPA for 24 h, they adopted a slightly activated morphology (Fig. 1). LPS treatment for 24 hours induced a rapid (within 30 min.) change of morphology, in that the processes appeared to become thicker and more ruffled. However, substantial change in morphology did not appear until at least 24 h after LPS priming, when the majority of the cells became amoeboid and the cell membranes ruffled. When the cells were treated with LPS in the presence of rtPA, the microglia became further activated in proportion to the concentration of rtPA.

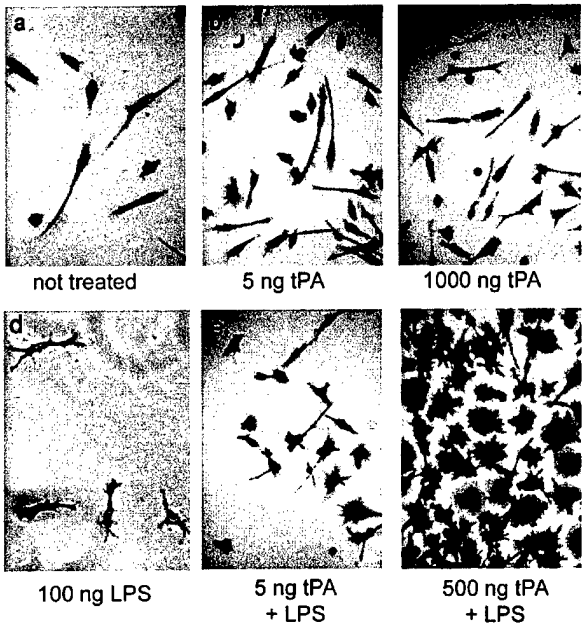


Figure 1: tPA mediates a dose-dependent activation response in primary $tPA^{-/-}$ microglia. Microglia were sub-cultured from mixed cortical cultures onto coverslips for 2 days, and then rtPA was added for 24 h. LPS was added for another 16 h, and then cells were fixed and stained for F4/80 expression. a. No treatment. b. 5 ng/ml rtPA. c. 1000 ng/ml rtPA. d. 100 ng/ml LPS. e. 5 ng/ml rtPA, LPS. f. 500 ng/ml rtPA, LPS.

Quantitation of microglial activation was performed both by western blot analysis for the F4/80 marker (the cell surface glycoprotein F4/80 is upregulated when cells of the monocyte/macrophage lineage are activated (Lawson et al., 1990)), and by detecting changes in NO production. Using a quantitative western blotting method, we detected an increase in F4/80 immunoreactivity after increasing amounts of rtPA were added to $tPA^{-/-}$ microglia (Fig. 2), consistent with the observed changes in cell morphology in Fig. 1. Since we collected data at one time point, other microglial factors released earlier or later may amplify or modify this response. Activated microglia secrete many molecules upon activation, including NO and TNF-α (Meda et al., 1995; Rogove and Tsirka, 1998).

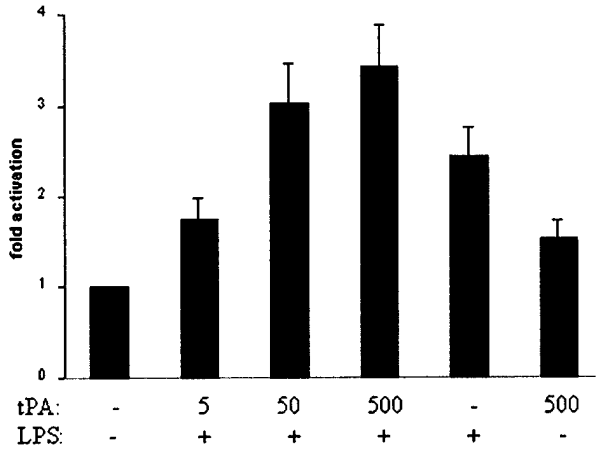


Figure 2. Quantitation of tPA-/- microglial activation by F4/80 western blot. Microglia treated as described in text were scraped into 0.25% Triton x-100 and the total protein amount determined. Equal amounts of protein was separated on 10% SDS-PAGE and then transferred onto PVDF. F4/80 expression was quantified and plotted as fold activation over untreated expression (column 1).

Therefore, in addition to changes in F4/80 expression, we measured the amount of nitrites released into the culture medium, and the levels of inducible nitric oxide synthase (iNOS) expression. Activated microglia also secrete higher amounts of NO (data not shown), consistent with the observed morphological and immunochemical changes, shown in Figures 1 and 2.

Both neurons and microglia can activate microglia by releasing tPA.

In order to determine the source of the tPA that activates microglia, we cultured microglial cells with conditioned media from wild-type or $tPA^{-/-}$ neurons stimulated with glutamate (nCM), or from wild-type or $tPA^{-/-}$ microglia primed with LPS and then washed (mCM). Activation was then evaluated using F4/80 immunocytochemistry (Fig. 3) and western blotting (data not shown). We simulated injury to neurons by incubating them in the presence of 25 μM L-glutamate, which results in low level neurotoxicity (Siao and Tsirka, manuscript in preparation), then added this conditioned medium to microglia. When we incubated microglia with wild-type nCM, these microglia became activated, presumably in response to both tPA released from injured neurons as well as other factors (e.g., NO). Activation was also observed in the presence of wild-type mCM from which the LPS had been removed. In contrast, $tPA^{-/-}$ neuronal and microglial CMs did not stimulate microglia to mount an activation response.

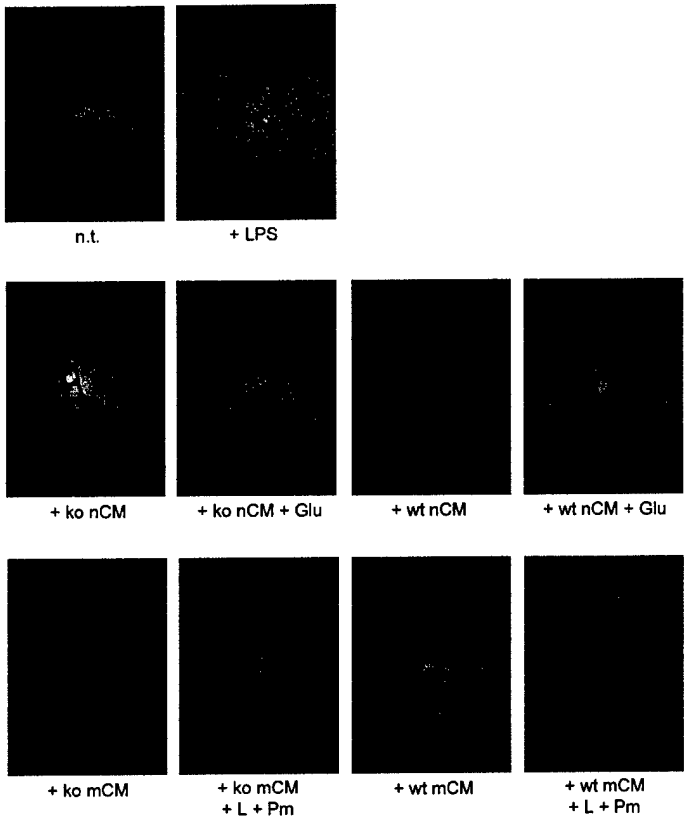


Figure 3. $tPA^{-/-}$ microglia in culture respond to injured neurons and microglia by becoming activated and changing morphology. Cells were treated as described in text, and the expression of F4/80 was examined. **N.t.** (Not treated); **+LPS** (100 ng/ml LPS); **+ko nCM.** (+untreated $tPA^{-/-}$ neuronal Conditioned Medium); **+ko nCM + Glu** (injured $tPA^{-/-}$ nCM); **+wt nCM** (+untreated wild-type nCM); . **+wt nCM+Glu** (injured wild-type nCM); **+ko mCM** (+untreated $tPA^{-/-}$ microglial CM); **+ko mCM +L+Pm** (+untreated $tPA^{-/-}$ microglial CM + LPS, where LPS was removed by polymyxin B (Pm) prior to addition to microglia); **+wt mCM** (+untreated wild-type mCM); **+wt mCM +L+Pm** (+untreated wt mCM+ LPS, polymyxin-treated).

Our results show that both neuronal and microglial sources of tPA are able to mediate microglial activation, suggesting both an autocrine and a paracrine aspect to tPA-mediated signaling, probably depending on the amount of tPA secreted by the different cell types.

The finger domain of tPA mediates microglial activation.

tPA mediates microglial activation via a non-proteolytic mechanism, possibly through protein-protein interactions with the microglial cell surface. We used tPA mutants which lacked individual domains [the finger (ΔF), growth factor (ΔGF), and kringle (ΔK1 or ΔK2) domains] to determine whether one or more of these domains mediate microglial activation. Figure 4 shows the results using western blotting (Fig. 4a) and NO detection (Fig. 4b). When microglia are cultured in the presence of ΔGF, ΔK1, or ΔK2 tPA proteins, they are able to activate to or beyond levels mediated by wild-type rtPA. However, note the failure of $tPA^{-/-}$ microglia to become activated in the presence of the tPA mutant deleted in the finger domain (ΔF). These results are represented in Figure 4c. These data

indicate that the finger domain of tPA is critical in mediating microglial activation, presumably via interaction with a cell surface molecule (receptor) expressed by microglia.

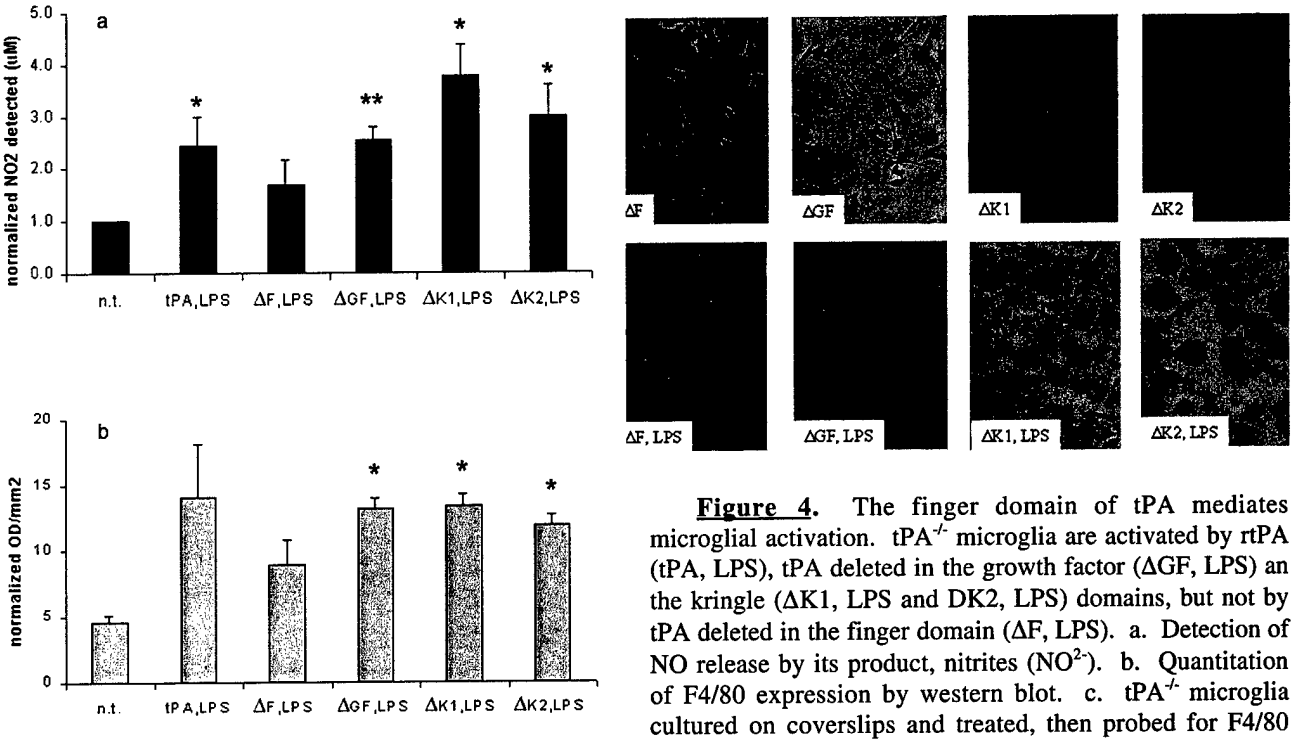


Figure 4. The finger domain of tPA mediates microglial activation. $tPA^{-/-}$ microglia are activated by rtPA (tPA, LPS), tPA deleted in the growth factor (ΔGF, LPS) an the kringle (ΔK1, LPS and DK2, LPS) domains, but not by tPA deleted in the finger domain (ΔF, LPS). a. Detection of NO release by its product, nitrites (NO^{2-}). b. Quantitation of F4/80 expression by western blot. c. $tPA^{-/-}$ microglia cultured on coverslips and treated, then probed for F4/80 expression as described.

Microglial activation by tPA's finger domain is receptor mediated.

One candidate for a microglial tPA receptor is the cell membrane-associated annexin II (Hajjar et al., 1994). It is first necessary to determine whether annexin II is expressed by microglial cells and whether this expression is unique on this CNS cell type, which would suggest specificity of its interaction with tPA. RT-PCR was performed using total RNA from pure neuronal or microglial cultures. This RT-PCR (Fig. 5a) and western blot analysis (Fig. 5b) show that annexin II is expressed by microglia and not by neurons in the mouse CNS. The quality of the neuronal cDNA was assessed by the amplification of the amyloid precursor protein (APP), used as a positive control (Fig. 5a, lane 4).

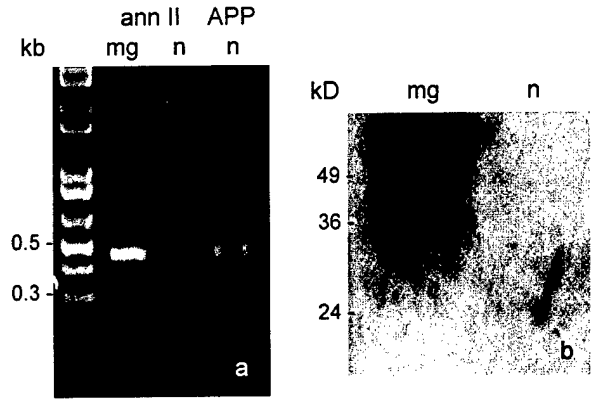


Figure 5. Primary microglia, but not neurons, express annexin II, a potential tPA receptor. A. RT-PCR results from microglia (mg) or neurons (n), amplified using either annexin II (ann II) or amyloid precursor protein (APP) primers. B. Western blot showing primary microglia (mg), but not neurons (n), express annexin II.

Since annexin II is expressed by microglia, and given the established interaction in endothelial cells between tPA (finger domain) and annexin II, we evaluated whether tPA also binds to annexin II in this cellular system, and how this potential

interaction affects microglial activation. A polyclonal anti-annexin II antibody was added to $tPA^{-/-}$ microglia prior to addition of either wild-type rtPA, deletion mutants, or the finger domain alone (Fig. 6).

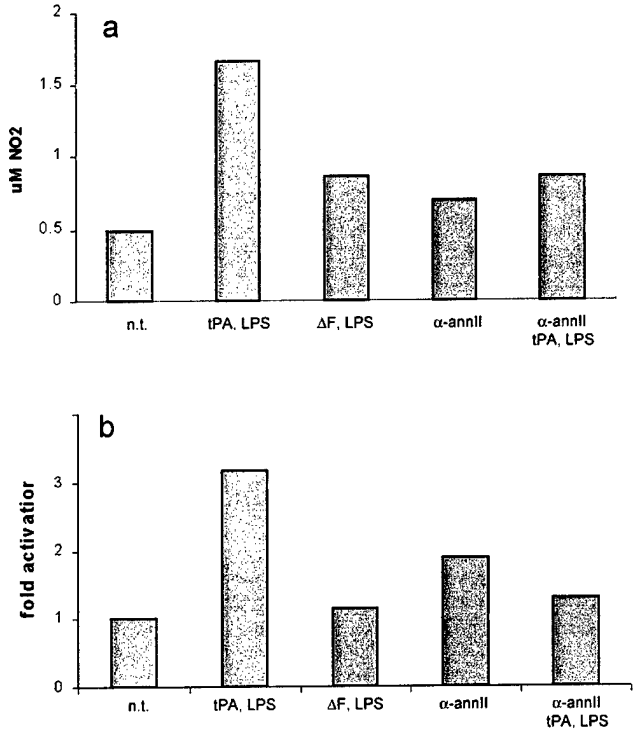


Figure 6. tPA-mediated microglial activation is blocked by antibodies against annexin II.A.Quantitation of NO release, measured as nitrites (NO^{2-}), from $tPA^{-/-}$ microglia cultured with medium only (n.t.), 500 ng/ml rtPA (tPA, LPS), 500 ng/ml ΔF mutant (ΔF, LPS), 1 ug/ml anti-annexin II (a-annII), or 1 ug/ml anti-annexin II and 500 ng/ml rtPA (a-annII, tPA, LPS). B.Measurement of F4/80 expression by western blot.

Addition of the antibody alone did not have any effect on microglia over untreated cells. Incubation with either rtPA or the ΔK2 mutant restored microglial activation, while the ΔF mutant failed to activate microglia above levels seen with LPS priming. Incubation with the ΔF mutant in the presence of the antibody also failed to activate microglia. These data suggest that the finger domain of tPA mediates microglial activation by interacting with the cell surface annexin II.

Discussion

tPA mediates neuronal death and microglial activation during excitotoxic injury. Our previous data showed that tPA's proteolytic activity is critical in promoting neurodegeneration, through activation of plasmin (Tsirka et al., 1997), and possible subsequent degradation of the ECM molecule laminin (Chen and Strickland, 1997). However, tPA's proteolytic role is not necessary for activating microglia (Rogove et al., 1999), suggesting a cell-signaling function for tPA in the CNS. We pursued this novel aspect of tPA function.

Using cultured primary cells from $tPA^{-/-}$ mice, we showed that LPS-primed microglia respond to tPA-mediated activation in a dose-dependent manner. The observed dose-response to rtPA treatment is significant by implicating the presence of a microglial tPA receptor which transduces an extracellular tPA-initiated signal into an intracellular response, resulting in microglial activation.

We examined which source, neuronal or microglial, of tPA was responsible for activating microglia in culture. By incubating neurons with glutamate, the subsequent release of neuronal soluble factors, including tPA, could induce microglial activation. tPA-mediated microglial activation can also further stimulate microglial release of factors, such as tPA and other pro-inflammatory molecules, that can activate other microglia, and ultimately lead to recruitment of microglia to the site of injury in the brain. This amplification of injury signals can promote both a timely resolution of insult as well as an overly sensitive inflammatory response.

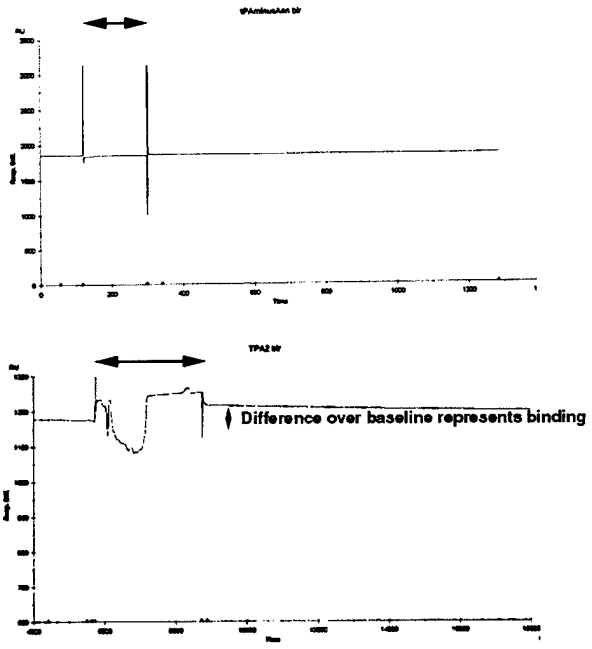
To further study the signaling role of tPA in microglial activation, we undertook a domain deletion approach to delineate which domain of tPA mediates microglial activation. Our results indicate that the finger domain of tPA plays a major role in activating microglia during excitotoxic injury, but do not rule out the possibility that the protease domain can also mediate activation. It was noted in the literature that tPA can bind to smooth muscle endothelial cells via its serine protease domain, but not its active site (Werner et al., 1999). Interestingly, deletion of the kringle domains appears to activate more microglial cells in culture. This may indicate an intramolecular control of domain participation in different cell-cell interactions. We are currently expressing the kringle domains by themselves to confirm this observation.

Annexin II, a Ca^{2+}-binding membrane-associated protein, has been shown to bind to tPA's finger domain and increase tPA activity on endothelial cells (Hajjar et al., 1994). Our data show that by sterically blocking tPA interaction with annexin II using antibodies against annexin II, we also blocked microglial activation. We are currently characterizing biochemically the interactions of the finger domain with annexin II.

There is a large body of research showing both neurotoxic and neuroprotective roles of activated microglia in various injury models (Dickson et al., 1993; Gehrmann et al., 1995; Benveniste, 1997; DiPatre and Gelman, 1997). It is highly likely that both roles are correct. In addition to cleaning up debris from dead and dying cells, phagocytes are also required to contain damage so other cells in the surrounding regions of the injury are not overly affected. Overzealous reactive microglia, however, can secrete too much pro-inflammatory molecules, thus worsening the initial injury. Identification of a microglial cell surface tPA receptor would suggest a potentially powerful method of interfering with microglial activation when such activation may be detrimental to the system as a whole.

The results described here are in a manuscript that is in preparation; we anticipate submission by the end of August 2001.

These results are also going to be presented in the form of a poster presentation at the Annual SFN meeting in San Diego (see attached poster abstract).



In addition to the biochemical experiments described above, we have initiated experiments using the BIACORE 2000 instrument, to obtain a more detailed understanding of the binding as well as to get information about the kinetics of the interaction. tPA was immobilized on the CM5 sensor chip at pH 5.5. We immobilized three different sets of resonance units for tPA (aiming for1000 RU we immobilized 987 RU, aiming for 2000 RU we immobilized 1849 RU and aiming for 4000 RU we immobilized 3120 RU) at a concentration of 10 μg/ml. Different amounts of membrane extracts isolated from the mouse microglial cell line N9 were then used to assay for a binding activity. These extracts were suspended in PBS (pH7.4) containing 0.005% NP40. They were delivered over a minute at a

flow rate of 20 μl/min. A strong binding activity was detected with a very high 'on' rate and a very slow 'off' rate. This binding pattern would be consistent with a tight binding partner. The Kd between tPA and annexin II has been determined in endothelial cells as 47 nM (Hajjar et al., 1998). To investigate the possibility that annexin II may be the binding activity, annexin II was immuno-depleted from the N9 membrane extracts. The efficiency of immunodepletion was determined to be detectably complete using western blot analysis against annexin II in the depleted extracts as well as in the immunoprecipitated material. The extracts were then passed through the BIAcore as described above. As shown in the Figure above, when N9 membrane extracts were delivered over the chip (lower panel), there was an increase of resonance units, indicating the presence of binding activity (the diamond, ◆, denotes the binding). When extracts immunodepleted for annexin II were delivered over the same chip (upper panel), the absence of detectable binding activity was evident. The double arrow over both panels denotes the injection period of the N9 extracts.

This experiment, although it needs to be repeated and confirmed, is quite encouraging in suggesting that annexin II may be the primary binding activity for tPA on microglial cells.

Gene expression changes during microglial activation:

We have also started evaluating the signal transduction cascades induced in microglia that result from tPA-mediated microglial activation.

The onset of the 'program' of neurodegeneration is accompanied by changes in gene expression in the injured and dying neuronal cells: using mRNA in situ hybridization and RT-PCR, we have observed both increased and decreased mRNA levels of several different markers in this setting. For example, the transcription factor CHOP, a marker for cells that are under stress, becomes rapidly upregulated in the hilar cells of the injected side of the hippocampus as compared to the uninjected side. This increase does not take place in tPA-deficient mice (Tsirka, unpublished). CHOP up-regulation may underlie the subsequently altered levels of plasminogen activator inhibitors-1 and 2 mRNA that we have observed, as well as the increase in monocyte chemoattractant protein-1 (MCP-1).

Similar changes have been observed for microglial cells upon their activation: in addition to the observed increase in cytokine mRNA levels, the mRNA levels for cell surface proteins such as annexin II and F4/80 are also up-regulated.

Given that these changes exist, we propose to identify other genes differentially regulated in this setting, which may lead to useful markers and novel insights into the relevant downstream effector pathways. We have performed preliminary experiments using the University Core Affymetrix System. We hybridized and compared cRNAs derived from wild-type hippocampi injected with kainate to tPA-deficient injected hippocampi. A set of very interesting genes is differentially expressed (see below Tables 1 and 2). As our internal control, the tPA expression level was markedly different, as well as a number of other genes that we had already identified using other methods (e.g., the chemokines MIP-1α and β). We are currently confirming some of those genes using real-time PCR (LightCycler, Roche) and characterizing them further.

Some of the genes obtained from these first studies have been shown to be involved in neuronal death (e.g., cell death activator CIDE-A and death adaptor molecule Raidd/Cradd) or in microglial activation [e.g., macrophage/monocyte specific transcription factor LRG-21 and MIP-1β (macrophage

inflammatory protein 1β)]. Furthermore, genes whose regulation is presumably linked to tPA were also identified, e.g. types 1 and 2 plasminogen activator inhibitor (PAI). Brain-derived neurotrophic factor (BDNF) was also found to be differentially expressed, as previously reported (Fiumelli et al., 1999). Chondroitin sulfate proteoglycan expression levels were also changed, result in agreement with our recent findings that phosphacan and neurocan are modified by the plasminogen activator system during neurite outgrowth (Wu et al., 2000).

TABLE 1: mRNAs decreased in tPA-deficient hippocampi as compared to wt hippocampi after kainate injection

Fold Change	mRNAs
~-9.2	transcription factor LRG-21
-8.8	Heat shock protein, 70
~-7.7	MIP-1b gene for macrophage inflammatory protein 1b
~-7.5	Plasminogen activator inhibitor, type II
-7	glucocortoid-regulated inflammatory prostaglandin G/H synthase (griPGHS)
-6.7	neuronal pentraxin 2 (Nptx2)
~-6.4	mRNA for DBY RNA helicase
-4.5	Aggrecan, proteoglycan
~-4.1	Syntaxin binding protein 2
-4	lymphocyte specific formin related protein (Frl)
-3.8	Calnexin
-3.8	Ras-like GTP-binding protein Rad
-3.7	mRNA for translation initiation factor eIF2 gamma
~-3.2	intracellular calcium-binding protein (MRP8)
~-2.9	S100A3 calcium binding protein
-2.9	proteoglycan core protein mRNA
~-2.6	Cytotoxic T lymphocyte-associated protein 2 beta
-2.6	Zinc finger protein 36
~-2.5	Protein tyrosine phosphatase, receptor-type, N polypeptide 2
-2.5	Brain derived neurotrophic factor

TABLE 2: mRNAs increased in tPA-deficient hippocampi as compared to wt hippocampi after kainate injection

Fold Change	mRNAs
4.1	beta chemokine TCA4 gene
~3.7	Extracellular matrix protein 2
~3.3	Interferon activated gene 202
~2.9	Mus musculus cell death activator CIDE-A (Cide-a)
~2.7	Kreisler (maf-related) leucine zipper protein
~2.6	Signal transducer and activator of transcription 1
~2.6	nuclear protein SA-1
~2.6	death adaptor molecule (Raidd/Cradd)
2.6	spalt transcription factor
2.6	Fc receptor, IgE, high affinity I, beta polypeptide
~2.5	interleukin 2 receptor gene
2.5	Actin, beta, cytoplasmic
2.3	Neuropeptide Y receptor Y2
~2.2	Alpha-2-macroglobulin
~2.1	Fibroblast growth factor inducible 16
2.1	Immunoglobulin heavy chain 8 (heavy chain of IgG3)
2.1	Mouse spi2 proteinase inhibitor (spi2/eb4)
~2.0	Zinc finger protein 101
1.8	Potassium inwardly-rectifying channel, subfamily J, member 9
1.8	plasminogen activator inhibitor (PAI-1) mRNA
1.8	phospholipase C-beta-1b mRNA, complete cds
1.7	bright and dead ringer gene product homologous protein Bdp
1.4	synaptotagmin XI

These data (along with results from plasminogen-/- injected animals will be analyzed by Cluster analysis. The analysis will be performed using the XCluster software, which uses Hierarchical Clustering for organizing the data. This software uses Average Linkage algorithm for clustering basically as is described by Eisen et al (Eisen et al., 1998).

Key Research Accomplishments

- Narrowed down the site of interaction between tPA and microglial cells. It is the finger domain
- Defined that the growth factor-like domain regulates the interaction, possibly acting as a dominant negative
- Determined that neuronal tPA is not sufficient to mediate neurotoxin-induced cell death, but needs the presence of higher concentrations of microglial tPA
- Determined that lipopolysaccharide-induced microglial activation and excitotoxin-induced microglial activation proceed via different signaling pathways
- Characterized initially the binding activity on microglial cells as annexin II

Reportable Outcomes

A. Manuscripts:

- Chia-Jen Siao, Jermel Watkins, and Stella E. Tsirka. 'Tissue plasminogen activator mediates microglial activation via its finger domain', to be submitted to Journal of Biological Chemistry
- C-J Siao, S.E. Tsirka (2001) Extracellular proteases and neuronal cell death. *Mol. Cell. Biol.*, in press.

B. Abstracts:

- CJ Siao, A. D. Rogove and SE Tsirka. "Neuronal- and microglial-specific tissue plasminogen activator play different but complementary roles in excitotoxicity-induced neurodegeneration in the mouse", 30th Annual SFN Meeting, New Orleans.
- CJ Siao, J. Watkins and SE Tsirka. 'Tissue plasminogen activator mediates microglial activation via a cell surface receptor', 31st Annual SFN Meeting, San Diego.

C. Funding applied for based on work supported by this award:

I have applied for a NIH grant on microglial activation (to be reviewed by November 2001) based on some of the results described here.

Conclusions

The major findings of our funded research up to now point to the finger domain of tPA as the domain responsible for binding and thus mediating microglial activation. It appears that the interaction is mediated via binding on to annexin II on the surface of microglia cells. Knowing the binding domain and interacting molecule/receptor will allow us to generate a reagent very useful for turning on and off microglial activation. Such an accomplishment will allow us to modulate the neurotoxic effects of microglial activation, which is involved in promoting neuronal death in various neurodegenerative diseases.

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RECEPTOR ANTAGONIST LIMITS PROGRESSIVE NECROSIS AFTER INJURY IN THE MOUSE. D.M. Inman*, O. Steward. *Reeve-Irvine Research ...versity of California, Irvine, College of Medicine, Irvine, CA, USA*

...injury initiates a wave of cell death in the central nervous system, the magnitude of ...the final extent of the lesion. This study investigated the contribution of excito... ...death to the progressive necrosis after spinal cord crush injury in the mouse. Three ...received an extradural crush injury of the spinal cord. One group then received the ...antagonist APV (5 mg/kg) 30 minutes after injury; one group received a 5 mg/kg ...per day for three days; and a third group served as the control. Open field walk... ...using the Basso Beattie Bresnahan rating scale once per week after injury. At three ...sacrificed, the mice were euthanized, and lesion areas and cavitation were assessed using ...morphometry. Lesion size was significantly smaller in animals that received either sin... ...doses of APV. There was no significant difference between groups that received 1 vs. ...doses. However, Behavioral scores did not differ significantly between APV and control groups. ...These data confirm previous findings in rats that treatment with the NMDA receptor antago... ...decreased final lesion size, suggesting that NMDA receptor-mediated excitotoxic cell ...contributes to the progressive necrosis seen after spinal cord crush injury in this strain of ...*Supported by: NIH NS32280.*

...ROPHIC FACTORS MAY POTENTIATE NEUROTOXICITY. A. Neuhaus-Folliní, ... *Neurosurgery Lab, Montefiore Medical Center, Bronx, NY*

...several decades, a host of factors that increase neuronal survival have been identified ...terized using cell culture systems. However, while brain levels of many of these factors ...after mechanical and chemical lesions in vivo and in affected brain areas of patients with ...degenerative disorders, this up-regulation does not seem to prevent neuronal loss. In this ...investigated the possibility that under some conditions, "neurotrophic" factors may exac... ...toxicity. We employed a cell culture model in which embryonic rat mesencephalic neu... ...treated with the glutamate agonist quisqualic acid (QA). We demonstrated that QA was ...dopaminergic neurons, identified by tyrosine hydroxylase immunocytochemistry, in a dose... ...dependent manner. Conditioned medium from QA-treated cultures potentiated QA tox... ...boiling the conditioned medium significantly reduced its effect, suggesting that the active ...is a protein. Interestingly, we observed that dopaminergic neurons exhibited a trophic ...before they died, as demonstrated by morphological changes such as increased neurite ...growth. Since basic fibroblast growth factor (bFGF) is induced in other models of neurode... ..., we determined whether it was induced in our cultures. ELISA data indicate that ...was induced 10-fold after 24 hours by 5 μM QA. Furthermore, co-incubation of 5 μM QA ...neutralizing antibody to bFGF for 48 hours inhibited both maturation and toxicity. Taken ..., these results suggest that bFGF may exacerbate non-NMDA receptor mediated neuro... ..., and may mediate the trophic response seen before cell death. *Supported by: in part, a Parkin... ...Foundation Summer Fellowship to ANF.*

...11

... GLUTAMATE AND NEURODEGENERATION IN THE RAT SPINAL CORD. M.S... ..., R.C. Rogers, G.E. Hermann, J.C. Bresnahan. *Dept Neurosci, Ohio State Univ, Colum... ...OH, USA*

...by our Spinal Cord group has suggested that the cytokine tumor necrosis factor [TNF] may ...important agent in the propagation of neuro- and glio-degenerative processes which occur ...subsequent to spinal cord trauma. Others have suggested that TNF entry to the cord is acceler... ...by injury and evokes the activation of glial cells which, in turn, produce more TNF and per... ...degeneration. The mechanism of TNF-induced neuro-degeneration is not clear. On the ...hand, glutamate action on both AMPA and NMDA receptors has been associated with a ...number of neurodegenerative models. Recent work in our Autonomic Group has shown that ...activates neurons associated with autonomic control [solitary nucleus], and potentiates glu... ...ergic afferent inputs. We hypothesized that TNF may activate neurons in the SC and that ...may potentiate the action of glutamatergic agonists producing significant neurodegeneration. ...injections of TNF [3.5 femtomoles] alone produce a zone of cFOS labeled neurons and glia. ...activated spinal neurons are closely associated with activated microglia which also demon... ...nuclear FOS. Tissue degeneration following TNF injection alone is not apparent. Kainate ...injections [17 nanomoles] produce a "ring" of FOS activated neurons and glia centered on a ...area [~300 microns] of neuronal degeneration. The combination of KA and TNF, however, ...produce a much larger area of degeneration [2-5 mm], again, surrounded by a "halo" of FOS acti... ...neurons and glia. These results suggest that the connection between TNF and propagated ...degeneration proposed in earlier studies may involve an interaction between TNF and glu... ...The precise mechanism of this interaction is under intense investigation. *Supported by: NIH ... 19079 and DK-52142.*

856.8

THE ANTIOXIDANT S-PBN PREVENTS NT 4/5 AND BDNF-MEDIATED POTENTIATION OF IRON-INDUCED INJURY IN RAT SPINAL CORD. X.Z. Liu*, M.M. Behrens, J.W. McDonald, D.W. Choi. *Ctr Study Nervous Sys Injury, Washington Univ Med Sch, St Louis, MO, USA*

We previously reported that pre-treatment with NT-3 or BDNF potentiated the vulnerability of cultured cortical neurons to excitotoxic or free radical-induced necrosis. This contrasts the ability of these neurotrophins to attenuate apoptosis. Here we examined 1) the dependency of such neurotrophin injury potentiation on trk receptor activation and 2) the relevance of the necrosis-potentiating effect of neurotrophins to in vivo models of CNS injury. BDNF or NT-4/5 potentiation of iron-induced neuronal injury in cortical cultures was attenuated by co-application of the trk kinase inhibitor Gö6976. In addition, pre-exposure to BDNF failed to potentiate iron-induced death in trk-B deficient neuronal cultures derived from single mouse embryos of lacking the trk B gene. Furthermore, in the in vivo model of oxidative injury induced by micro-stereotaxic iron injections (Fe^{3+}-citrate, 0.75 nmol in 0.5 μl, pH 7.4) into the spinal cord gray matter of adult female Long-Evans rats, prior injection (24 h before iron) into the cord of BDNF (1 μg/0.5 μl) or NT 4/5 (0.5 μg/0.5 μl) markedly increased the iron-induced lesion area. This injury-potentiating effect of neurotrophins in vivo was blocked by co-treatment with the antioxidant S-PBN (200 mg/kg, given i.p. twice daily beginning with neurotrophin treatment). These data suggest that the necrosis-potentiating effect of neurotrophins is mediated by trk B receptor activation, and is relevant to the injured adult CNS. *Supported by: NIH grant NS 32636 to D.W.C and NIH grant NS 01931 to J.W.M.*

856.10

NEUBLASTIN/ARTEMIN AND GDNF PROTECT CA1 PYRAMIDAL CELLS AGAINST NMDA MEDIATED EXCITOTOXICITY IN HIPPOCAMPAL SLICE CULTURES. C. Bonde[1], M. Meyer[1], B.W. Kristensen[1], T.E. Johansen[2*], J. Zimmer[1]. [1]*Anatomy and Neurobiology, SDU-Odense University, Odense, Denmark,* [2]*NsGene A/S, Ballerup, Denmark*

Neublastin/Artemin (NBN) is a neurotrophic factor belonging to the glial cell line-derived neurotrophic factor(GDNF) family. Recently GDNF was found to protect against ischemic neurodegeneration in rats. Here we investigated the neuroprotective potential of both NBN and GDNF against N-methyl-D-aspartate (NMDA)-induced neuronal degeneration in hippocampal slice cultures, prepared from 7-day-old rats and grown on semiporous membranes for 3-4 weeks in serum-free medium. Neuronal degeneration was examined by densitometric measurements of cellular uptake of propidium iodide(PI), according to established protocols, and showed that exposure of cultures to 10 μM NMDA induced a relative selective degeneration of CA1 pyramidal cells. For testing the neuroprotective effect of NBN, 3-week-old hippocampal slice cultures were transferred to serum-containing medium, conditioned for 2-4 days by growth of transfected, NBN-releasing HiB5 cells or non-transfected HiB5 cells. For testing the neuroprotective effect of GDNF, slices were transferred to serum-free medium containing rhGDNF (25-100 ng/ml). After 1h 10 μM NMDA was added. In cultures treated with medium from NBN-transfected HiB5 cells or rhGDNF, the PI-uptake in the CA1 pyramidal cell layer induced by 10 μM NMDA was significantly reduced after both 24 and 48h of exposure. We conclude that both NBN and GDNF, besides their known trophic effects on dopaminergic neurons, also can protect against NMDA excitotoxicity in the hippocampus. The findings thereby have implication for neuroprotection against cerebral ischemia. *Supported by: The Danish MRC and Neuroscience Pharmabiotec.*

856.12

NEURONAL- AND MICROGLIAL-SPECIFIC TISSUE PLASMINOGEN ACTIVATOR PLAY COMPLEMENTARY ROLES IN EXCITOTOXICITY-INDUCED NEURODEGENERATION IN THE MOUSE. C.-J. Siao[1*], A.D. Rogove[1], S.E. Tsirka[1,2]. [1]*Pharmacol Sci, State Univ New York at Stony Brook, Stony Brook, NY, USA,* [2]*Psychiatry, State Univ New York at Stony Brook, Stony Brook, NY, USA*

Microglia, considered the immune cells of the brain, play a critical role in the neurodegeneration seen after excitotoxicity. During excitotoxic injury induced by kainic acid (KA) injection into the hippocampus, pyramidal neurons undergo necrotic and apoptotic death. In order to understand how microglia are involved in this process, we focus on the protease tissue plasminogen activator (tPA). tPA was shown to mediate neuronal degeneration in the hippocampus after mice were injected with KA. In addition, tPA is involved in activating microglia during excitotoxicity. Therefore, we have taken two approaches to understand whether there is a temporal or source-specific role of tPA in mediating neuronal degeneration and microglial activation. Specifically, we have cultured primary neurons and microglia together, but separated by a porous membrane, so that only diffusible molecules can pass through. We show that microglial, but not neuronal, tPA is responsible for neuronal death after glutamate excitation. tPA from neurons and microglia is responsible for microglial activation. To confirm this data, we have generated transgenic mice that express neuronal- or microglial-tPA on a tPA-null background. We subjected these mice to unilateral hippocampal KA injection, and then examined neuronal degeneration and microglial activation five days later. Both neuronal- and microglial-tPA-expressing mice are susceptible to excitotoxic neurodegeneration. The degree of susceptibility is dependent on the amount of tPA secreted by cells. *Supported by: US Army MCMR.*

Tissue plasminogen activator mediates microglial activation via a cell surface receptor.

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Tissue plasminogen activator (tPA) is a serine protease which converts the inactive zymogen plasminogen into the active serine protease plasmin. In addition to their role in the fibrinolytic system, tPA and plasmin promote neurodegeneration during excitotoxicity. Excitotoxic neuronal death is accompanied by activation of microglia, the immune cells of the CNS. When microglia sense a CNS insult, they become activated and acquire neurotoxic properties and a stereotypical phenotype. We have shown that tPA plays a role in activating microglia during excitotoxicity. However, preventing tPA's proteolytic ability does not attenuate microglial activation, thus indicating a chemokine function for tPA in the CNS. Since tPA is produced by both neurons and microglia in the mouse CNS, we undertook a cell culture approach to study its role in activating microglia. Our results suggest that tPA, released by both neurons and microglia, is capable of activating microglia, possibly by both paracrine and autocrine mechanisms. This activation is characterized by changes in cell morphology, increases in cell number, and release of NO. Furthermore, tPA activates microglia via interactions between its finger domain and a cell-surface receptor on microglia. This specific interaction provides a possible route for interfering with microglial activation when such activation may be detrimental to the system as a whole.

The Journal of Neuroscience, May 1, 2002, 22(9):3352–3358

Tissue Plasminogen Activator Mediates Microglial Activation via Its Finger Domain through Annexin II

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Microglia are the immunocompetent cells of the CNS, and their activation is thought to play an important neurotoxic role in many diseases modeled by glutamate-induced excitotoxicity. One molecule whose expression is upregulated after excitotoxic injury is tissue plasminogen activator (tPA), a serine protease with dual roles in the CNS. The catalytic activity of tPA, which converts plasminogen into plasmin, leads to neuronal death during excitotoxicity. Via a nonproteolytic mechanism, tPA also mediates microglial activation. We show here in culture studies that stimulated wild-type neurons and microglia can release the tPA that elicits the activation, and that tPA acts in combination with other factors. We also show that the finger domain of tPA is necessary to trigger the activation and identify annexin II as its probable binding partner–receptor. Together, these findings suggest that tPA released by either neurons or microglia can act as a neural cytokine, signaling through annexin II to activate microglia in settings of disease and injury. Developing methods to inhibit the interaction of tPA with annexin II would offer a new and selective approach to interfere with microglial activation for therapeutic purposes.

Key words: nonproteolytic; cell signaling; cytokine; receptor; domain deletions; F4/80 glycoprotein

Microglia, derived from the monocyte/macrophage lineage, are considered the immune cells of the brain (Kreutzberg, 1996). After injury, they become activated and acquire a stereotypical phenotype (Streit et al., 1999) that includes proliferation and increased expression of specific markers [e.g., nitric oxide (NO), interleukin-1 (IL-1), tumor necrosis factor (TNF)-α, proteases, and the surface glycoprotein F4/80], changes in morphology (from ramified to amoeboid), migration toward injured cells, and acquisition of antigen presentation and phagocytosis properties.

Activated microglia accumulate in many CNS diseases. They cluster around amyloid plaques in Alzheimer's disease (Giulian, 1999), are found in the brains of animals with scrapie (Russelakis-Carneiro et al., 1999), and have been implicated in the progression of multiple sclerosis and human immunodeficiency virus dementia (Gonzalez-Scarano and Baltuch, 1999). Ischemic events induce vigorous and prolonged microglial activation (for review, see Stoll et al., 1998), which increases the ensuing neurotoxicity by exacerbating the initial insult into the surrounding region (Yenari and Giffard, 2001).

Microglial activation in part regulates the neuronal death pathway known as excitotoxicity (Rogove and Tsirka, 1998). Excitotoxicity, which is observed after overstimulation of neurons with excitatory neurotransmitters, is a component in several neuropathologies (Dickson et al., 1993; DiPatre and Gelman, 1997) and is modeled in animals by injection of glutamate analogs such as kainate (KA) (Olney, 1986). Pharmacological delay of microglial activation protects neurons from KA-induced excitotoxicity (Rogove and Tsirka, 1998).

Tissue plasminogen activator (tPA) is a serine protease; its activation of plasmin is critical for the progression of excitotoxicity (Tsirka et al., 1995, 1997). Plasmin degrades laminin (Chen and Strickland, 1997) and possibly other extracellular matrix (ECM) molecules, and the loss of this substratum promotes neuronal death.

Microglia from tPA$^{-/-}$ mice show attenuated activation after KA injection. Infusion of either catalytically active or catalytically inactive tPA into these mice before KA injection restores microglial activation (Rogove et al., 1999). These data indicate that contrary to its proteolytic role in neurotoxicity, tPA activates microglia via a nonproteolytic mechanism. In addition to its catalytic domain, tPA also contains a fibronectin type-3 finger domain (F), an epidermal growth factor-like (GF) domain, and two kringle domains (K1 and K2), which are thought to mediate protein–protein interactions. The finger domain binds fibrin (van Zonneveld et al., 1986) and annexin II (Hajjar et al., 1994). The GF domain, well characterized in other proteins, is necessary for urokinase plasminogen activator, the other mammalian plasminogen activator, to bind to and activate its receptor (Rabbani et al., 1992). The kringle domains bind fibrin (van Zonneveld et al., 1986), and the second kringle also binds lysine (Gething et al., 1988). Moreover, in addition to mediating the proteolytic function of tPA, the catalytic domain binds to vascular smooth muscle cells (Werner et al., 1999). The catalytic domain also mediates binding to inhibitors such as the plasminogen activator inhibitors (van Zonneveld et al., 1986).

In this report, we use deletion analysis of tPA to define the mechanism through which tPA activates microglia. We find that tPA released from neurons or microglia activates microglia via its finger domain, which most likely interacts with annexin II, a cell-surface receptor, to initiate an intracellular signaling cascade.

Received Sept. 19, 2001; revised Jan. 24, 2002; accepted Jan. 29, 2002.

This work was supported by a Neurotoxin Research Program grant from the United States Army Medical Research to S.E.T. We thank Drs. Michael Frohman, Lincoln Stein, and Chuck O'Neal for critically reviewing this manuscript. We are also grateful to Genentech Inc. for providing the deletion domain mutant tPA proteins.

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MATERIALS AND METHODS

Recombinant human tPA and mutants lacking the finger (ΔF), epidermal growth factor (ΔGF), kringle 1 (ΔK1), or kringle 2 (ΔK2) domains were a generous gift from Genentech Inc. (San Francisco, CA). The tPA proteins were used at a concentration of 500 ng/ml unless otherwise noted. Bacterial lipopolysaccharide (LPS; strain O55:B5; Sigma, St. Louis, MO) was used at 100 ng/ml.

Animal studies and cell culture

C57BL/6 (wild-type) and tPA$^{-/-}$ (Carmeliet et al., 1994) mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained in the Stony Brook Department of Laboratory Animal Research with access to food and water *ad libitum*. The tPA$^{-/-}$ animals were further backcrossed to C57BL/6 mice for nine generations, and progeny of those mice were used for the experiments. All procedures followed guidelines set by the National Institutes of Health and were approved by Stony Brook University. Cultured cells were incubated at 37°C with 5% CO_2. All tissue culture media and additives were from Invitrogen (San Diego, CA) except as noted.

Neuronal cultures were made as described previously (Rogove and Tsirka, 1998). Briefly, the hippocampi from embryonic day 17.5 mouse embryos were dissected and gently trypsinized (0.25% trypsin in HBSS) at 37°C for 15 min and then triturated to form single-cell suspensions. The cells were plated onto poly-L-lysine precoated coverslips at a density of 100,000 cells/cm^2 in neurobasal medium with B27 supplements, 25 μM glutamate, 0.5 mM L-glutamine, and 10 gm/l gentamycin sulfate. The medium was changed 4 hr after the initial plating, and the glutamate was removed after 4 d in culture. Neurons were used after at least 7 d *in vitro*. Neuronal conditioned medium (CM) was obtained from cells stimulated with 25 μM L-glutamate for 4 hr.

Microglial cultures were made from mixed cortical cultures as described previously (Rogove and Tsirka, 1998) except that single cells, obtained by passage through a 70 μm nylon mesh, were plated into poly-L-lysine-precoated 75 cm^2 tissue culture flasks at a density of two brains per flask. The mixed cortical cultures were used to isolate microglia after 14 d *in vitro*, when the cells were selectively released from the flasks by incubation with 15 mM lidocaine for 7 min at room temperature, followed by gentle shaking for 3 min by hand. After low-speed centrifugation, the cell pellet was resuspended in complete medium and plated onto poly-L-lysine-precoated 48 well plates (0.25 ml culture volume per well). The microglia were used at least 2 d after subculture, by which time their morphology resembled that of resting microglia. To obtain microglial CM with the LPS removed, microglia were primed with 100 ng/nl LPS overnight, and then the medium was collected, passed over a polymyxin B-agarose column as directed by the manufacturer (Sigma), and sterilized through a 0.22 μm syringe filter. Endotoxin removal was assayed using the Pyrogent Plus kit (BioWhittaker, Walkersville, MD). Ten endotoxin units (E.U.) of residual LPS were detected, compared with the 25 E.U. originally added. Blocking molecules (see below), when used, were added to the cell cultures 1 hr before addition of tPA. After the addition of tPA, the cultures were continued for 1–24 hr before adding LPS for a final overnight culture period. We found no difference in microglial activation regardless of whether the tPA was added for short (1 hr) or long (24 hr) periods before the addition of LPS. For some experiments, tPA at a 500 ng/ml final concentration was added to the tPA$^{-/-}$ CM, or α-tPA antibody (clone N-14, 600 ng/ml; Santa Cruz Biotechnology, Santa Cruz, CA) was added to the wild-type CM, before the addition of these CM to the microglial cultures (Fig. 1).

Microglial activation

Immunocytochemistry. Microglia were fixed on their coverslips with 4% paraformaldehyde–20% sucrose and then permeabilized using 0.1% Triton X-100. After blocking with goat serum (10% in PBS), the rat anti-F4/80 antibody (Serotec, Indianapolis, IN) was used at a 1:200 dilution, followed by incubation with biotinylated anti-rat IgG (1:1000; Vector Laboratories, Burlingame, CA). The signal was amplified using the Vector Laboratories avidin–biotin complex Elite kit (avidin coupled with horseradish peroxidase) and detected using diaminobenzidine–peroxide (Sigma), after which the coverslips were successively dehydrated in ethanol, delipidated in xylenes, and mounted using Permount (Fisher, Houston, TX).

Quantitative Western blot. Cultured microglia were lysed in 0.25% Triton X-100 in PBS. After centrifuging to remove cell debris, the total protein concentration was measured using the Bio-Rad (Hercules, CA) Bradford detergent-compatible (Dc) assay. Equal amounts (20 μg) of protein from each sample were separated on 10% SDS-PAGE, transferred onto polyvinylidene difluoride membrane, blocked with 5% nonfat dry milk in PBS/0.5% Tween 20 (PBS-T), and incubated with F4/80 antibody (1:200 dilution) overnight in milk/PBS-T. Biotinylated secondary anti-rat antibodies were used (1:500 dilution) and were detected using FITC-labeled ExtrAvidin (1:200; Sigma). Fluorescence was detected by a FluorImager (Molecular Devices, Menlo Park, CA) and quantified using the ImageQuant (Amersham, Sunnyvale, CA) software.

Nitrite detection. The production of nitric oxide from activated cells was measured in the form of nitrites according to the method of Si et al. (1997). CM from treated microglia was collected and refrigerated before detection, after which 20 μl of 0.05 mg/ml diaminonaphthalene (Molecular Probes, Eugene, OR) in 0.28N HCl was added to 100 μl of CM per well in a 96 well black plate (Nunc, Naperville, IL), which was then incubated at room temperature for 10 min. The reactions were terminated by the addition of 100 μl of 0.28N NaOH followed by incubation for 10 min. The generation of nitrites was measured using a microplate fluorescence reader (Titertek, Huntsville, AL) with 365 nm excitation and 450 nm emission filters.

Annexin II analysis

Reverse transcription-PCR. Total RNA was isolated from cultured microglia and neurons using Trizol (Invitrogen) according to the manufacturer's directions. Reverse transcription (RT) was performed using poly-T primers and Moloney murine leukemia virus (Invitrogen) according to the manufacturer's directions. PCR for annexin II was performed using the following primers: 5′-ATGTCTACTGTCCACGAAATC-3′ (forward) and 5′-CAGGTAGAGCCACTTCTGGG-3′ (reverse). For amyloid precursor protein (APP), the primers used were 5′-CGATGGGGGATGCTTCTTGTG-3′ (forward) and 5′-GCTATCATGGCATAAGCAATG-3′ (reverse).

Western blot analysis. Total protein from cultured neurons or microglia was lysed in 0.25% Triton X-100 and clarified by centrifugation. Total protein (5 μg) from the supernatant, quantified using the Bio-Rad Bradford Dc assay, was separated on a 10% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. α-Annexin II was detected using a monoclonal antibody (1:1000 final dilution; Transduction Laboratories, Lexington, KY). To verify cell-specific blotting, α-microtubule-associated protein-2 (a neuronal marker; 1:700; Sigma) antisera and F4/80 (a macrophage/microglial marker) were used. To show equal loading of proteins, an α-actin antibody (1:250; Sigma; a generous gift from Dr. M. Berrios, State University of New York at Stony Brook, Stony Brook, NY) was used.

Antibody blocking assays. Primary tPA$^{-/-}$ microglia were plated as above. Antibodies were added in the following amounts (in μg): 1 α-annexin II, 1 mouse IgG1 (Sigma), 5 α-low-density lipoprotein receptor-related protein (LRP) (rabbit anti-human antiserum; a generous gift from Dr. G. Bu, Washington University, St. Louis, MO). After incubation for 1 hr, tPA was added for another hour, and finally LPS was added and cells were incubated overnight before analysis.

RESULTS

Both neurons and microglia can activate microglia in a paracrine and tPA-dependent manner

Purified wild-type microglia stimulated with LPS become activated, whereas tPA$^{-/-}$ microglia do not unless exogenous tPA is added to the culture (Rogove et al., 1999). However, addition of exogenous tPA in the absence of LPS does not result in activation. These findings suggest that LPS priming stimulates several events required for activation, one of which could be the release of tPA, which would then further stimulate the microglia in an autocrine–paracrine manner. Alternatively, we have proposed that neuronal release of tPA could also activate the primed microglia at this step. To address these hypotheses, we developed a system to assess whether defective tPA$^{-/-}$ microglial activation can be rescued using CM from glutamate-stimulated purified neurons and LPS-primed microglia.

In the absence of manipulation, the cultured tPA$^{-/-}$ microglia used for the experiments displayed primarily a resting phenotype, which is characterized by small cell bodies with long, thin pro-

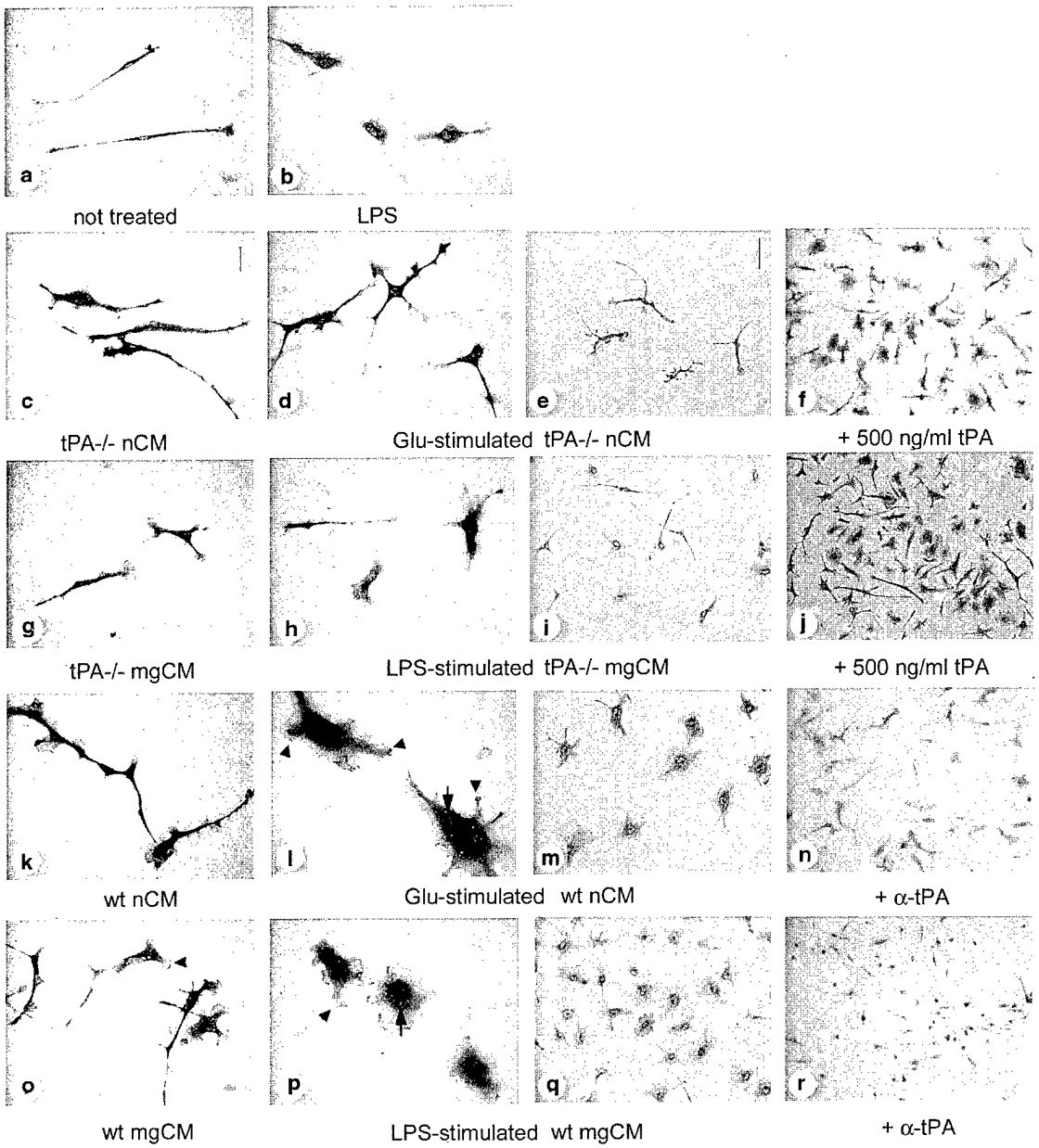

Figure 1. $tPA^{-/-}$ microglia in culture respond to signals released from injured neurons and microglia by becoming activated and changing morphology. Cells were treated as described in Results, and the expression of F4/80 was examined. *a*, Control, untreated $tPA^{-/-}$ microglia (400× magnification). *b*, 100 ng/ml LPS (400× magnification). *c*, CM prepared from quiescent $tPA^{-/-}$ neurons (400× magnification). *d*, CM prepared from glutamate (*Glu*)-stimulated $tPA^{-/-}$ neurons (400× magnification). *e*, Same as *d* (100× magnification). *f*, CM prepared from glutamate-stimulated $tPA^{-/-}$ neurons additionally containing tPA at 500 ng/ml (100× magnification). *g*, CM prepared from quiescent $tPA^{-/-}$ microglia (400× magnification). *h*, CM prepared from LPS-stimulated $tPA^{-/-}$ microglia. The LPS was partially removed using a polymyxin B column before addition of the CM to the responding $tPA^{-/-}$ microglia (400× magnification). *i*, Same as *h* (100× magnification). *j*, CM prepared from LPS-stimulated $tPA^{-/-}$ microglia additionally containing tPA at 500 ng/ml. The LPS was partially removed using a polymyxin B column before addition of the CM to the responding $tPA^{-/-}$ microglia (100× magnification). *k*, CM prepared from quiescent wild-type neurons (400× magnification). *l*, CM prepared from glutamate-stimulated wild-type neurons (400× magnification). *m*, Same as *l* (100× magnification). *n*, CM prepared from α-tPA antibody of glutamate-stimulated wild-type neurons (100× magnification). *o*, CM prepared from quiescent wild-type microglia (400× magnification). *p*, CM prepared from LPS-stimulated wild-type microglia. The LPS was partially removed using a polymyxin B column before addition of the CM to the responding $tPA^{-/-}$ microglia (400× magnification). *q*, Same as *p* (100× magnification). *r*, CM prepared from α-tPA antibody of LPS-stimulated wild-type microglia. The LPS was partially removed using a polymyxin B column before addition of the CM to the responding $tPA^{-/-}$ microglia (100× magnification). *Arrowheads*, Membrane ruffles or pseudopods. *Arrows*, Phagocytic vacuoles.

cesses (Fig. 1*a*). With LPS stimulation, these cells began the process of activation in that their cell bodies and processes became slightly increased in volume (Fig. 1*b*).

In the presence of CM prepared from unstimulated wild-type or $tPA^{-/-}$ neurons or microglia, the $tPA^{-/-}$ microglia similarly exhibited very limited signs of activation (Fig. 1*c,g,k,o*). However, dramatic activation was observed in the presence of CM prepared from wild-type neurons subjected to simulated injury (66.5% of the microglia were activated, as judged by altered morphology) [Fig. 1, *l* (higher magnification) and *m* (lower magnification)]. The

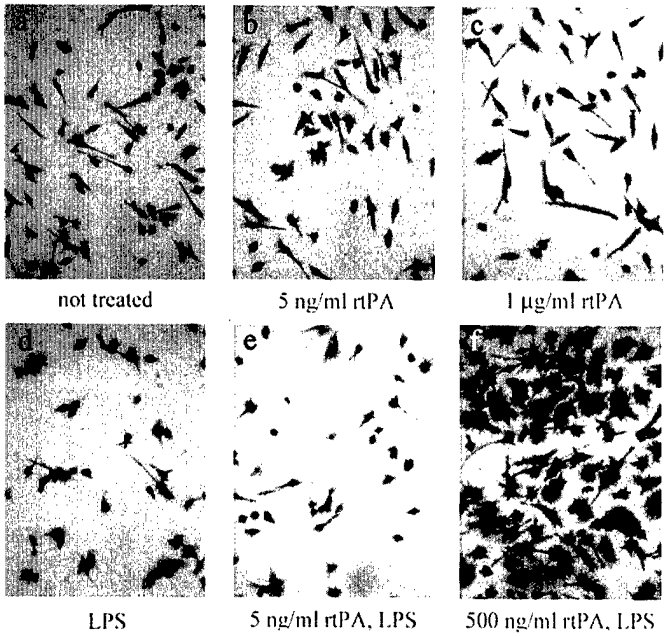

Figure 2. tPA and LPS are required to activate $tPA^{-/-}$ microglia. Microglia were cultured as indicated and then fixed and immunostained for F4/80 expression. *a*, Not treated; *b*, 5 ng/ml tPA; *c*, 1 μg/ml tPA; *d*, 100 ng/ml LPS; *e*, 5 ng/ml tPA, LPS; *f*, 500 ng/ml tPA, LPS. All panels are shown at 100× magnification.

cell membranes became ruffled (Fig. 1, *arrowheads*), and phagocytic vacuoles were observed in the cell bodies (Fig. 1, *arrows*). In contrast, CM prepared from injured $tPA^{-/-}$ neurons did not trigger activation (6.8%) (Fig. 1*d,e*). CM prepared from LPS-stimulated wild-type microglia similarly triggered activation (92.6%) (Fig. 1*p,q*), whereas CM prepared from LPS-stimulated $tPA^{-/-}$ microglia did not (10.6%) (Fig. 1*h,i*). However, the $tPA^{-/-}$-stimulated neuronal or microglial CM could be made functional with the addition of tPA (Fig. 1*f,j*). Finally, addition of an α-tPA antibody to the stimulated wild-type neuronal or microglial CM impaired the ability of the CM to fully activate the responding $tPA^{-/-}$ microglia (Fig. 1*n,r*).

Together, these results show that wild-type but not $tPA^{-/-}$ microglia and neurons, once primed or injured, release a factor(s) in the medium that activates $tPA^{-/-}$ microglia. Because this factor can be replaced by purified tPA and can be blocked by anti-tPA antiserum, it is most likely tPA. The results demonstrate that both neurons and microglia are capable of mediating microglial activation through the release of tPA.

tPA activates microglia in synergy with other stimuli

Cultured microglia often adopt a slightly activated morphology [probably attributable to the culture preparation and conditions (Fig. 2*a*) (Nakajima et al., 1989)]. Stimulation of $tPA^{-/-}$ microglia with tPA does not induce significant morphological changes (Fig. 2, compare *a* with *b* and *c*). LPS treatment alone also induces a rapid (within 30 min) but quite limited change in morphology in many cells (Fig. 2*d*). However, when the cells are treated with 500 ng/ml tPA plus LPS, the microglia become highly activated (Fig. 2*f*). This finding indicates that tPA alone does not suffice to activate microglia; instead, a second signal is required, which can be provided by LPS priming or by factors other than tPA released by stimulated microglia and neurons (Fig. 1).

Microglial activation as a function of tPA and LPS addition was quantified by Western blot analysis. The expression of the glycoprotein F4/80 is upregulated when cells of the monocyte–macrophage lineage are activated (Lawson et al., 1990). We detected an increase in F4/80 immunoreactivity after culturing $tPA^{-/-}$ microglia with LPS and increasing concentrations of tPA. From baseline unstimulated levels, we found a 0.8-fold increase with the lowest dose of tPA added (5 ng/ml), which increased to 2-fold with 50 ng/ml tPA and to 2.4-fold with 500 ng/ml tPA, indicating that the level of activation is dependent on the concentration of tPA (data not shown). Microglia secrete many other molecules during activation, including NO and TNF-α (Meda et al., 1995). The activated microglia were also found to secrete higher amounts of nitrites (2.5 ± 0.2 μM at 500 ng/ml tPA, compared with 0.9 μM from control cells), consistent with the immunochemical data shown in Figure 2 (data not shown).

The finger domain of tPA mediates microglial activation

tPA mediates microglial activation via a nonproteolytic mechanism, possibly through interactions with protein(s) on the microglial cell surface. We used tPA mutants lacking the finger (ΔF), growth factor (ΔGF), and kringle (ΔK1 or ΔK2) domains to determine whether the absence of one or more of these domains abolished microglial activation. The ΔGF, ΔK1, and ΔK2 proteins activated $tPA^{-/-}$ microglia as well as or better than full-length tPA, as judged by F4/80 upregulation (Fig. 3*a*), NO release (Fig. 3*b*), and morphological change (Fig. 3*c*). However, the ΔF mutant did not elicit significant activation (see also Fig. 5*a*). The ΔF mutant did not act as a dominant-negative or toxic factor, because wild-type levels of activation were observed when full-length tPA and ΔF were used together in the activation assay (data not shown). Thus, the presence of the tPA finger domain is critical for mediating microglial activation.

Microglial activation by the finger domain of tPA is receptor-mediated through annexin II

One candidate for a microglial tPA receptor is annexin II (Hajjar et al., 1994). The Ca^{2+}- and phospholipid-dependent annexin II is a known receptor for tPA in endothelial cells (Hajjar et al., 1994). We found by RT-PCR and Western blot analysis that annexin II is expressed by microglial cells but not neurons (Fig. 4), suggesting that there could be a microglial-specific tPA signaling pathway.

To assess whether this pathway was functional, we preincubated $tPA^{-/-}$ microglia with a monoclonal α-annexin II antibody for 1 hr before tPA/LPS activation (Fig. 5*a*). Addition of the antibody alone (Fig. 5*A*, *column 4*) did not affect unstimulated cells (Fig. 5*A*, *column 1*). However, the antibody did block (Fig. 5*A*, *column 5*) the activation normally elicited by tPA and LPS (Fig. 5*A*, *column 2*), as assessed by F4/80 upregulation. Similar results were observed by morphology (Fig. 5*b–d*) but not in the presence of an IgG1 isotype control (Fig. 5*e*). Finally, incubation with an antibody directed against LRP, which is a known receptor for tPA in liver cells (Bu et al., 1992), did not prevent microglial activation (Fig. 5*f*). These results suggest that the finger domain of tPA mediates microglial activation by signaling through the cell surface protein annexin II.

DISCUSSION

tPA mediates neuronal death and microglial activation during excitotoxic injury. We have reported previously that the proteolytic activation of plasmin by tPA is required for neurodegeneration to progress (Tsirka et al., 1997). The role of plasmin has been

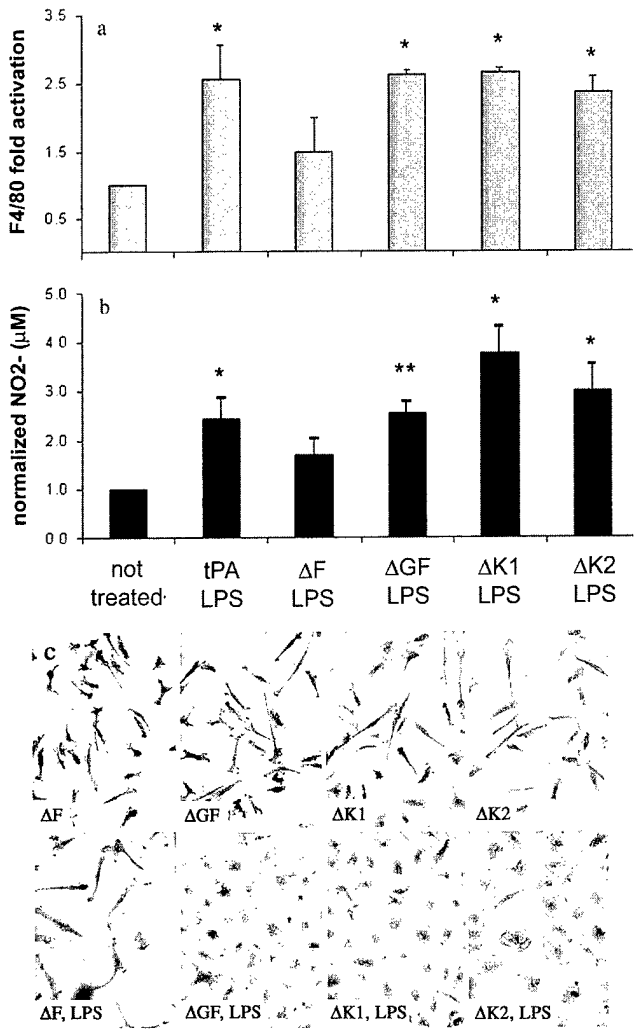

Figure 3. The finger domain of tPA mediates microglial activation. $tPA^{-/-}$ microglia were cultured with LPS in combination with full-length tPA (*tPA LPS*), tPA lacking the finger domain (*ΔF LPS*) or the growth factor domain (*ΔGF LPS*), or either of the kringle domains (*ΔK1 LPS* and *ΔK2 LPS*). *a*, Quantification of F4/80 expression by Western blot. *b*, Detection of NO release by its product, nitrite (NO_2^-). $^*p < 0.01$; $^{**}p < 0.05$. Data expressed are averages ± SEM of triplicate experiments; all analyses were conducted using Student's *t* test relative to the control cells that did not receive additions to the culture. *c*, $tPA^{-/-}$ microglia were cultured on coverslips under the indicated conditions and then immunostained for F4/80 expression; panels are shown at 200× magnification.

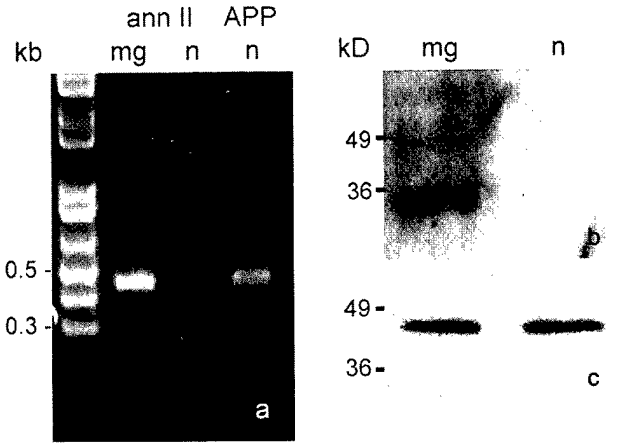

Figure 4. Primary microglia, but not neurons, express annexin II, a potential tPA receptor. *a*, RT-PCR results from microglia (*mg*) or neurons (*n*), amplified using either annexin II (*ann II*) or APP primers. The quality of the neuronal cDNA was established using the APP as a positive control (*lane 4*). *b*, Western blot confirming that primary microglia but not neurons express annexin II. The cell lysates used for the Western blot were also probed with the neuronal marker microtubule-associated protein 2 and the microglial marker F4/80 to confirm that the respective cell types had been highly enriched as intended and indicated (data not shown). *c*, An anti-actin antibody was used to ascertain equal protein loading for the lysates analyzed on the Western blot.

proposed to involve subsequent degradation of ECM molecules, such as laminin (Chen and Strickland, 1997), resulting in loss of neuronal contact with the ECM, which may lead to apoptosis (Frisch and Ruoslahti, 1997; Frisch, 2000). However, the proteolytic activity of tPA is not necessary to activate microglia, suggesting an additional, cell-signaling function for tPA in the CNS (Rogove et al., 1999). Because tPA is secreted by both neurons and microglia, we addressed first whether either cell type released enough tPA into CM under conditions of stimulation to activate microglia. We found that stimulated $tPA^{-/-}$ neurons or microglia failed to induce activation in $tPA^{-/-}$ microglia but that this result was reversed by addition of tPA into the cultures (Fig. 1). Finally, incubation with an antibody that sequesters the released tPA present in the stimulated wild-type CM partially (neuronal CM) to fully (microglial CM) inhibits the ability of the CM to mediate activation (Fig. 1; also see below for further discussion). These experiments confirm that one of the factors responsible for microglial activation after stimulation in our system is soluble tPA.

The finding that glutamate-injured neurons can release sufficient tPA to activate $tPA^{-/-}$ microglia suggests a paracrine mechanism of microglial activation *in vivo*, in which the injured neurons would signal the immune cells of the CNS (i.e., the microglia) to scavenge damaging molecules secreted from the dying cells. In addition, we show that microglial activation can further stimulate release of microglial factors, such as tPA, that can in turn activate neighboring microglia in an autocrine or paracrine manner. This signal amplification ultimately leads to recruitment of microglia to the site of injury in the brain and can promote both a timely resolution of cellular injury and an overly sensitive inflammatory response. The data presented here support the role of tPA as a cytokine in this setting.

Our dose–response results indicate that there is a threshold for microglial reactivity to injury. It is widely accepted that microglia are the sensors of injury in the brain (Kreutzberg, 1996), and we propose that tPA acts as a microglial sensor molecule. Normally, the level of tPA released into the extracellular space is tightly regulated. tPA is stored in intracellular vesicles and is released only by Ca^{2+} influx during neuronal depolarization (Gualandris et al., 1996). Another level of control is afforded by the presence of several serine protease inhibitors in many regions of the brain [e.g., plasminogen activator inhibitors-1 and -2 and neuroserpin (Akiyama et al., 1995; Krueger et al., 1997)]. We and others have shown that tPA plays an important role in physiological processes such as neurite outgrowth (Seeds et al., 1999; Wu et al., 2000). This highly localized secretion of tPA, and hence localized activation of plasmin, is probably necessary for neurites to degrade ECM structures so that they can reach their targets. Therefore, neurons must be able to tolerate significant local increases in tPA activity surrounding them without sending out injury/death and inflammatory signals. To this end, we have shown that tPA alone

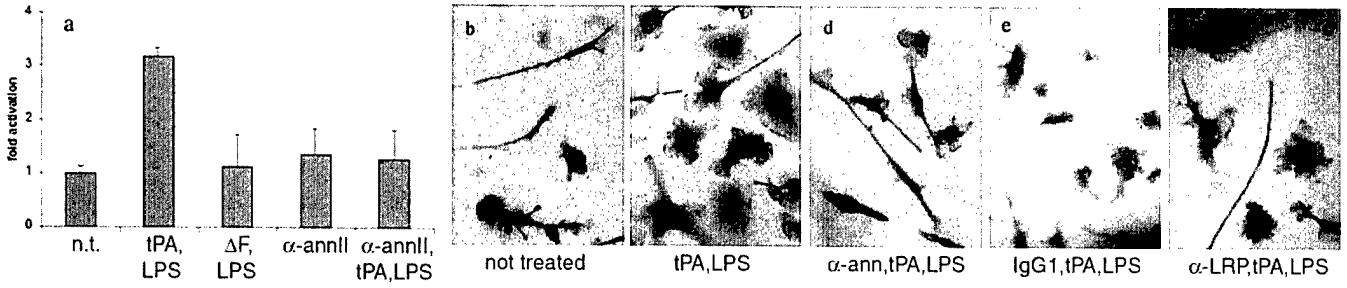

Figure 5. tPA-mediated microglial activation is blocked by α-annexin II antibodies. *a*, Measurement of F4/80 expression by Western blot. $tPA^{-/-}$ microglia were cultured with medium only [not treated (*n.t.*)]; LPS and 500 ng/ml tPA (*tPA, LPS*); LPS and 500 ng/ml ΔF mutant (*ΔF, LPS*); 1 μg/ml anti-annexin II (*α-annII*); or LPS, 1 μg/ml anti-annexin II, and 500 ng/ml tPA (*α-annII, tPA,LPS*). Data from two independent experiments were pooled and plotted as fold activation over the control cells, which received no additions. Error bars indicate range of data points. *b–e*, $tPA^{-/-}$ microglia were cultured alone or with α-annexin II or α-LRP antibodies for 1 hr, 500 ng/ml tPA was added for another hour, and then LPS was added and the cells were incubated overnight. Activation was examined using F4/80 immunocytochemistry on fixed cells as described in Materials and Methods. The panels are shown at 400× magnification.

is not neurotoxic (Tsirka et al., 1996) (C.-J. Siao and S. E. Tsirka, unpublished observations). Like neurons, microglia must also be able to differentiate between high levels of tPA alone (which may be physiological) and high levels of tPA and some other factor, the combination of which is now an injury signal for microglia to become activated. In most of our experimental conditions, that other factor is bacterial LPS, whose presence is uncommon in the normal CNS. Physiological candidates would include proinflammatory molecules such as TNF-α and IL-1β that play important roles in activating microglia in culture (Meda et al., 1995). We believe that this cell-culture approach will help to define the identity of this other factor or factors. Indeed, our data using antibodies to block tPA binding to microglia and subsequent activation support this hypothesis (Fig. 1). Figure 1*n* (wild-type neuronal CM plus α-tPA) shows more activated microglia than those in Figure 1*r* (wild-type microglial CM plus α-tPA). Because the same concentration of antibody was used and the same amount of tPA was released from each cell type (data not shown), an additional factor other than soluble tPA presumably is responsible for the advanced activation using neuronal CM. Recent data from our laboratory suggest that excitotoxic stimulation of hippocampal neurons results in the upregulation and secretion of active monocyte chemoattractant protein-1, which promotes microglial activation–migration (A. D. Rogove, J. Z. Sheehan, Y.-P. Wu, Tsirka, unpublished observations).

The use of CM in a cell-culture setting is artificial, because of, among other reasons, the absence of cell types such as astrocytes, which can contribute to both amplification of tissue injury and containment of damage (Cui et al., 2001; Mandell et al., 2001). In addition, although LPS is widely used to prime cells of the monocyte/macrophage lineage for activation *in vitro*, this priming step would presumably be mediated *in vivo* by unknown endogenous factors. Finally, our findings show that microglia and neurons can release sufficient tPA *in vitro*, but not that they take up this role *in vivo*. All of these issues will be resolved by developing methods to address the questions using transgenic animal models, and these experiments are currently in progress.

To explore the mechanism of receptor-mediated microglial activation by tPA, we undertook a domain-deletion approach. Our results suggest that the finger domain of tPA plays a key role in activating microglia during excitotoxic injury. The slight activation of microglia in the cells treated with the ΔF mutant and LPS is probably attributable to LPS priming. We would not predict that addition of the finger domain alone would completely reconstitute microglial activation; evidence suggests that the finger–growth factor domains interact with the catalytic domain (Novokhatny et al., 1991). Thus, the protease domain could also be involved (noncatalytically) in physically mediating activation. Recent data indicate that the catalytic domain may mediate tPA binding to human vascular smooth muscle cells (Werner et al., 1999). Mutants with catalytic domain deletions could be used to answer this question.

Annexin II, a Ca^{2+}- and phospholipid-binding protein, belongs to a family of proteins characterized by a highly conserved set of α-helical repeats in the C terminus that mediate membrane binding (Mollenhauer, 1997). Annexin II is usually found as a heterotetramer consisting of two annexin II (p36) and two S100A10 (p11) subunits. A hexapeptide sequence in the N terminus of annexin II has been shown to bind to the finger domain of tPA and to increase tPA activity on endothelial cells (Hajjar et al., 1998). Because annexin II is a membrane-associated protein to which tPA and plasminogen presumably only dock, it is unclear how it may transduce inside the cells the activation signal resulting from the binding of extracellular tPA. Members of the annexin family are secreted to the cell surface via an unknown mechanism (Mollenhauer, 1997). Annexin II binds phosphatidylserine, so it could be cotranslocated across the membrane during early stages of apoptosis, although how and why this might occur during microglial activation is not at all clear.

Once inside the cell, the tPA/annexin II complex could signal in various ways. For example, the site at which tPA binds to annexin II is also where S100A10/p11 binds annexin II. One could envision a displacement and release of S100A10/p11 to function as an intracellular signal molecule. Alternatively, another as yet unidentified molecule associated with annexin II may mediate signaling cascades to activate microglia when tPA binds to annexin II. Annexin II is also a prominent intracellular signaling protein found in caveolas and lipid rafts, and it is phosphorylated by several kinases at key residues. Finally, annexin II is known to mediate interaction between cholesterol-rich membrane domains and the actin cytoskeleton (Filipenko and Waisman, 2001). This observation may underlie the changes in morphology during microglial activation. We are currently characterizing biochemically the interactions of the finger domain with annexin II and examining how this interaction mediates signal transduction.

tPA is used clinically as a thrombolytic agent during acute

stroke. However, its neurotoxic effects in a mouse ischemia model suggest caution (Wang et al., 1998), because it can also activate microglia and lead to an exacerbation of neuronal death (Rogove and Tsirka, 1998; Rogove et al., 1999). Identification and further characterization of the tPA domain that activates microglia may aid in reducing inflammation and neurotoxicity. Mutating this binding region could lead to a clinically useful form of tPA that retains its thrombolytic properties yet does not trigger microglial activation.

A large body of research suggests that microglia can undertake either neurotoxic or neuroprotective roles in different disease or model settings (Dickson et al., 1993; Gehrmann et al., 1995; Benveniste, 1997; DiPatre and Gelman, 1997). It is highly likely that both roles exist. In addition to cleaning up debris from dead and dying cells, phagocytes are also required to contain damage so that other cells in the regions surrounding the injury are not damaged. However, overzealous reactive microglia can secrete excessive proinflammatory molecules, thus worsening the initial injury. Identification of annexin II as a microglial cell surface tPA receptor and characterization of the detailed interaction between the finger domain of tPA and annexin II would allow a potentially powerful method of interfering with microglial activation when such activation may be detrimental to the system as a whole.

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ISSN 0145-5680

March
Vol. 48/ No. 2 2002

CELLULAR AND MOLECULAR BIOLOGY™

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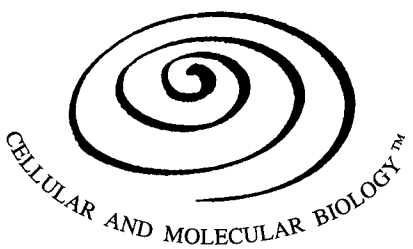

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Founder : Raymond J. WEGMANN, Paris, France

Publishing, Subscription, Advertising, Inquiries :
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2002 Cellular and Molecular Biology™, N° Commission Paritaire: 51379AS
Director of the Publication: R.J. Wegmann

Cellular and Molecular Biology™ **48** (2), 151-161
Printed in France.

0145-5680/02
2002 Cell. Mol. Biol.™

Review

EXTRACELLULAR PROTEASES AND NEURONAL CELL DEATH

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Received November 28, 2000; Accepted December 20, 2000

Abstract - Neuronal cell death occurs during development of the central nervous system as well as in pathological situations such as acute injury and progressive degenerative diseases. For instance, granule cells in the developing cerebellum and neuronal precursor cells in the cortex undergo programmed cell death, or apoptosis (40). There is currently strong debate concerning the mechanism of death in many degenerative events such as ischemia, blunt head trauma, excitotoxicity and neurodegenerative diseases, i.e. Alzheimer's disease. Neurons can die a necrotic death when the initial insult is too great; apoptosis requires "planning." For example, the cell death seen in the core of an ischemic infarct is necrotic, while in the surrounding penumbra region the death is probably apoptotic (87). Regardless of the degenerative pathway, damaged or dead neurons are a hallmark of many diseases including Alzheimer's, Parkinson's, glaucoma, ischemia and multiple sclerosis. Molecules such as cytokines, chemokines, reactive nitrogen/oxygen species, and proteases play an important role in promoting and/or mediating neurodegeneration. Proteases have been implicated in both physiological and pathological events, suggesting their intervention in key points when things go awry. In this review we will summarize recent findings linking extracellular proteases with neuronal cell death in both human diseases and their animal models.

Key words: Tissue plasminogen activator, neurodegenerative diseases, thrombin, metalloproteinase, excitotoxicity

PROTEASES AND THE PHYSIOLOGIC BRAIN

Before addressing the role of extracellular proteases in neuronal cell death, we will give a short overview of the potential physiological roles performed by some of these proteases. (Table 1.)

Tissue plasminogen activator

Tissue plasminogen activator (tPA), an extracellular serine protease that cleaves the zymogen plasminogen into the active, broad-spectrum protease plasmin, plays an important role in fibrinolysis (blood clot removal). It is increasingly recognized to be involved in brain development and function as well.

Expression of tPA very early in mouse central nervous system (CNS) development (around E7.5) suggests that the protease plays a role in tissue remodeling (15). Several pieces of evidence indicate that tPA regulates neuronal plasticity and migration. tPA is released from growth cones of granule cells in culture, suggesting that protease activity is required for neurite outgrowth or synaptic plasticity (47,48). tPA mRNA levels are high in the murine hippocampus (79), which is the site of learning and memory integration. tPA expression is upregulated during motor learning in the rat cerebellum (83). In the developing cerebellum of mice deficient in tPA ($tPA^{-/-}$), granule neurons migrate into the molecular layer at only half the rate of wild-type neurons (83). However, by the end of postnatal development, these granule neurons catch up, and eventually no difference is evident between wild-type and $tPA^{-/-}$ cerebellar cells (84).

$tPA^{-/-}$ mice show no overt developmental or adult phenotype, which would distinguish them from wild-type mice (14). However, they do show reduced and different late stage of long-term potentiation (32,37), suggesting a deficiency in learning and memory consolidation. This finding is supported by abnormal learning and completion of memory-related tasks (13), and by some deficits in either memory retention or behavioral inhibition in a cocaine self-administration experimental paradigm (74). In a transgenic mouse model, where tPA was overexpressed in neurons (under the control of the Thy1.2 promoter), the mice exhibited

Abbreviations: **CNS**: central nervous system; **ECM**: extracellular matrix; **IL**: interleukin; KA: kainic acid; **LPS**: lipopolysaccharide; **MCP**: macrophage chemotactic protein; **MMP**: matrix metalloproteinase; **MSP**: myelencephalon-specific protease; **PAI**: plasminogen activator inhibitor; **PN-1**: protease nexin-1; **TNF**: tissue necrosis factor; **TPA**: tissue plasminogen activator

Table 1 Expression patterns of proteases in the mammalian CNS

Protease	CNS Regional Expression	Citation
Tissue Plasminogen Activator	Neurons, microglia (mouse, rat)	88
(pro)Thrombin	Neurons of olfactory bulb, cortex, superior and inferior colliculi, corpus striatum, thalamus (rat)	23
MMP-9 (Gelatinase B)	Pyramidal neurons (human)	5
	Oligodendrocytes (human)	92
	Macrophages (stroke, MCAO); endothelial cells	4
		77
	Astrocytes, microglia (normal); reactive astrocytes (active MS)	20
MMP-2 (Gelatinase A)	Macrophages (MCAO), white matter microglia, leukocytes (acute MS lesions)	77
		4
ADAM 8 (CD 156)	Cortical neurons, granular neurons of cerebellum and dentate gyrus, spinal cord motoneurons	79
Neuropsin	Hippocampus, cerebra cortex, limbic system	56
Myelencephalon-specific Protease	Oligodendrocytes (rat spinal cord)	78
Neurotrypsin	Neurons of cerebral cortex, hippocampus and amygdala	32
Cathepsin-G	Astrocytes	16
Cathepsin-S	Microglia and macrophages (rat)	67

enhanced LTP, and performed better than their wild-type counterparts in learning tasks that evaluated spatial orientation (52). Our recent work demonstrates that the tPA/plasmin system regulates neurite outgrowth along the mossy fiber pathway, indicating a potential role for tPA's proteolytic function during learning and memory formation (103). All the current data point to the important role that tPA plays in synaptic plasticity and memory formation.

Thrombin

Thrombin is another serine protease, which was first described as a factor of the coagulation system. In the CNS the proenzyme prothrombin has been localized in the postnatal rat olfactory bulb, cortex, superior and inferior colliculi, corpus striatum and thalamus (25). In culture, thrombin has been shown to induce neuronal and glial process retraction, and glial proliferation, results that suggest a role for thrombin in CNS development and plasticity (101). Most of the cellular functions of thrombin are effected through its receptor, a member of the G-protein coupled receptor superfamily (97). Although there is no current evidence that prothrombin is activated to thrombin in the brain, its receptor has been localized *in vivo* and in culture to both neurons and glia (61).

Metalloproteinases

The family of metalloproteinases comprises both extracellular and membrane-bound proteases which require divalent cations such as Zn^{2+} for activity. Under this heading, we will discuss both matrix metalloproteinases and the disintegrin-metalloproteinases.

Matrix metalloproteinases or MMPs, play an important role in remodeling of both central and peripheral tissues by normal and invading cancer cells. Their substrates include almost all of the extracellular matrix proteins, including collagens, gelatins, proteoglycans and other MMPs (16). MMP-9 (gelatinase B) is synthesized by neurons (6) as well as glia (Table 1). In contrast, other MMPs appear to localize to the CNS only after injury, presumably through a disrupted blood-brain barrier. In the CNS, MMP action has been linked to mediating neurite outgrowth (6), astrocytic process formation (95), and oligodendrocyte process outgrowth (64). Interestingly, the tissue inhibitors of MMPs (TIMPs) are also specifically localized in the same regions as MMPs, suggesting that proteases and inhibitors in the CNS are normally balanced with each other.

The disintegrin-metalloproteinases (the ADAM proteins) are characterized as cell surface proteins with the following structure (from amino- to carboxyl-terminus, outside to inside the cell): pro-domain, metalloproteinase, disintegrin, cysteine-rich pro-adhesion domain, EGF-like domain, transmembrane region, and a short cytoplasmic tail (72). Of the nearly 30 members, only ADAM 8 (CD 156) is found in microglia, the monocytic cells of the CNS (104). ADAM family proteases are also important in processes such as fertilization, protein shedding, myogenesis, and neurogenesis (72). Recently, it was demonstrated that metalloproteinase inhibitors such as IC-3 promote dorsal spinal cord axonal outgrowth in the presence of netrin-1, a chemoattractant (33). The receptor for netrin-1, deleted in colorectal cancer (DCC), appeared to be cleaved extracellularly (shed) by metalloproteinases. The regulation of receptors to chemoattractants can thus help shape axonal pathfinding in the developing CNS (33).

Neuropsin, myelencephalon-specific protease and neurotrypsin

Neuropsin is a serine protease with substrate specificity very closely related to that of tPA and thrombin (19). It is expressed in the rodent CNS in regions of the limbic system, hippocampus and cerebral cortex (58). It becomes up-regulated in mice after any direct stimulation of the hippocampus (19,88), and presumably affects learning and memory. Neuropsin has been suggested to play a role in modulating the extracellular matrix during plasticity-related processes (105); as supporting evidence, it has been found to degrade fibronectin (85). Furthermore, neuropsin is post-translationally modified by glycosylation (89), which implicates this protease in protein-protein interactions and regulation of neurite outgrowth.

Another serine protease expressed in the CNS is myelencephalon-specific protease (MSP), which was cloned based on its similarity to neuropsin, trypsin and kallikrein (80). It is expressed primarily by oligodendrocytes (81) and is present at higher levels in the brain and spinal cord compared to the rest of the tissues (54). After excitotoxic insult to the rat, the levels of MSP mRNA increased and remained high compared to uninjured controls. MSP mRNA localization in the spinal cord white matter glia suggests that MSP may play an important role in such degenerative spinal cord diseases as amyotrophic lateral sclerosis (ALS) (80). It can also modify the extracellular matrix by degrading structural proteins such as fibronectin and gelatin, but not laminin, collagen I and IV or elastin (52).

Neurotrypsin, mapped to human chromosome 4 (44), is a newly cloned multi-domain serine protease with expression restricted to the cerebral cortex, hippocampus and amygdala. Due to its expression pattern, neurotrypsin is proposed to play a role in learning and memory formation (34).

Cathepsins

The cathepsins form a family of intracellular / lysosomal and secreted cysteine proteases expressed by cells of the monocyte / macrophage origin (69). In the CNS, these cells are the phagocytic microglia, which express cathepsins B (78) and S (69). Cathepsin G is primarily expressed by astrocytes and binds to them via a cell surface expressed receptor which has affinity for the cathepsin G: α1 anti-chymotrypsin complex (17). Suppressing the activity of lysosomal cathepsins B and L pharmacologically has led to the formation of meganeurites (10). Meganeurites are found positioned between neuronal cell bodies and axons, suggesting that they may interfere with axonal transport; thus cathepsins may regulate neurite outgrowth and synaptic plasticity. Cathepsin G is also implicated in disposing of oxidatively modified mitochondria in the aging and degenerating nervous system (17).

tPA AND EXCITOTOXICITY

Rothman and Olney (65) introduced the theory of excitotoxicity which suggested that excess extracellular neurotransmitters, such as the central excitatory amino acid glutamate, can over-stimulate specific neurons so that the cells become hyper-depolarised and degenerate. Since then, a substantial amount of research has been conducted to show that excitotoxicity is an important cause of neuronal death in a variety of pathological events. Extracellular glutamate levels are tightly controlled in the normal CNS by astrocytes and microglia. Astrocytes are the primary CNS cells which convert glutamine to glutamate and subsequently store it. When damaged, astrocytes can release excess glutamate, which can act as an excitotoxin. Microglia can also release large amounts of glutamate through their glutamate transporters (63).

Excess extracellular glutamate is linked to many pathological diseases. Glutamate toxicity has been suggested to mediate neurodegeneration in Alzheimer's disease (43,55). Intra-amygdala injections of kainate, an analogue of glutamate, induce seizures that mimic epileptic events and cause hippocampal CA3 pyramidal neurons to undergo apoptosis (71). High levels of glutamate are found in the vitreous bodies of glaucomatous eyes, suggesting an excitotoxic cause of death of the optic nerve and retinal ganglion (28). Finally, glutamate-induced excitotoxicity is implicated in the neuronal loss observed in autoimmune demyelination (70).

We and others have shown that intra-hippocampal injection of kainate kills pyramidal neurons in wild-type mice (3), but not in $tPA^{-/-}$ mice (91). The resistance to neuronal death in $tPA^{-/-}$ mice is due to tPA's proteolytic cleavage of plasminogen, since mice deficient in plasminogen are also resistant to kainate (93) (Fig. 1). Infusion of recombinant tPA into the brains of $tPA^{-/-}$ mice restored neuronal sensitivity to kainate, indicating that it is the acute action of tPA / plasmin, and not any developmental abnormality of the mutant mice, that mediates this neurotoxic role (93). In a different paradigm of neuronal death, namely in the model of focal ischemia / reperfusion injury, mice deficient in tPA were shown to have approximately 50% smaller infarct size that their wild-type counter-parts (when a no-clot-inducing, siliconized suture was used for ischemia). Intravenous delivery of recombinant tPA resulted in increase of the infarct size to wild-type levels (99). Furthermore, the potential of intracerebral hemorrhage was augmented by 85% in a rat focal ischemia model, suggesting that tPA promotes hemorrhage during reperfusion injury (5).

Interestingly, tPA and plasmin are not involved in kainate-induced motoneuron degeneration *in vitro* (96), suggesting that other factors may be affecting the excitotoxin death in the

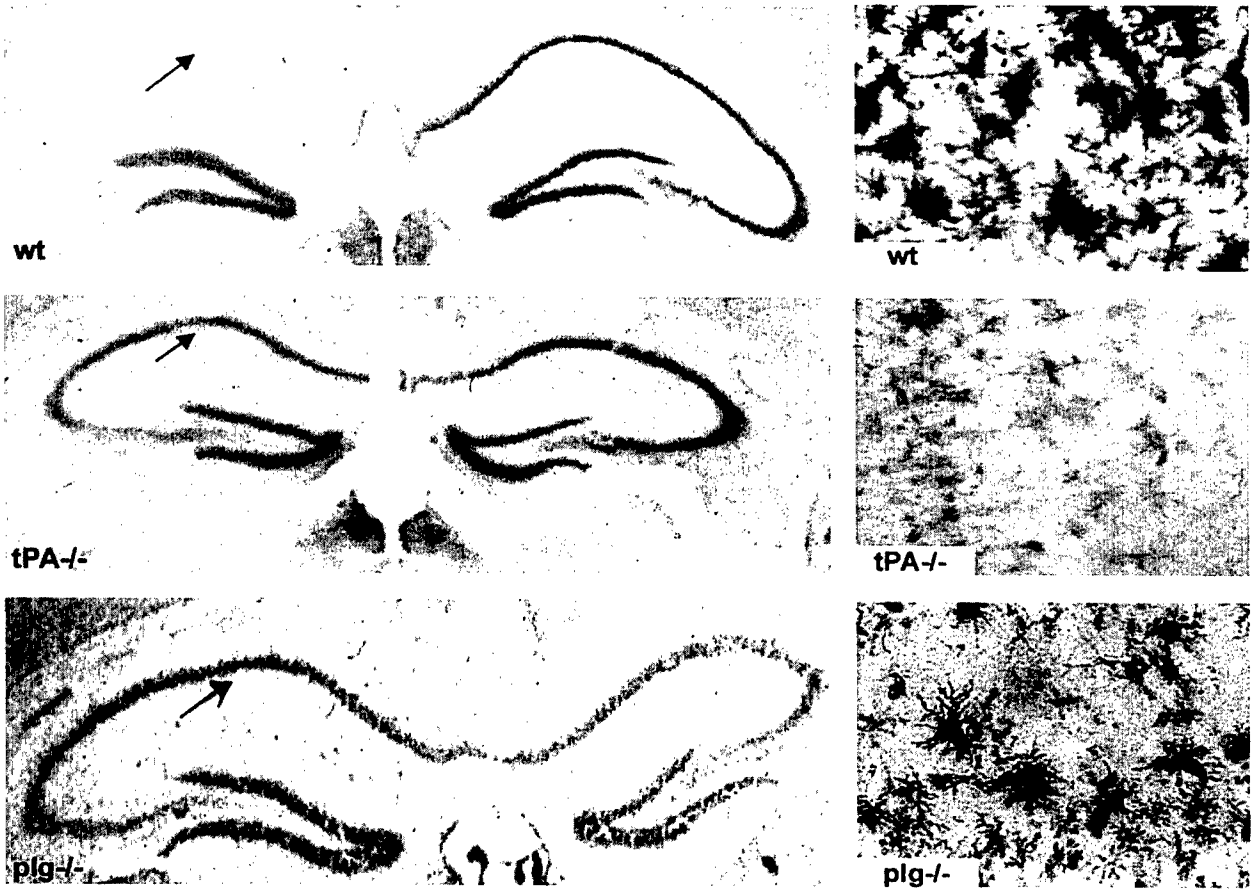

Fig. 1 tPA mediates neuronal death and microglial activation in wild-type mice using two different mechanisms. When kainate, a glutamate analogue, is injected unilaterally into the hippocampus (arrow) of a wild-type mouse (top left), extensive neuronal death is evidenced by the lack of cresyl violet staining. At the same time, microglia are activated (top right). In a tPA-deficient mouse, limited neurodegeneration is seen (middle left) and microglial activation is attenuated (middle right). The proteolytic activity of tPA is required for neurodegeneration, since mice deficient in plasminogen, tPA's substrate, are also resistant to excitotoxic death (bottom left). Meanwhile, tPA activates microglia in a non-proteolytic fashion, since in plg-deficient mice the microglia are still activated in response to the kainate insult (bottom right).

peripheral nervous system (PNS). In fact, increased axonal degeneration was detected in tPA- or plasminogen-deficient mice after sciatic nerve crush (1). The extent of the injury was ameliorated by genetic or pharmacological depletion of fibrin(ogen), identifying fibrin as the plasmin substrate in the PNS under inflammatory axonal damage (1). This result suggests that fibrin deposition could be a regulator of neuronal injury in the PNS. However, it does not affect the acute neuronal death observed during excitotoxicity in the CNS, since fibrin is not normally detected in the brain parenchyma (92). Mice deficient in both plasmin(ogen) and fibrin(ogen) are resistant to excitotoxin-induced neuronal death, similar to plasminogen-deficient mice (92).

Furthermore, tPA does not affect the cerebellar neuronal death seen in the *weaver* mouse (56), even though elevated tPA activity was detected in these mutant mice at the time of neuronal death (59). However, the serine protease inhibitor aprotinin was shown to rescue the dying *weaver* neurons from cell death, and to even restore their resting potential (59). The cell death in this mouse has been attributed to a mutation in the GIRK2 potassium channel, which renders the channel overly excitable (66,86). Since it has been shown that serine protease inhibitors have a three-dimensional structure similar to potassium channel blockers (49), we could hypothesize that the neuroprotection by aprotinin may be attributed to this blockage of the mal-functioning potassium channel. In this case the increased tPA activity was just coincident with the presence of dying neurons.

In a different setting of neurotoxicity, in zinc-mediated neuronal cell death in the rat, tPA was reported to protect neurons from degeneration via a non-proteolytic mechanism (42).

tPA is released by both neurons and microglia in the mouse CNS (91). Since tPA promotes neuronal death during excitotoxicity, and literature suggests that microglia may have a role that can be either neuroprotective or neurotoxic, we investigated the role of microglia in neuronal death.

MICROGLIA: FRIEND OR FOE?

Deriving from the bone marrow, microglia migrate across the immature blood-brain barrier and become resident brain cells. Of the macrophage/monocyte lineage, microglia exhibit many of the phenotypes of these immune cells, including the ability to phagocytose and present antigens.

Resting microglia in the brain exhibit a ramified morphology, characterized by short, thin processes and a small cell body. When they sense damage or injury to the brain, they become activated, a state which includes several changes in cell behavior. They migrate towards the site of damage or injury and proliferate around the injured neurons. They also change their gene expression, which includes the production of excitatory amino acids (i.e. glutamate and quinolinic acid) and factors such as cytokines, proteases and reactive nitrogen/oxygen species. Microglial morphology changes, as evidenced by the enlargement of the cell body and concomitant thickening of the processes. Finally, activated microglia acquire phagocytic abilities. The activation state and recruitment of microglia depend on their proximity to the site of injury, and may be triggered by molecules secreted by damaged neurons or glia in a gradient fashion.

Activated microglia secrete a plethora of molecules, at concentrations that render them toxic to neurons (45). For example, the inflammatory factors TNF-α, IL-1β, IL-6 and the chemokine MIP-1α are associated with demyelination and CNS inflammation (41). Chemokines, such as MCP-1, secreted by locally activated astrocytes and microglia, can recruit more microglia to the site of injury, thus potentially exacerbate the neurotoxic effects of microglial cells. Activated microglia also secrete large amounts of nitric oxide, which has been shown in several studies to potentiate neuronal death (57,68). Microglia can be stimulated, probably in a paracrine manner, by activation of glutamate receptors, particularly those of the AMPA subtype; depolarization of these cells can lead to release of many toxic factors including TNF-α (63). Excess glutamate is also released by activated microglia (67), supporting the role of these cells in NMDA receptor-mediated neurotoxicity, as seen in cerebrovascular neuronal damage and HIV-associated dementia (7). Finally, activated microglia can convert L-tryptophan to quinolinic acid, an analog of NMDA, which is highly neurotoxic (35).

Interestingly, activated microglia are often seen clustered around injured neurons and neurites in acute or chronic diseases, for example, around the dystrophic neurons and plaques of Alzheimer's disease (24,26). This accumulation can be interpreted in two ways, both of which may be true: that microglia are recruited to / by the injured neurons to phagocytose the debris, or that their presence exacerbates the injury due to the toxic molecules they secrete. One potential way to differentiate the two possibilities is to prevent microglial activation, but allow their recruitment to the site of injury.

Activated microglia secrete higher amounts of tPA than resting microglia (76). Since proteases are implicated in

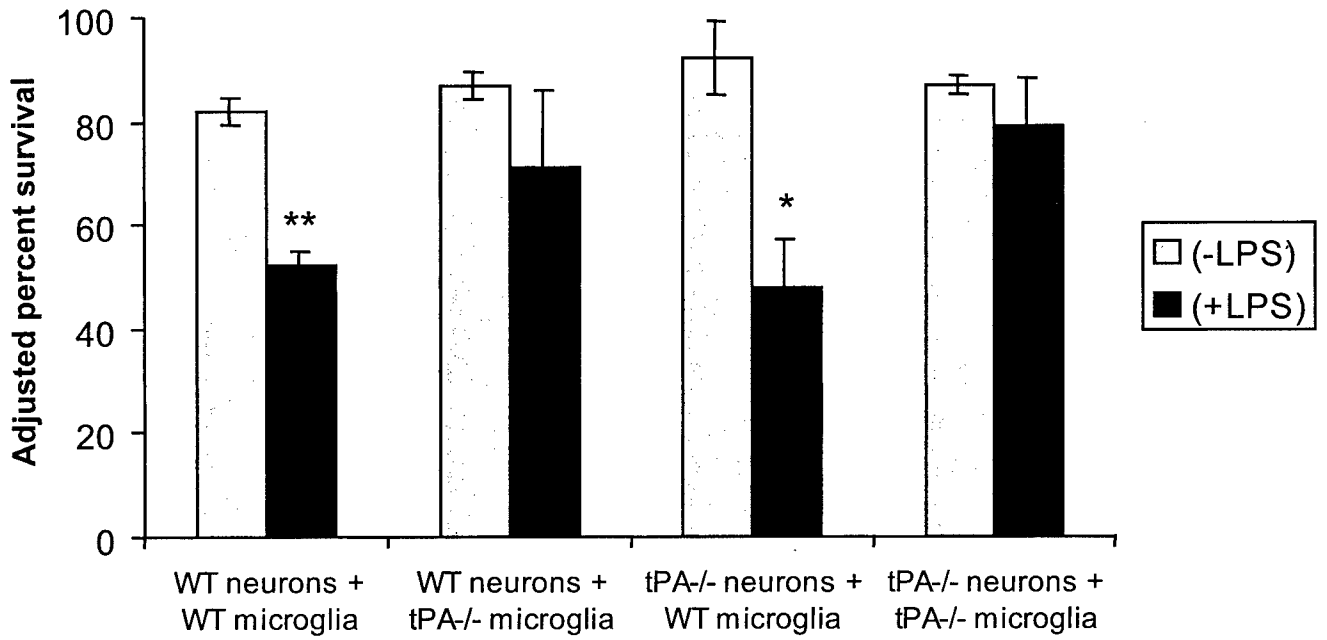

Fig. 2 Co-culturing wild-type and tPA-/- neurons and microglia shows that microglial tPA is required for neuronal death *in vitro*. Microglia were stimulated with 100 ng/ml LPS, and neurons were primed with 25 μM glutamate prior to co-culture. Cells were separated from each other by a 0.4 μm membrane and co-cultured for 3 hr. LPS alone did not injure neurons above baseline levels in our hands. Statistical significance within +LPS and –LPS sets was measured by Student's t-test. *$p<0.005$; **$p<0.001$.

neuronal injury during pathological events, we sought to determine whether tPA is involved in activating microglia. Using the unilateral intra-hippocampal kainate injection model, we determined that $tPA^{-/-}$ microglia showed only attenuated microglial activation compared to wild-type microglia (91). Full activation was restored when recombinant tPA was infused back into the $tPA^{-/-}$ brain (90). Furthermore, inhibiting wild-type microglial activation pharmacologically with the tripeptide macrophage inhibitory factor (MIF) (75) inhibited tPA release and prevented neuronal death after the excitotoxin injection, indicating that, in this experimental paradigm of excitotoxicity, microglial cells exhibit neurotoxic properties. These neurotoxic properties may be attributed to the secretion of excess glutamate, tPA, and other cytotoxic factors that are intimately involved with killing neurons.

We and others have tested this effect using isolated neurons and glia in culture (31). We found that co-culturing wild-type or $tPA^{-/-}$ neurons and wild-type microglia together caused significant neuronal death (approximately 50% in 3 hr), while co-culturing wild-type or $tPA^{-/-}$ neurons with $tPA^{-/-}$ microglia did not (Fig. 2). The neurons must be primed first with a low dose of glutamate (25 μM glutamate for 3 hr resulted in approximately 23% neuronal death), so that they may become more susceptible to excitotoxic death (31). This degeneration is due to (a) diffusible factor(s), since the two cell types (neurons and microglia) are separated from each other by a 0.4 μm membrane. The microglia must be pre-activated with bacterial lipopolysaccharide (LPS) to induce neuronal death. These data support the *in vivo* results we obtained, which show resting microglia or tPA alone are not neurotoxic. In our model, tPA mediates neurodegeneration only in the presence of excitatory amino acids. This finding is in agreement with a recent report by the Vassalli group (52), that overexpression of tPA in the mouse brain did not result in spontaneous neurodegeneration.

The proteolytic activity of tPA is not required for its ability to activate microglia (76). When a catalytically inactive form of tPA (S478A) is infused into the brain of a $tPA^{-/-}$ mouse prior to kainate injection, hippocampal pyramidal neurons do not degenerate, but microglia are fully activated (76). This result suggested that tPA is mediating microglial activation by a pathway different than the conversion of plasminogen to plasmin. Furthermore, this piece of data excluded the presence of another tPA substrate in the CNS, since all the potential tPA substrates [hepatocyte growth factor, HGF (53); NMDA receptor subunit 1, NR1 (62)] require tPA's proteolytic activity for their activation. tPA may mediate microglial activation by interacting with a microglial surface receptor. There are several candidates for such a receptor, for example, the endothelial tPA receptor annexin II. Identifying and characterizing this receptor may provide a new pharmacological approach to protect neurons during excitotoxicity from activated, over-zealous microglia.

tPA-INDUCED LAMININ DEGRADATION LEADS TO NEURONAL DEATH

Recent results have shown that proteolysis of laminins in the hippocampus by tPA-generated plasmin leads directly to neuronal death after KA injection (18,93). The laminins are a group of glycoproteins, components of the extracellular matrix (ECM), which consist of three heteromeric chains, α, β and γ (102). It is believed that degradation of laminin and the loss of neuronal contact with the ECM (anoikis) signals the termination of neuronal support by the ECM and subsequent death for the neurons.

The working model for plasmin-mediated excitotoxic neurodegeneration is as follows (Fig. 3): The injury (i.e. excitotoxin injection) leads to an increase in tPA expression and tPA's extracellular release (9). tPA cleaves plasminogen and the resulting plasmin degrades ECM molecules. Signaling from the injured neuron causes hypersensitivity to direct attacks by tPA [there may be a second tPA binding partner on neurons, such as the low-density lipoprotein-related protein, LRP (106)], recruitment and activation of microglia, subsequent release of microglial neurotoxic factors, and further degradation of ECM molecules by other proteases activated by plasmin.

Nakagami *et al.* (60) showed that incubation of hippocampal slices with plasmin leads to loss of maintenance of long-term potentiation, and that this loss is probably due to the degradation of laminin. Other ECM molecules such as type IV collagen and fibronectin were not affected. These results suggest that extracellular proteases are not always detrimental to neuronal survival, but could also modulate the extracellular milieu in order to facilitate synaptogenesis, neurite extension and memory formation.

OTHER PROTEASES MAY PLAY A ROLE IN NEURODEGENERATION

There is growing evidence that MMPs and the tissue inhibitors of metalloproteinases (TIMPs) are involved not only in tissue remodeling (95), but also in neurodegeneration. For example, laminins can be degraded by MMP-2, -3 and -7 (38), suggesting that MMPs can also play a role in modification of ECM, and development of anoikis. During traumatic brain injury, it was shown that MMP-9 contributes to brain damage (98), either by damaging the vascular integrity and thus increasing edema, or by disrupting cell-matrix interactions beyond the vascular compartment. Inpatients suffering relapsing-remitting multiple sclerosis (RRMS), MMP-9 levels increase while levels of its inhibitor,

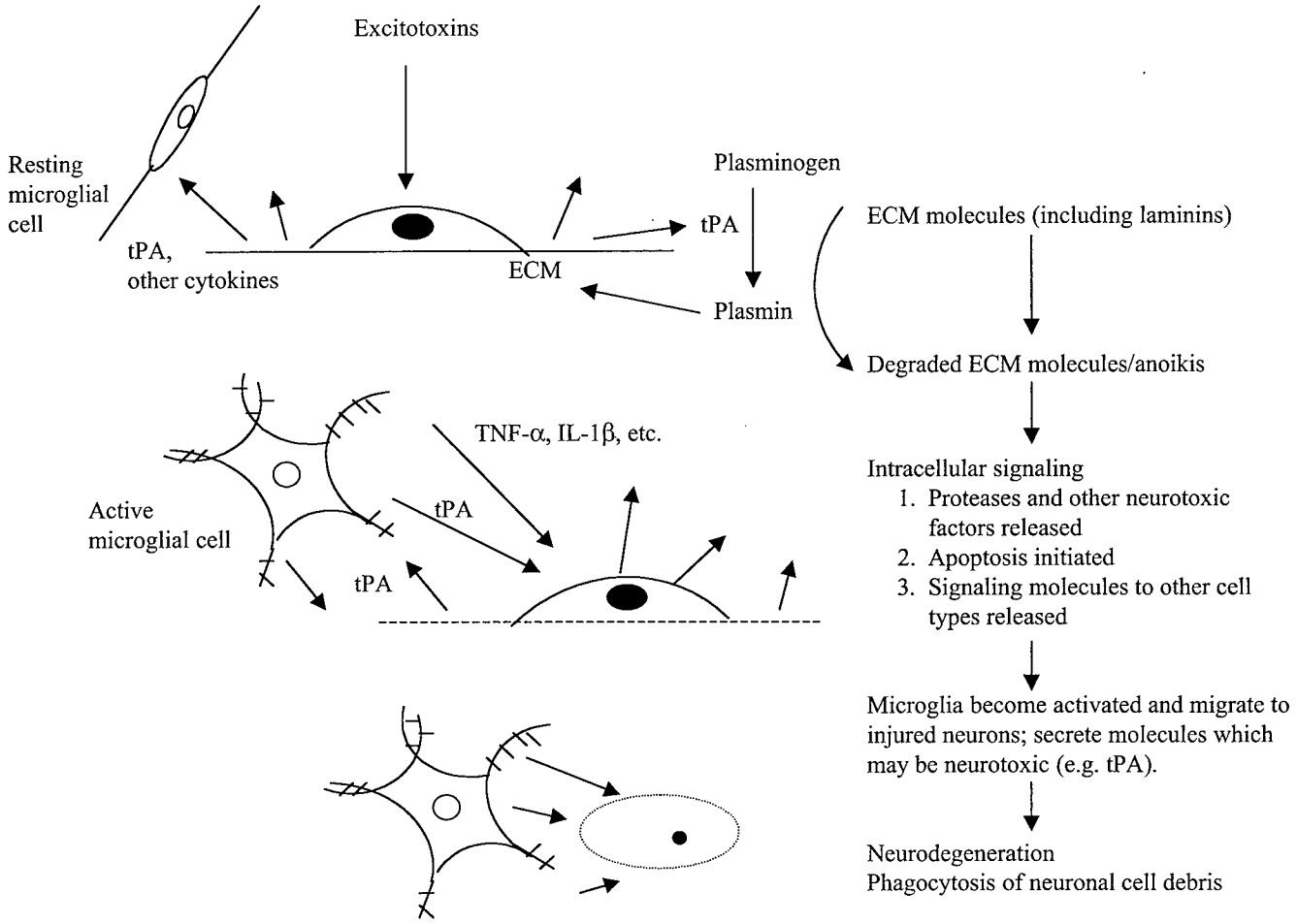

Fig. 3 A model for the neurotoxic actions of neuronal and microglial tPA in the CNS. When an excess of an excitatory neurotransmitter, such as glutamate, is released in the vicinity of a neuron, it becomes hyperdepolarized. Molecules such as tPA are released from intracellular vesicles into the extracellular environment. Among other molecules, tPA mediates activation of microglia, the primary immune cells of the brain. tPA also cleaves extracellular plasminogen into active plasmin, causing a cascade of protease activity which ultimately degrades the extracellular matrix. The neuron becomes detached and dies from lack of signaling from other cells in its environment. Microglia may play both a sanitary role (in cleaning up the dying neuron) and a neurotoxic role (in secreting tPA and other cytokines to kill the stressed neuron).

TIMP-1, remain the same, making MMP-9 an important predictor, if not promoter, of new MS lesions (100). Other MMPs may be involved in facilitating tissue invasion by T cells into the CNS in experimental allergic encephalomyelitis, an animal model of multiple sclerosis (8). In a model for neonatal pneumococcal meningitis, MMPs and TNF-α collaborate to perpetuate neuronal degeneration that leads to devastating long-term problems such as hearing impairment, obstructive hydrocephalus, and damage to the brain parenchyma (50).

A useful mouse model for neurodegenerative disease is the *wobbler* mouse (82), whose phenotype is characterized by forelimb muscle atrophy due to neurodegeneration, gliosis, and microglial activation in the brain stem and spinal cord (29,73). An ADAM protease, ADAM 8, was found to be highly expressed in monocytes in the lymphatic system and in microglia of the CNS (104). In particular, ADAM 8 mRNA levels are eight to ten times higher in the brainstem and spinal cord, respectively, of the *wobbler* mouse compared with wild-type mice (82). TNF-α was found to regulate ADAM 8 expression in astrocytes, possibly by inducing the transcriptional factor IRF1 to bind to a consensus site on the ADAM 8 promoter (82).

Where there is no appropriate animal model for a disease, cell culture techniques are very useful for defining how a protease may trigger cell death. Addition of thrombin to serum-starved neuroblastoma cells causes a rapid cell rounding and neurite retraction (39). The cell rounding is transient, but the retraction is long-lasting; it can be reversed only if thrombin is removed from the culture medium. A similar observation is made in cultured chick motoneurons, where brief exposure to thrombin or to a thrombin receptor

agonist leads to apoptosis (94). Treatment with protease nexin-1 (PN-1, a protease inhibitor), rescues apoptotic chick motoneurons as well as axotomized mouse motoneurons from death (36). Interestingly, thrombin-induced neurodegeneration can be prevented by culturing cells with muscle extracts containing neurotrophic factors, supporting a tightly regulated balance between proteases, their inhibitors, and trophic factors in the normal nervous system (94).

Finally, both tPA and uPA (urokinase) were found to potentiate thrombin-induced intracerebral hemorrhage, as evidenced by increased edemas and higher incidence of death (30). The authors postulated that the presence of both thrombin and the plasminogen activators in the brain leads to their competition for endogenous inhibitors such as PAI-1 and PN-1 (see below), causing an enhancement in brain injury which would have been ameliorated by these inhibitory molecules acting on one protease only.

PROTEASE INHIBITORS IN NEURONAL CELL DEATH

The physiological control of proteases comes from many endogenous inhibitors. For tPA, the astrocytic proteins PAI-1 (2), neuroserpin (11,46), PAI-2 (23) and PN-1 (51,107) have all been found to inhibit its activity. The selective expression of protease inhibitors in various regions of the brain may reflect a method of tightly controlling the spatial expression of proteases involved in many physiological processes.

Recently, PAI-1 was shown to mediate the neuroprotective ability of transforming growth factor (TGF)-β1 during NMDA-induced excitotoxic death (12) and cerebral ischemia (27), suggesting that, not only are serine proteases intimately involved in killing neurons, but that their actions may also be modified or inhibited completely by judicious use of protease inhibitors.

On the other hand, a striking example of protease inhibition gone awry is seen in neuroserpin, a serine protease inhibitor found in the CNS. Its targets are plasminogen activators and plasmin, but particularly tPA (46). Many neurodegenerative dementias, such as Alzheimer and Parkinson's diseases, are characterized as containing inclusion bodies. Neuroserpin is found in the inclusion bodies of a newly described familial encephalopathy with associated dementia (21,22). This autosomal dominant disease, which was identified in two unrelated families, manifests itself in the fifth decade, and progresses along similar degeneration pathways as other neuropathologies (e.g. Alzheimer's disease; (22). It is likely that, due to heritable mutations in the neuroserpin gene, the protein misfolds, aggregates, and accumulates intracellularly. Therefore, it is postulated to be unable to inhibit tPA, which then acts as a mediator of neuronal death during excitotoxicity, as described earlier.

In summary, extracellular proteases are important not only in the developing nervous system, pathfinding, and synapse formation, but they also play a critical role in mediating cell death during neurotoxic events such as acute trauma and chronic degenerative diseases. It is clear that physiological and tightly regulated control of proteases by their inhibitors is vital to normal function and survival. However, when the control is lost, by some yet-unknown pathological event, intra- and inter-cellular signaling cascades are initiated, which ultimately result in neuronal death.

Acknowledgements – We are grateful to Drs. Lincoln Stein (Cold Spring Harbor Laboratory), Michael Frohman and members of the Tsirka lab for critical reading of the manuscript. This work was funded by an NIH grant and a US Army Medical Research grant (to SET).

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CELLULAR AND MOLECULAR BIOLOGY™

2002, Volume 48, Number 2

CELL DEATH IN NEUROLOGICAL DISEASES

Content

Indexed/Abstracted in:
Current Contents, Index Medicus, MEDLINE/PubMed, BIOSIS Database, PASCAL/CNRS Database, Cam. Sci. Abstr., CAB Inter., Chem. Abstr. Service, RIS in References Update

Continued on the inside backcover

ISSN 0145-5680
CMBIDI 48(2) 125-218 (2002)

Published by C.M.B.-ASSOCIATION
Editorial Office: 1bis, Avenue du Pavé Neuf
F-93160 Noisy-le-Grand (France)

Printed by PEETERS - FRANCE S.A.R.L.
52, Boulevard Saint-Michel
F-75006 Paris (France)

Paper No. CDD 1086SA

Cell Death and Differentiation (2002) 00, 000–000

www.nature.com/cdd

Microglial activation and recruitment, but not proliferation, suffice to mediate neurodegeneration

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Received 12.10.01; revised 13.12.01; accepted 31.1.02
Edited by CJ Thiele

Abstract

Microglial activation occurs during excitotoxin-induced neurodegeneration. We have reported that microglia can exhibit neurotoxic behaviors after injection of excitotoxins into the hippocampus. It is not known, however, whether microglial proliferation, which is part of the activation response, is required for neurodegeneration to be observed, or whether activation of the pre-existing resident microglia suffices. Using osteopetrotic (*op/op*) mice, in which injury-induced microglial proliferation does not take place, we demonstrate that only the microglia initially residing in the CNS are adequate to promote neurodegeneration. Our data suggest that there is a threshold at which a maximal microglial contribution to neurotoxicity is observed. This threshold appears to be sufficiently low, such that activation of just 40% of the microglia present in wild-type mice serves to trigger neurodegeneration. Furthermore, since the decrease in microglial numbers coincides with a decrease in tissue plasminogen activator's activity, we suggest that tissue plasminogen activator can be used as a marker for microglial proliferation.

Cell Death and Differentiation (2002) **00**, 000–000. DOI: 10.1038/sj/cdd/4401041

Keywords: microglia; neurotoxicity; osteopetrotic mouse; tissue plasminogen activator

Abbreviations: tPA, tissue plasminogen activator; op, osteopetrotic; CSF-1, macrophage colony stimulating factor

Introduction

When an excitotoxin is introduced into the mammalian hippocampus, neuronal death soon follows. This neuronal injury is accompanied by activation of the resident microglial cells.[1] Onset of the microglial activation process is followed by their migration to the site of injury, local proliferation, changes in gene expression, presentation of class II major histocompatibility antigens, and phagocytosis. We and others have shown that microglia can exhibit neurotoxic properties when neuronal injuries are elicited by local injections of excitotoxins,[1,2] autoimmune inflammation,[3] or ischemia.[4,5] Cytokines and neurotoxins secreted by microglia may help regulate the responses to injury in the central nervous system.[6–8]

In mice deficient for tissue plasminogen activator (tPA), a secreted serine protease normally present in the brain, hippocampal neurons are resistant to excitotoxic glutamate analogs and the microglia display attenuated activation.[9] This attenuation is evident both as a decrease in the number of activated microglia present and as a decrease in the levels of expression of various microglial markers and cytokines.[10] Evidence in wild-type mice suggests that additional tPA is generated by microglia shortly after their activation and this tPA contributes to the ensuing neurotoxicity.[2] Accordingly, strategies to decrease the amount of tPA released for the purpose of achieving neuroprotection could include blocking microglial activation, and specifically microglial recruitment or proliferation. In this study, we describe experiments designed to evaluate if a decrease in the number of activated microglia reduces excitotoxic neural injury using the mutant osteopetrotic (*op/op*) mouse.

Op/op is a spontaneous mutation in C57Bl6 mice that results in a deficiency of the macrophage colony stimulating factor (CSF-1) gene product. A single base pair insertion within this gene causes a frameshift mutation and creates a truncated protein that is non-functional.[11] In cell culture, microglia isolated from *op/op* mice do not proliferate unless the medium is supplemented with CSF-1.[12] Furthermore, microglial proliferation is dramatically reduced in the nucleus of the transected facial nerve after axotomy in *op/op* mice.[13] However, the microglia that were present following axotomy in *op/op* mice displayed similar changes in morphology and gene expression as control *op* heterozygous (*op/+*) mice, indicating that they do respond to activation signals from injured neurons. Witmer-Pack *et al.* reported that microglial cells are not completely dependent on CSF-1, since they can be found in the brain.[14] Quantitatively, it has been reported that during cerebral cortical ischemic lesion the numbers of microglia per mm^2 on the contralateral side (which would also represent normal, uninjured numbers of microglia in that region) are similar between wild-type and *op/op* mice. However, around the injury side, the numbers of microglia remain unchanged in *op/op* mice, whereas those of wild-type animals increase by 2.9 fold.[15]

Accordingly, the *op/op* mouse offered us the opportunity to determine if the proliferation aspect of microglia activation plays a critical role in microglial-mediated excitotoxic neurodegeneration.

Results

Decreased proliferation but wild-type-like morphological activation of *op/op* microglia after kainic acid injection

After injection of kainic acid into the hippocampus of control (C57/Bl6) mice, microglial cells undergo activation. The kinetics of this activation process has been determined.[1] Although the process of microglial activation begins shortly after injury, maximum levels of microglial activation, as evidenced by dramatic morphological changes, are reached between 5 and 14 days after the injection.[1] Therefore we chose to examine the levels of microglial activation in *op/op* mice (identified by genotyping as described in Materials and Methods, Figure 1) 5 days after kainate injection when the morphological changes are most evident. To identify activated microglia, immunohistochemistry using the monoclonal antibodies F4/80 (Figure 2) and Mac-1 (data not shown) was performed on sections from control and *op/op* mice. Microglia in *op/op* mice acquire the morphological characteristics of activation after kainate injection (compare Figure 2E and F). However, only ~30–40% of the number of activated microglia are observed in the hippocampus of *op/op* mice in comparison to control mice (see Table 1), in agreement with the literature.[15] To further assess the relative quantity of activated microglia in *op/op* and control mice we evaluated the levels of TNF-α present in brain lysates from each genotype by Western blot analysis (data not shown). TNF-α is a cytokine that is produced by microglia as part of the activation process. Kainate-injected brain lysates from *op/op* mice contained 48% of the amount of TNF-α present in lysates from control mice. These quantitative data agree with the qualitative observation that smaller numbers of activated microglia are present in the injured hippocampus of *op/op* mice.

The migration of microglia in the *op/op* mice to the injury site was comparable to that of microglia in control mice (note the presence of activated microglia in the CA1 hippocampal subfield mainly in the injected sides for both genotypes, Figure 2A and B). Even at higher magnifications (Figure 2E

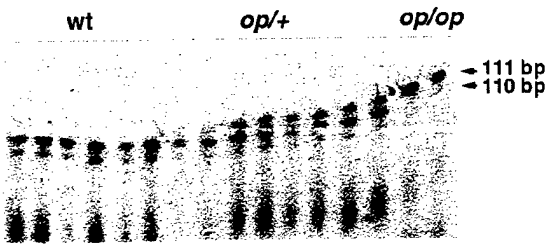

Figure 1 PCR-genotyping of *op/op* mice. Wild-type (C57/Bl6), *op/+*, and *op/op* mice were genotyped from tail DNA by a one-step PCR reaction as described in Materials and Methods. The PCR products were analyzed on a 6% polyacrylamide gel and visualized by autoradiography. C57/Bl6 mice amplify a single 110 base pair product (see arrow), *op/+* mice amplify 110 and 111 base pair products, and the *op/op* mice amplify a single 111 base pair product (see arrow)

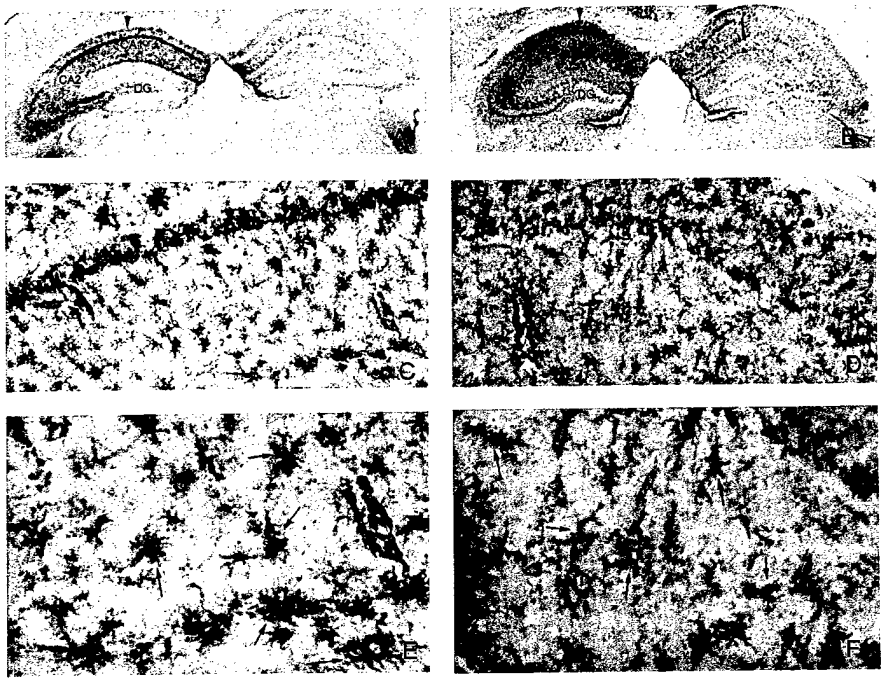

Figure 2 Microglial proliferation is deficient after excitotoxic injury, but microglial activation proceeds normally in *op/op* mice. Kainate was injected unilaterally into the hippocampi of control (*op/+*; *n*=11) and *op/op* mice (*n*=12) to induce microglial responses (arrows indicate sites of injection on **A** and **B**). Control mice respond with activation of their microglia on the injected side (**C** and **E**). Such activation also includes a strong microglial proliferative response (seen best in **C**). While the proliferation of microglia in the injected side of the hippocampus of the *op/op* mice is defective (seen best in **D**), a higher powered photomicrograph indicates that the microglia that migrate to the site of injury have the characteristics of activated microglia (**D** and **F**). The hippocampal subfields CA1, CA2 and CA3 as well as the dentate gyrus (DG) are labeled

and F), the microglia in the control and *op/op* mice appeared similar aside from minor morphological differences visualized using F4/80 staining, as reported previously.[16]

To evaluate the levels of microglial proliferation after excitotoxin injection, we used double immunofluorescence against F4/80 (detecting microglia) and BrdU (for proliferating cells). As shown in Figure 3, microglial proliferation was clearly evident in wild-type mice (wt), as well as in $tPA^{-/-}$ ones (even though the overall number of microglial cells was significantly reduced). Proliferation was not observed for $F4/80^+$ microglia in *op/op* mice, as was earlier reported.[15] Therefore, in this excitotoxin-injection model, microglia in *op/op* mice display the phenotypic characteristics of microglial activation and are recruited to the sites of injury but do not mount a proliferative response (as evident by the presence of fewer activated $F4/80^+$ cells and the observation that the $F4/80^+$ cells are not BrdU labeled). This observation is in agreement with the data obtained in the facial nerve axotomy model.[13]

Pyramidal neurons in *op/op* mice undergo excitotoxic cell death

We previously showed that the microglial activation normally observed in wild-type mice following kainate injection is significantly attenuated in tPA-deficient mice.[9] Furthermore, the pyramidal neurons in $tPA^{-/-}$ mice, as well as in wild-type mice in which microglial activation has been delayed by macrophage-microglial inhibitory factor,[2] are resistant to excitotoxin-induced neuronal death. To evaluate whether eliminating the proliferative component of the microglial response was sufficient to confer protection against neuronal cell death, we assessed neuronal survival in control (*op/+*) and *op/op* mice after the unilateral injection of kainate. As seen in Figure 4, the pyramidal hippocampal neurons were sensitive to excitotoxin injection in both genotypes, suggesting that there were sufficient activated microglia present in *op/op* mice to mediate neuronal death.

Combined with the results above, these data suggest that although microglia in *op/op* mice are unable to respond to CSF-1 by proliferating, they can respond to the signals produced by injured neurons to cause their activation and ultimately promote neuronal death.

Table 1 Decreased numbers of activated microglia and lower levels tPA activity in the brains of *op/op* mice

Genotype	Activated microglia in CA1 ± s.d.*	tPA (ng/μg protein ± s.d.)**
Control	73 ± 7	4.63 ± 0.01
op/op	26 ± 10	1.99 ± 0.21

Activated microglia in the CA1 region of kainate-injected control (C57/B16) and *op/op* mice were counted as decribed in the text. Activated microglia were identified based on intensity of immunostaining by F4/80 and characteristic morphological changes. The data are presented as the average number of microglia present in four microscopic fields from cryostat coronal 14 μm sections of brain around the injection site. Few if any activated microglia were present in the non-injected side in both control and *op/op* mice. Brains from control (C57/B16) and *op/op* mice were homogenized, as listed in the Materials and Methods section, and the levels of tPA activity were measured by the amidolytic assay at 30 and 90 min, 12 and 24 h. tPA activity was calculated from initial rates in the amidolytic assay. Extracts from $tPA^{-/-}$ brains were used as negative controls. *$P<0.01$, value associated with Student's two-tailed *t*-test. **$P<0.05$, value associated with Student's two-tailed *t*-test

Decreased levels of tissue plasminogen activator in *op/op* mice

Tissue plasminogen activator (tPA) is a serine protease that catalyzes the conversion of the zymogen plasminogen to the active protease plasmin. Both neurons and microglia in the mouse brain express this protease. The synthesis of tPA is rapidly upregulated after microglial activation in cell culture. We have previously shown that tPA can promote excitotoxic cell death in the mouse brain and suggested that secreted microglial-derived tPA may contribute to the neurotoxic properties of these cells.[2] Given that the mutation that causes to the *op/op* phenotype results in defective proliferation of microglial cells only (but does not affect the numbers of neurons), we performed both zymographic and amidolytic assays to determine the levels of tPA. As shown in Table 1, there is a significant difference between the levels of tPA in the kainate-injected brains of control mice (4.63 ng of active tPA/μg of brain extract), and *op/op* mice (1.99 ng of active tPA/μg of brain extract) as demonstrated by the amidolytic assay. Similar differences in tPA levels were noted by zymographic analysis (data not shown). It is interesting to note in the *op/op* forebrain there is ~43% tPA activity present compared to that of control mice, a number close to the difference in activated microglia between the

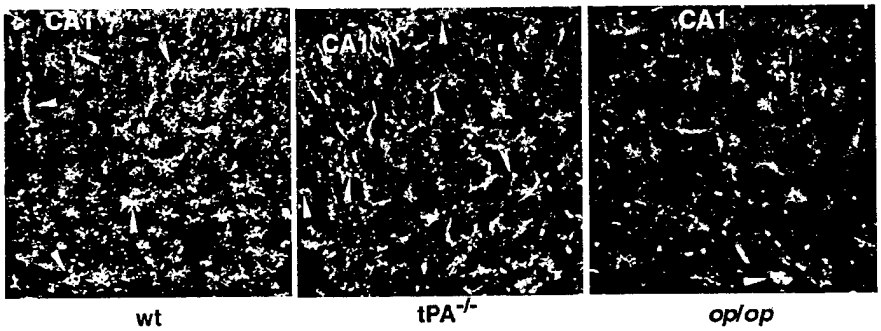

Figure 3 Microglial proliferation is nearly absent in *op/op* mice during excitotoxin-induced neuronal death. Microglial proliferation was evaluated by double immunofluorescence in wild-type, tPA-deficient and *op/op* mice on 14 μm cryostat coronal sections. Arrows indicate the presence of doubly labeled (proliferating) microglia

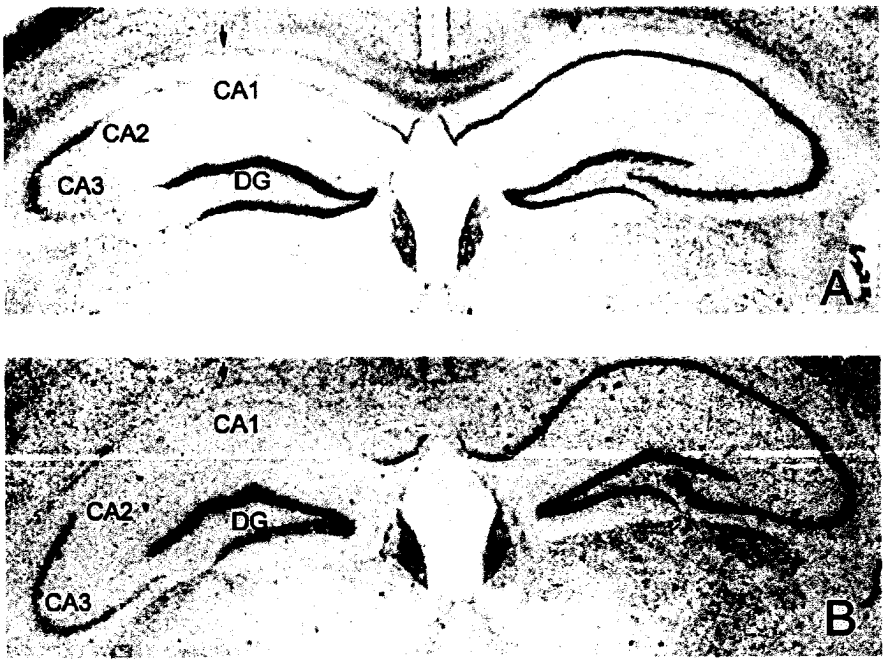

Figure 4 *Op/op* mice are sensitive to excitotoxin-induced neuronal death. The viability of pyramidal neurons in the hippocampus of injected control (*op/+*, *n*=11) and *op/op* mice (*n*=12) after unilateral intrahippocampal kainate injection was evaluated by cresyl violet staining. In kainate-injected *op/op* mice (**B**), the neurons at the injected side were eliminated to the same extent as those in control (**B**) mice. The arrows indicate the site of kainate injection. CA1, CA2 and CA3 indicate the hippocampal pyramidal subfields, and DG denotes the dentate gyrus of the hippocampal formation

two genotypes (see Table 1). Since the amount of tPA correlated well with the numbers of microglia present in the injured hippocampus, and hippocampal neurons (the other producer of tPA in the mouse CNS parenchyma) were eliminated by the excitotoxin, we propose that tPA may be useful as a marker for microglial proliferation in the mouse forebrain.

Discussion

The *op/op* mouse carries a frameshift in the CSF-1 gene[11] that results in defective microglial proliferation both in cell culture and *in vivo*.[12,13] Previously, we reported that retarding microglial activation in response to excitotoxin injection could protect against neuronal death.[2] This suggested a neurotoxic role for microglia in the mouse brain during excitotoxicity. However, it was uncertain which component(s) of microglial activation is necessary for excitotoxin-mediated cell death. We set out therefore to ascertain whether impairment of microglial proliferation would confer protection from neuronal death, or if the initial number of activated microglia present would suffice to promote neuronal death. Intrahippocampal delivery of kainic acid into the brain parenchyma of the *op/op* mice resulted in neuronal cell death, a result consistent with the observed vulnerability of *op/op* cortical neurons to ischemic insult.[15] These results suggest that despite the diminished proliferative capability of microglia in *op/op* mice, the microglia that become activated are able to promote neurotoxicity and to phagocytose the debris of injured neuronal cells. In addition it is suggested that a threshold may exist for the number of activated microglial cells above which they cease to become a rate-limiting factor in this neurodegeneration pathway.

These neurotoxic properties of microglia may be the result of the up-regulation and secretion of several factors that these cells express, such as tPA and TNF-α.[10,17] Interestingly, at least TNF-α (and potentially tPA) lies at the beginning of potent signal cascades that can result in cell death.[18] This provides a rationale through which even a small number of activated microglia could provide the necessary downstream signals to effect neuronal cell death. We have already shown that the tPA/plasminogen proteolytic cascade promotes excitotoxic cell death.[19,20] Furthermore, microglial-derived tPA may initiate this cascade (C-J Siao, personal communication). The data presented here demonstrate that the number of activated microglia is diminished in the kainate-injected *op/op* mouse (36% of control), and these data correlate with the levels of decreased enzymatic activity of tPA in kainate-injected *op/op* mice (43% compared to those of control mice). This result further suggests that microglial tPA is a major source of tPA locally at the site of injury. The potential for signal amplification present in a proteolytic cascade initiated by tPA and plasmin may account for the strength of the neurotoxic effects of the few activated microglia in the *op/op* mouse. Moreover, we propose that tPA can be used as a marker for microglial proliferation following injury induced by kainate.

Additionally, TNF-α has been implicated to play a role in the neurotoxic properties which microglia possess.[21,22] TNF-α is secreted by activated microglia and can inhibit the re-uptake of glutamate by astrocytes, thereby allowing higher and potentially toxic concentrations of extracellular glutamate.[21] TNF-α lies at the beginning of a biochemical cascade that potentiates neuronal death following kainate injection.[18] In fact, we report here that there is approximately half the level of TNF-α present in the kainate-injected *op/op* mouse, yet excitotoxin-induced neurodegeneration proceeds normally. This may occur because TNF-α can stimulate the generation of IL-1β, IL-6 and other cytotoxic cytokines.[23] However, we have also previously shown that in tPA-deficient mice (where microglial activation is attenuated)[9] neurons are resistant to excitotoxicity and the microglia in these mice secrete 43% less TNF-α than wild-type mice in response to activation stimuli.[10] This amount of TNF-α is comparable to that secreted by *op/op* microglia. It is tempting to speculate that the different effects of kainate in tPA-deficient and *op/op* mice lie in the ability of activated microglia in *op/op* mice to produce sufficient tPA to cross a threshold and successfully initiate the biochemical cascades that lead to cell death.

Activated microglia have been implicated to play a role in several neuropathological conditions such as Alzheimer's disease, stroke and multiple sclerosis. Previously, Raivich *et al*.[13] reported that microglia in *op/op* mice underwent normal activation in the facial nerve axotomy model. In the present study we show that the activation of microglia, rather than their proliferation, is critical for effecting neuronal death. We and others previously demonstrated that microglia possess neurotoxic properties,[2,5,6,24] and that inhibition of microglial activation can be neuroprotective against kainate-induced neuronal injury and death. Taken together with the data presented here, we suggest that the toxic mediators, secreted by microglia after neuronal injury, are sufficiently potent that even decreased numbers of activated microglia are able to promote neuronal death. Therefore, any potential neuroprotective therapy involving suppression of microglial activation must take their potency into account.

Materials and Methods

Animal procedures

All experiments performed on mice were done in accordance to the NIH guide for the care and use of laboratory animals as well as the institutional guidelines set by the IACUC Committee and the Division of Laboratory Animal Research at Stony Brook. All efforts were made to minimize the use of animals and to ensure minimal suffering of those animals used.

Mice

The osteopetrosis (*op/op*) mouse arose form a spontaneous mutation in the CSF-1 gene in C57/Bl6 mice. This single base insertion within the coding region of CSF-1 leads to a truncated, non-functional protein.[24] Since homozygote *op/op* mice do not breed well, *op/+* heterozygotes were mated to expand the *op/op* colony. The genotypes of the offspring were determined using a PCR-based assay. Primers that flanked the mutation were designed (primer 1: 5'-CAGCTGGATGATCCTGTTTGC-3'; primer 2: 5'-CTCGGTGGCGTTAGCATTGGG-3') such that genomic DNA from a homozygote *op/op* mutant would yield a 111 base pair product whereas wild-type DNA would yield a 110 base pair product. Genomic DNA was prepared from the tails of mice using standard procedures. The genomic DNA was PCR-amplified using the above mentioned primers using the following protocol: 94°C 5 min followed by 30 cycles of 94°C 45 sec, 58°C 1 min, 72°C 1 min, with a final extension at 72°C for 7 min. ^{32}P-dATP was included in the PCR reactions. The products were separated on a 6% polyacrylamide gel and subjected to autoradiography to visualize the bands (Figure 1).

Intrahippocampal injection of kainate

Adult C57Bl6/J, *op/+* and *op/op* male mice, between 20 and 25 grams, were injected intraperitoneally with atropine (0.6 mg/kg of body weight) and deeply anesthetized with 2.5% avertin (0.02 ml/gram of body weight). The mice were then injected with 1.5 nmol kainic acid (in 300 nl phosphate buffered saline) unilaterally into the hippocampus using stereotaxic coordinates (bregma −2.5 mm, medial/lateral 1.7 mm and dorsoventral 1.6 mm). The excitotoxin was delivered over 30 sec, and the injection needle remained in place for two additional min to prevent reflux of fluid. Five days after the injection, the brains of the injected mice were analyzed for neuronal survival and microglial activation.

BrdU labeling of proliferating microglial cells

At 48 h following kainate injection, BrdU (200 mg/kg) was delivered to the mice by intraperitoneal injection. These injections occurred once daily for 3 days. At day 5 post-kainate injection, the mice were sacrificed and subjected to immunohistochemistry as described below.

Immunohistochemistry

Coronal sections (30 μm) of the brains of the injected mice were cut at the level of the hippocampus. Non-specific immunoreactivity was blocked by incubation of the brain sections with 5% goat serum. The sections were incubated with antibodies either to the mature macrophage/microglia specific antigen F4/80 (1:100, Serotec) or Mac-1 (1:10, Roche Biochemicals). Biotinylated secondary antibodies were used (Vector Laboratories) and the avidin-biotin-peroxidase complex (ABC reaction) was visualized with diaminobenzidine and hydrogen peroxide (Vector Laboratories) as described previously.[20] For BrdU immunostaining the tissue sections were treated with 2N HCl prior to preincubation with goat serum. Fluorescent-labeled secondary antibodies (anti-rat FITC for F4/80 and anti-mouse TRITC for BrdU) were used to identify proliferating microglial cells.

Amidolytic assay for tPA activity

For quantitative determination of tPA activity, the amidolytic assay was performed as previously described.[25] Briefly, the tissue was lysed in 0.25% Triton X-100 and incubated at 25°C in a mix containing 0.3 mM S-2251 and 0.42 μM plasminogen in 0.1 M Tris, pH 8.1, 0.1% Tween-80). The change in absorbance (ΔA) at 405 nm was measured at different time-points. Known concentrations of recombinant tPA protein were used to generate a standard curve.

Quantitation of microglial cell numbers

F4/80$^+$ and Mac-1$^+$ microglia in the CA1 hippocampal subfield were counted in four successive cryostat coronal sections (cut at 14 μm)

around the injection site, and the numbers were averaged, as previously described.[26] Microglial cell counting occurred 5 days after the excitotoxic injury, when microglial activation (evident by F4/80 immunostaining) reaches peak levels.[1] Activated microglia were characterized as cells with a cell body larger than 10 μm in diameter, with short, thick processes and intense immunoreactivity. No resting microglia (characterized by small cell body, long processes and weak immunoreactivity) were observed at that time-point on the sections.

Quantification of microglial activation

Whole brain lysates were prepared by dounce homogenization in phosphate buffered saline from kainate-injected control and *op/op* mice. To estimate the relative amounts of activated microglia present in each brain, relative tumor necrosis factor alpha (TNF-α) levels were assessed by Western blot analysis. Briefly 20 μg of total lysate was separated through a 15% polyacrylamide gel and transferred to a PDVF membrane. TNF-α was detected using a rat-anti-mouse TNF-α antibody (clone MP6-XT3) at a dilution of 1:500 (Boehringer Mannheim) followed by biotinylated goat-anti-rat secondary antibody at a 1:3000 dilution (Vector Laboratories). Finally, the avidin-biotin-peroxidase complex (ABC Elite, Vector Laboratories) was visualized by chemiluminescence (LumiGLO, KPL). The relative amounts of TNF-α were determined using a Bio-Rad densitometer.

Acknowledgements

We would like to thank Dr. S Strickland for advice, and are grateful to Dr. M Frohman for critical reading of the manuscript and the members of the Tsirka laboratory for helpful advice and discussions. This work was supported by fellowships from the Medical Scientist Training Program (AD Rogove), National Institutes of Health and an Army Medical Research Grant (SE Tsirka).

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