Cell, Vol. 91, 917–925, December 26, 1997, Copyright ©1997 by Cell Press

Neuronal Death in the Hippocampus Is Promoted by Plasmin-Catalyzed Degradation of Laminin

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Summary

Excess excitatory amino acids can provoke neuronal death in the hippocampus, and the extracellular proteases tissue plasminogen activator (tPA) and plasmin (ogen) have been implicated in this death. To investigate substrates for plasmin that might influence neuronal degeneration, extracellular matrix (ECM) protein expression was examined. Laminin is expressed in the hippocampus and disappears after excitotoxin injection. Laminin disappearance precedes neuronal death, is spatially coincident with regions that exhibit neuronal loss, and is blocked by either tPA-deficiency or infusion of a plasmin inhibitor, both of which also block neuronal degeneration. Preventing neuron–laminin interaction by infusion of anti-laminin antibodies into tPA-deficient mice restores excitotoxic sensitivity to their hippocampal neurons. These results indicate that disruption of neuron–ECM interaction via tPA/plasmin catalyzed degradation of laminin sensitizes hippocampal neurons to cell death.

Introduction

The mechanism of neuronal death in the adult mammalian central nervous system (CNS) is an area of intense medical interest. The loss of neurons in stroke, epilepsy, and Alzheimer's disease, for example, can lead to profound cognitive impairment. Therapeutic intervention that would prevent neuronal degeneration is therefore a holy grail of neurobiology.

The loss of neurons in many neuropathologies is thought to be stimulated by an excess of the excitatory amino acid glutamate (Coyle and Puttfarcken, 1993; Lipton and Rosenberg, 1994). In this model, overabundant glutamate caused by injury, ischemia, inappropriate electrical activity, or disease promotes entry of increased calcium, which in turn initiates downstream events leading to cell death. Consistent with this idea, injection of glutamate agonists into the CNS induces neuronal cell death in the hippocampus resembling that seen in various neuropathologies (Coyle et al., 1978).

The extracellular serine protease tissue plasminogen activator (tPA) and its physiological zymogen substrate plasminogen participate in excitotoxic neuronal cell death in the hippocampus. Mice deficient for tPA or plasminogen are resistant to neuronal destruction induced by excitotoxins (Tsirka et al., 1995, 1997a). The proteases are required at around the time of insult, because infusion of protease inhibitors into adult mice just prior to excitotoxin administration can protect wild-type mice from degeneration (Tsirka et al., 1996). These results indicate that an extracellular proteolytic cascade leads to plasmin generation, which then promotes neuronal death (Strickland et al., 1996). A central question arising from this model is the nature of the plasmin substrate(s), whose cleavage would sensitize neurons to degeneration.

Plasmin is a trypsin-like protease that has long been associated with fibrinolysis, and genetic evidence has indicated that the primary, and perhaps exclusive, physiological role of this enzyme is the degradation of fibrin (Bugge et al., 1996). In light of these studies, fibrin was investigated as a possible target for plasmin in the CNS. Experiments using mice deficient for both plasminogen and fibrinogen showed that fibrin is not the primary plasmin substrate in the hippocampus (Tsirka et al., 1997b).

Plasmin is a potent protease and is capable of degrading proteins other than fibrin. For this reason, it could be involved in many processes due to cleavage of growth factors, receptors, and other extracellular targets. For example, extracellular matrix (ECM) proteins might be cleaved by plasmin, which could affect cell survival; disruption of cell–ECM interaction via proteolytic degradation of the matrix (Boudreau et al., 1995) or spatial restriction (Coucouvanis and Martin, 1995) can lead to cell death. In cell culture in the absence of ECM interactions, human endothelial cells rapidly undergo programmed cell death (Meredith et al., 1993). Moreover, loss of laminin, a component of the ECM, has been implicated in neuronal cell death in the mouse weaver mutation (Murtomäki et al., 1995).

Because plasmin can degrade ECM proteins, such as laminin, fibrin, and fibronectin (Alexander and Werb, 1989), and because loss of ECM induces cell death, we investigated whether plasmin degradation of ECM proteins results in neuronal loss after excitotoxic lesion. Expression of various extracellular matrix proteins in the hippocampus was examined before and after excitotoxic lesion. Laminin is expressed strongly in the hippocampal neuronal cell layers and disappears rapidly after excitotoxin injection. Laminin disappearance occurs before neuronal cell loss, is spatially identical to regions that exhibit cell death, and is blocked by either tPA deficiency or infusion of a plasmin inhibitor, both of which also block neuronal degeneration. Furthermore, interference of neuron–laminin interaction with anti-laminin antibodies allows neuronal degeneration even in the absence of protease. These results indicate that laminin degradation is a critical event in determining neuronal death after excitotoxic lesion in the hippocampus.

Results

Laminin Is Expressed in the Hippocampal Neuronal Cell Layers

As a first step in identifying plasmin substrates in the hippocampus that might be involved in excitotoxin-induced neuronal degeneration, the expression of various ECM

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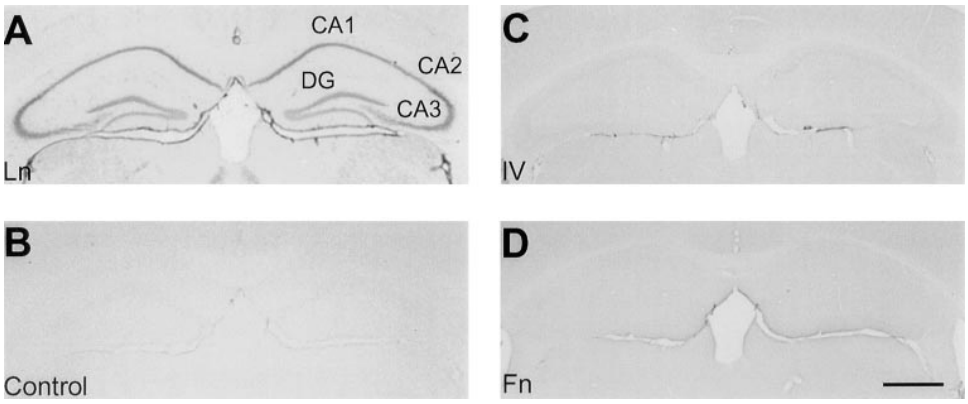

Figure 1. Laminin Is Expressed in the Hippocampal Neuronal Cell Layers

Coronal brain sections were stained with antibodies against various extracellular matrix proteins. (A) Laminin (Ln) staining; (B) sections stained with laminin antibody preabsorbed with laminin antigen (Control); (C) collagen type IV staining (IV); (D) fibronectin (Fn) staining. Scale bar, 750 μm.

proteins was examined using immunohistochemistry. As shown in Figure 1A, laminin was expressed strongly in the hippocampal neuronal cell layers, including the CA1–3 subfields and dentate gyrus. This expression was not uniform, with more intense immunoreactivity in CA3 and less in CA1, CA2, and the dentate gyrus (see below; Figure 3). When the antibody was preabsorbed with laminin antigen, the immunostaining was abolished completely (Figure 1B), demonstrating that the immunostaining was laminin-specific. Similar staining was also obtained with monoclonal antibodies directed against the laminin β1 chain or γ1 chain. These results are consistent with previous reports, which have detected laminin expression in the rat hippocampus (Yamamoto et al., 1988; Hagg et al., 1989). In contrast to laminin, expression of fibronectin and collagen type IV was not detected in the hippocampus (Figures 1C and 1D). Nidogen (entactin), which is often found associated with laminin in the ECM, was not detected in the hippocampal extracts (Figure 5) and has been reported not to be produced by hippocampal neurons (Niquet and Represa, 1996).

Laminin Disappears Rapidly in the Hippocampus after Kainate Injection in Regions Identical to Those that Exhibit Neuronal Cell Death

The expression of laminin in the hippocampus, coupled with the role of the ECM in mediating cell survival in other systems, suggested that this protein might play a role in neuronal degeneration. To investigate this possibility, neuronal loss and laminin expression were examined at various times after excitotoxin treatment. Thirty minutes after unilateral kainate injection, there was no obvious neuronal loss by cresyl violet staining, and there was also no detectable change in laminin expression in the injected side as compared to the uninjected side (data not shown). However, 2 hr after injection these two parameters became discordant; neurons had normal morphology (Figures 2A and 2C), but laminin had almost completely disappeared in the CA1 and CA3 subfields of the ipsilateral hippocampus (Figures 2B and 2E). Two days after injection, neurons in CA1, CA2, and CA3 of the ipsilateral hippocampus have degenerated (Figure 2G) and laminin appeared increased slightly compared to the early time points (Figure 2H), possibly due to glial synthesis. Laminin degradation was also evident using monoclonal antibodies against the β1 chain or γ1 chain, and a similar loss of laminin was observed when neuronal degeneration was induced by injection of NMDA (data not shown). As a control, PBS-injected hippocampi did not show any changes in laminin staining at any time points examined, indicating laminin disappearance was induced specifically by excitotoxin injection. Other extracellular matrix proteins such as fibronectin and collagen type IV showed no changes after injections (data not shown).

A comparison between early laminin disappearance and later neuronal death revealed that these two parameters were spatially coincident (compare Figures 2B and 2G). To analyze further the spatial correlation between laminin disappearance and neuronal death, a lower dose of kainate (0.33 nmol compared to 1.5 nmol in previous experiments) was injected unilaterally into the hippocampus. Lower doses of kainate induce limited neuronal cell death within the hippocampus (Andersson et al., 1991). Examination of many experiments revealed that the limited CA1 regions showing neuronal death were identical to the limited regions exhibiting laminin disappearance (data not shown).

These results indicate that laminin degradation precedes neuronal loss after kainate treatment and occurs in exactly the same hippocampal regions that eventually experience neuronal degeneration.

Increased Expression of Laminin in Hippocampal Subfields Correlates with Resistance to Excitotoxin-Induced Neuronal Cell Death

The above results demonstrate a good correlation between early laminin disappearance and later neuronal cell death. A further connection between expression of this ECM protein and neuronal survival was inferred from the laminin expression pattern in the hippocampus. Laminin expression in CA3a was stronger than in CA1

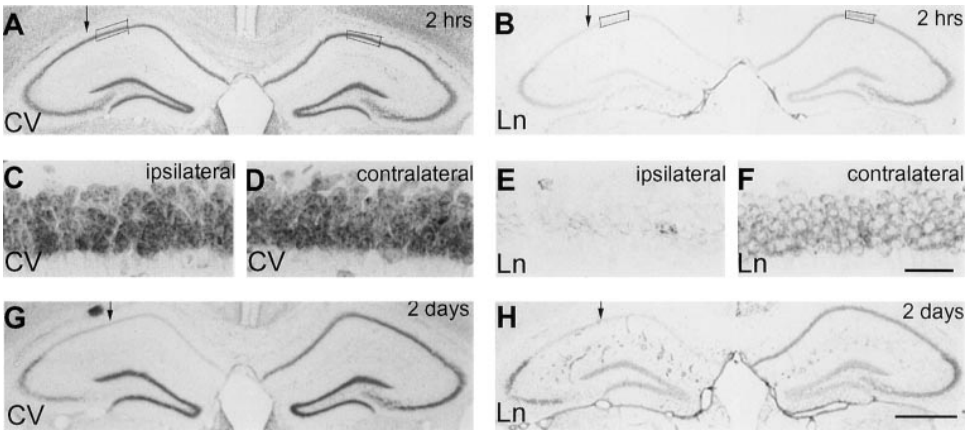

Figure 2. Laminin Disappearance Precedes Neuronal Degeneration after Kainate Injection

After unilateral kainate injection (1.5 nmol), the animals were sacrificed at the indicated time points, and their brains were removed for cresyl violet staining and laminin immunostaining (see Experimental Procedures). Higher magnification of the boxed areas in the 2 hr time point of (A) and (B) are shown in (C–F). At this early time point the neuronal morphology in the injected side is intact (C) compared to that in the uninjected side (D), but laminin has disappeared (E) in the injected side, while the uninjected side remains intact (F). CV, cresyl violet staining; Ln, laminin immunostaining; arrows, kainate injection sites. Scale bars: (A), (B), (G), and (H), 750 μm; (C)–(F), 45 μm.

and CA2 (Figures 1A and 3B). This difference in laminin immunostaining was not due to differences in regional cell densities, because the CA3a subfield with more laminin immunostaining showed less cell density compared to the adjacent CA2 or CA1 regions (Figures 3E and 3F). It is intriguing that the CA3a region that showed more laminin expression was more resistant to excitotoxin-induced neuronal cell death (Figures 3C and 3D). Two days after excitotoxin injection, the neurons in CA1, CA2, and CA3b had completely degenerated, whereas in CA3a some neurons exhibited normal cellular morphology as examined under high power (data not shown) and continued expressing laminin (Figure 3D). This correlation between high laminin expression and neuronal resistance to excitotoxin further implicated laminin as an important factor in maintaining cellular viability.

Abolishing Plasmin Activity by tPA Deficiency or a Plasmin Inhibitor Blocks Both Laminin Degradation and Neuronal Death

After pharmacological or electrical stimulation in the hippocampus, the synthesis of tPA is induced rapidly (Qian et al., 1993; Carroll et al., 1994). The enzyme is also released from storage vesicles after depolarization of neurons (Gualandris et al., 1996; Parmer et al., 1997). This increase in tPA would lead to increased levels of plasmin via activation of plasminogen in the hippocampus (Sappino et al., 1993; Tsirka et al., 1997a). Since plasmin can degrade laminin (Liotta et al., 1981; Salonen et al., 1984; Paulsson et al., 1988), disappearance of hippocampal laminin after kainate injection could be due to this enzyme. However, there are other extracellular proteases that could participate in this process. For example, neuropsin is an extracellular serine protease that is also expressed in the mouse hippocampus and whose expression exhibits neuronal activity–dependent changes (Chen et al., 1995).

If laminin disappearance is due to proteolysis via the tPA/plasmin cascade, then tPA deficiency or infusion of a plasmin inhibitor should be able to block laminin degradation. When $tPA^{-/-}$ mice were injected unilaterally with kainate, neuronal cell death was almost undetectable, as shown previously (Figures 4A and 4C; Tsirka et al., 1995). Moreover, laminin changes in the injected hippocampus were minimum at either 2 hr or 2 days (Figures 4B and 4D). Control wild-type mice injected in parallel with the $tPA^{-/-}$ mice exhibited laminin decreases and neuronal degeneration equivalent to that shown in Figure 2. Thus, tPA deficiency blocked both early laminin degradation and later neuronal degeneration. It is worth noting that tPA deficiency itself does not affect laminin expression in the hippocampal neurons (compare Figure 1A and Figures 4B and 4D).

Since most effects of tPA are thought to be mediated via its activation of plasminogen, the inhibition of laminin degradation in tPA-deficient mice was most likely due to the inability of these mice to generate plasmin. To explore this point, wild-type mice were first infused for 2 days with the plasmin inhibitor α_2-antiplasmin or PBS, then injected with kainate and infused further with α_2-antiplasmin or PBS for 2 hr or 2 days until analysis. The plasmin inhibitor blocked laminin degradation (Figures 4F and 4H) and neuronal degeneration (Figures 4E and 4G), but mice infused with PBS exhibited the expected neuronal degeneration and decrease in laminin expression (data not shown).

These results indicate that plasmin is required for laminin degradation, and they strengthen the correlation between disruption of this ECM protein and neuronal survival.

Biochemical Analysis of Laminin in Hippocampal Extracts after Excitotoxin Injection

To examine further the degradation of laminin in the hippocampus, we analyzed extracts of this region for laminin expression after kainate injection. To maximize the difference between kainate-injected and PBS-injected sides, the region of the hippocampus receiving maximal

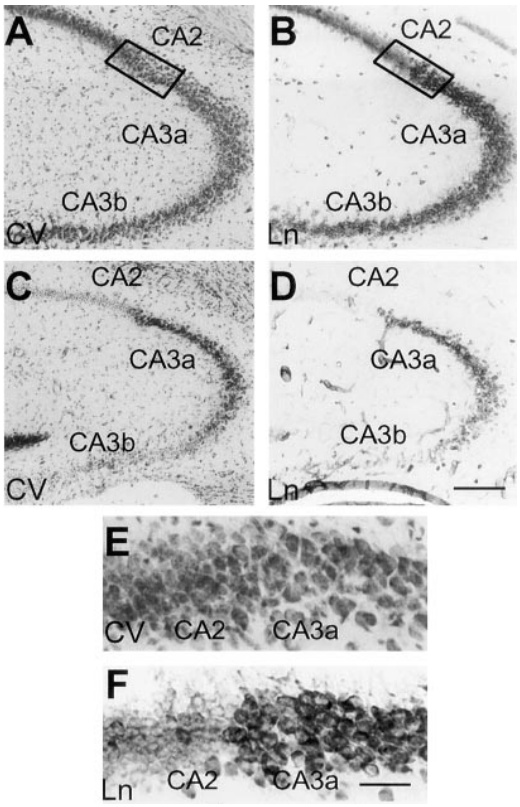

Figure 3. Differential Expression of Laminin in Hippocampal Subfields Correlates with Different Susceptibility to Neuronal Cell Death

Neuronal density and laminin staining were examined in wild-type mice before and after kainate injection. Cresyl violet staining (A) and laminin immunostaining (B) before kainate injection. Mice were injected unilaterally with kainate (1.5 nmol), and 2 days later the brains were analyzed for neuronal cell death (C) and laminin expression (D) in the hippocampus. The boxed areas in (A) and (B) are shown at higher magnification in (E) and (F), respectively; the boundary of increased laminin staining is apparent in (F). CA2, CA3a, and CA3b denote the hippocampal subfields. Scale bars: (A)–(D), 200 μm; (E) and (F), 50 μm.

excitotoxin was evaluated (primarily CA1; see Experimental Procedures); this region should show maximal changes in neuronal layer laminin expression.

We compared four samples by Western analysis with affinity-purified anti-laminin antibodies (Figure 5A): purified laminin with and without partial plasmin digestion and CA1 extracts from kainate- and PBS-injected hippocampi. The purified laminin showed the characteristic α (440 kDa) and β/γ (220 kDa) chains and a contaminating nidogen (entactin) band at approximately 150 kDa. Brief digestion of this material with plasmin resulted in a decrease in the laminin and nidogen bands and several intermediate degradation products. For the CA1 extracts, no protein the size of intact α chain was observed, an observation reported previously in analysis of adult rat, mouse, and human brains (Yamamoto et al., 1988; Liesi and Risteli, 1989; Murtomäki et al., 1992). Laminin subunits that were slightly smaller than the size of conventional β/γ chains were detected, as reported previously (Yamamoto et al., 1988), as well as other unusual subunits at approximately 125 and 80 kDa. All of these proteins reacted with monoclonal antibodies against the β1 and γ1 subunits. These results indicate that the laminin isoforms in the CNS are distinct from those normally found in the ECM.

To determine the relative expression of laminin after kainate or PBS injection, extracts from the two sides were analyzed for protein content, and equal amounts of protein were analyzed. Representative lanes stained with Coomassie blue before transfer confirmed that equal total protein had been loaded (Figure 5B). The amount of laminin detected on the kainate-injected side was reproducibly less than that on the PBS-injected side (Figure 5A). Depending on the experiment and the band analyzed, the reduction after kainate injection was from 5- to 18-fold.

The detection of a laminin-intermediate degradation product characteristic of plasmin action was not observed in the kainate-injected extracts. Since we know that plasmin is necessary for laminin disappearance (blocked by α_2-antiplasmin, Figures 4F and 4H), there are many possibilities for the lack of a plasmin intermediate. Plasmin could cleave laminin directly; however, other proteases in vivo could further degrade this intermediate, or the digestion products from the unusual CNS laminin isoform might be distinct from those with the laminin-1 standard. In either of these cases, the products might be undetectable. Alternatively, plasmin could be activating a second protease, which could then degrade laminin directly. These possibilities are considered further in the Discussion.

Disruption of Laminin–Neuron Interaction Makes Neurons Sensitive to Excitotoxic Death in the Absence of Plasmin

One explanation for the above results was that plasmin-catalyzed degradation of laminin makes neurons more sensitive to excitotoxic insult. If this model is correct, then blocking neuron–laminin interaction should have the same effect as degradation of the ECM protein. Therefore, one explicit prediction of the model is that tPA-deficient mice, which can not degrade laminin and are resistant to neuronal death, would be converted to sensitivity if their neurons were prevented from interacting properly with the ECM. This strategy has been used in other systems to demonstrate the importance of cell–ECM interactions (e.g., Boudreau et al., 1995; Coucouvanis and Martin, 1995).

To test this prediction, $tPA^{-/-}$ mice were infused bilaterally with an affinity-purified polyclonal antibody directed against laminin and then injected unilaterally with kainate. Control $tPA^{-/-}$ mice were infused with nonimmune antiserum or bovine serum albumin (BSA). The control $tPA^{-/-}$ mice showed the expected resistance to neuronal degeneration (Figures 6A and 6B). However, $tPA^{-/-}$ mice infused with the anti-laminin antibody exhibited increased neuronal death (Figure 6D). Pretreatment of the antibody with protein A-agarose (data not shown) or immobilized laminin (Figure 6C) abolished the effect of the antibody. In addition, neuronal degeneration was only observed on the kainate-injected side, demonstrating that the antibody is not toxic by itself (Figure 6D). This experiment shows that blocking interaction of neurons with laminin is functionally equivalent

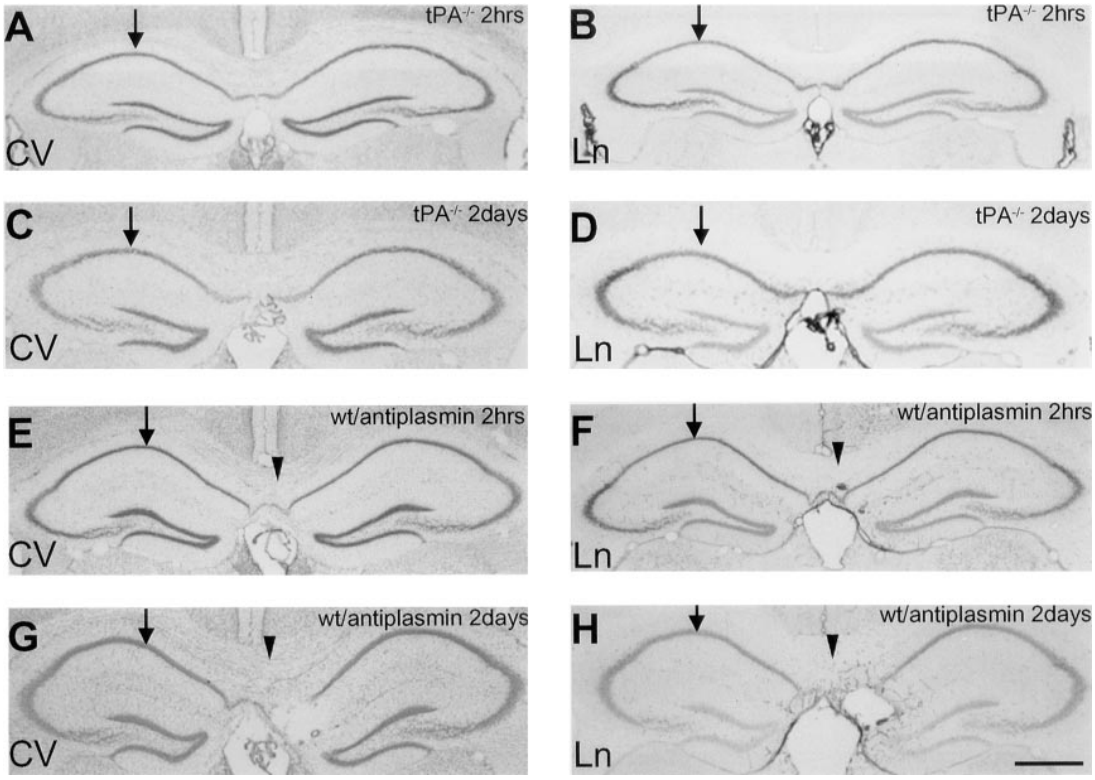

Figure 4. tPA Deficiency and α_2-Antiplasmin Block Laminin Degradation and Neuronal Cell Death after Excitotoxin Injection

Cresyl violet or laminin immunostained coronal sections through the hippocampus of $tPA^{-/-}$ (A–D) or α_2-antiplasmin-infused wild-type mice (E–H) 2 hr and 2 days after kainate injection. $tPA^{-/-}$ mice were injected unilaterally with kainate (1.5 nmol) and analyzed for neuronal survival and laminin expression. Wild-type mice were infused with α_2-antiplasmin for 2 days, kainate was injected, the infusion continued, and the mice brain sections analyzed for neuronal cell death and laminin expression. CV, cresyl violet staining; Ln, laminin immunostaining. Arrows, kainate injection sites; arrowheads, α_2-antiplasmin infusion sites. Scale bar, 750 μm.

to degradation of laminin in conferring sensitivity to excitotoxin-induced death.

Discussion

Mechanism of Excitotoxin-Induced Neuronal Death

The results presented in this paper document a critical role for laminin in the survival of CNS neurons after excitotoxic insult. A working model for degeneration in this system can be formulated as follows (Figure 7): (1) after exposure to excess kainate, neurons are massively depolarized leading to influx of calcium; (2) this depolarization results in two phases of tPA induction, a rapid phase (Z.-L. C., unpublished observations) caused by release of presynthesized tPA from vesicles (Gualandris et al., 1996; Parmer et al., 1997) and a slower phase resulting from stimulation of tPA gene transcription (Qian et al., 1993); (3) increased tPA activates CNS plasminogen to plasmin (Tsirka et al., 1997a), and this protease degrades laminin in the ECM (Liotta et al., 1981; Salonen et al., 1984; Paulsson et al., 1988); (4) the metabolic alterations associated with depolarization coupled with the loss of cell attachment sites leads to death of the neurons. While loss of laminin attachment is deleterious in combination with kainate injection, it is not by itself significantly toxic. Infusion of excess tPA into the mouse hippocampus does not kill neurons in the absence of excitotoxin treatment (Tsirka et al., 1996), and infusion of anti-laminin antibodies is only toxic in conjunction with kainate injection (Figure 6D, kainate-injected vs. noninjected side).

It is important to note two areas of uncertainty in this model. First, our results do not exclude other substrates for tPA and plasmin in the CNS. There are numerous extracellular proteins, such as neurotransmitter receptors and cell adhesion molecules, that might also be degraded by plasmin. It is therefore possible that excess proteolysis could affect a variety of proteins, leading to increased cell death. However, our experiments do identify laminin as one critical substrate, because blocking cell attachment to laminin with specific antibodies renders neurons sensitive to kainate even in the absence of protease.

Secondly, although our evidence demonstrates that plasmin is required for laminin degradation, it does not exclude that plasmin activates another protease, which then alone or in conjunction with plasmin effects laminin degradation. For example, plasmin is known to activate certain metalloproteinases (Kleiner and Stetler-Stevenson, 1993), and MMPs can degrade laminin molecules (Giannelli et al., 1997). If this class of enzyme plays a role in the degeneration process, then MMP inhibitors

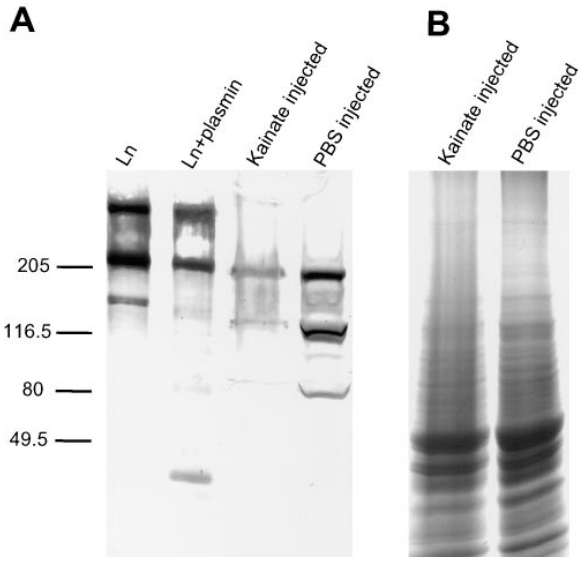

Figure 5. Western Blot Analysis of Laminin Expression in the Hippocampus after Excitotoxin Injection

(A) One microgram purified mouse laminin (Ln) or plasmin-digested laminin (Ln + plasmin) and extracts from kainate-injected hippocampi or PBS-injected hippocampi were applied to a 4%-20% polyacrylamide-SDS gel under reducing conditions, blotted to nitrocellulose membrane, and stained with affinity purified anti-laminin antibody, biotinylated secondary antibody, and a peroxidase stain. Laminin can be degraded by plasmin, and kainate-injected hippocampi show less laminin than PBS-injected hippocampi. Molecular weight markers in kilodaltons are shown on the left.

(B) Coomassie blue–stained gel after electrophoresis of extracts from kainate- and PBS-injected hippocampi.

might protect laminin and neurons after excitotoxin injury.

Laminin and Neuron Function

Laminin is a trimeric glycoprotein, composed of an α chain, β chain, and γ chain (Burgeson et al., 1994), that is present in the ECM (Timpl et al., 1979). All three chains of purified laminin can be digested to smaller fragments by plasmin (Liotta et al., 1981; Salonen et al., 1984; Paulsson et al., 1988). In addition, ECM laminin is especially sensitive to degradation by plasmin; when denuded human amnion basement membrane is incubated with plasmin, laminin immunoreactivity is completely removed, whereas collagen type IV immunoreactivity remains intact (Liotta et al., 1981).

It has been demonstrated that ECM proteins are important cell survival factors for many cell types, a phenomenon termed anoikis. If certain epithelial cells (Talhouk et al., 1992; Meredith et al., 1993; Frisch and Francis, 1994; Khwaja et al., 1997) and neurons (Kalcheim et al., 1987; Ernsberger et al., 1989) are prohibited from interacting with the ECM, their viability is impaired. This cell death can play a normal role in development. During cavitation in the mouse embryo, interior cells are spatially prevented from contact with the ECM, leading to death and formation of a cavity, whereas the outer ring of cells attached to the matrix survives (Coucouvanis and Martin, 1995). Another mechanism of blocking ECM interaction is proteolytic degradation of matrix proteins. For example, overexpression of the matrix metalloproteinase stromelysin-1 in the mammary gland degrades the ECM and induces apoptosis of epithelial cells (Boudreau et al., 1995).

Laminin has also been implicated directly in the function and survivability of neurons (Rogers et al., 1986; Kalcheim et al., 1987; Yamamoto et al., 1988; Hagg et al., 1989; Murtomäki et al., 1992, 1995; Liesi and Wright, 1996). This protein is involved in neurite outgrowth, growth cone guidance, pathfinding, and synapse formation (see Venstrom and Reichardt, 1993; Luckenbill-Edds, 1997). Laminin has also been linked to neuronal death. In an axotomy model of neuronal injury, the number of laminin-staining cells in the medial septum decreased after severing the nerve; NGF administration preserves both neurons and laminin staining (Hagg et al., 1989). This finding is consistent with a role for laminin in the survival of CNS neurons.

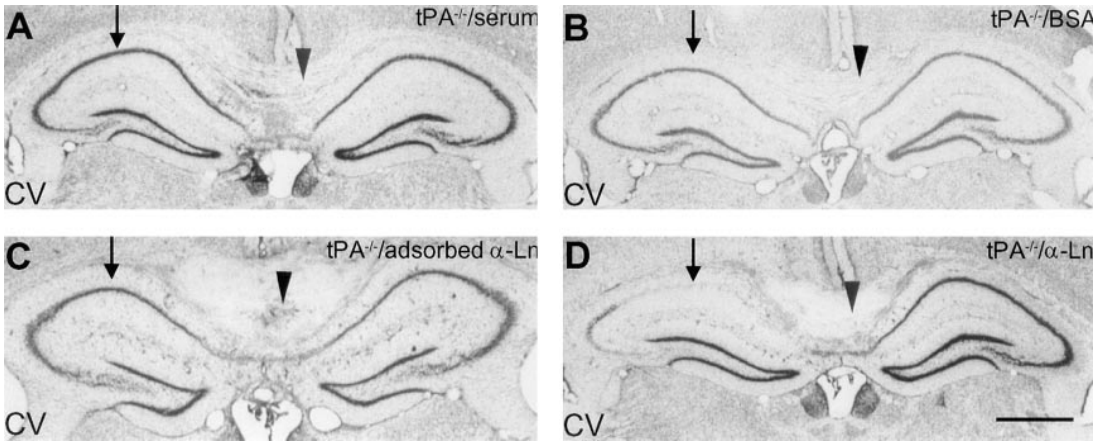

Figure 6. Disruption of Laminin–Neuron Interaction Makes Neurons Sensitive to Excitotoxic Death in the Absence of Plasmin

Cresyl violet–stained coronal sections through the hippocampus of tPA-deficient mice. The mice were infused with normal rabbit serum (A), 1% BSA in PBS (B), antigen preabsorbed rabbit anti-mouse laminin antibodies (C), or affinity-purified rabbit anti-mouse laminin antibody (D), kainate was injected, the infusion continued further for 2 days, and the mice brain sections were stained with cresyl violet. Arrows, kainate injection sites; arrowheads, antibody or other control reagents infusion sites. Scale bar, 750 μm.

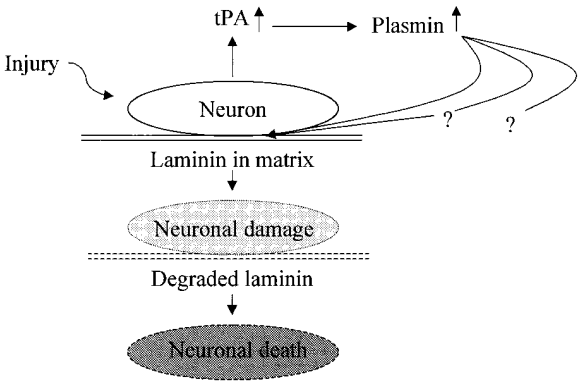

Figure 7. Model for Mechanism of Neuronal Degeneration after Excitotoxic Injury

Excess stimulation of glutamate receptors with excitotoxins leads to increased release and synthesis of tPA by neurons and microglia and consequently increased plasmin generation from neuronal plasminogen. Plasmin initiates laminin degradation, because blocking plasmin generation genetically or biochemically blocks loss of laminin. The loss of laminin destabilizes the neurons and promotes their death. Plasmin may (1) directly degrade laminin; (2) activate another protease (for example, an MMP), which would then act on laminin (e.g., Gianelli et al., 1997); or (3) work via both mechanisms. Also, since plasmin is a potent protease, it may have substrates other than laminin that contribute to death. Both excitotoxic injury and this protease cascade are important for cell death; degeneration caused by excitotoxins is reduced dramatically in the absence of tPA or plasmin activity, and excess tPA activity is not significantly cytotoxic in the absence of injury.

The cellular binding proteins in the hippocampus that interact with laminin are not fully identified. Integrin subunit α_8 is expressed in the hippocampus (Einheber et al., 1996), but $\alpha_8\beta_1$ does not appear to mediate laminin binding (Schnapp et al., 1995). Subunit $\beta 1$ is a component of most laminin-binding integrins, but its expression in the resting hippocampus is very low (Z. -L. C., unpublished observations). Therefore, the downstream signaling pathways that are induced by hippocampal neuron interactions with laminin remain to be clarified.

Laminin Degradation in the CNS: Normal Role and Therapeutic Potential

One question that emerges from our studies is whether limited laminin degradation occurs normally in the CNS. tPA activity is present in the resting hippocampus before any stimulation (Qian et al., 1993; Sappino et al., 1993) and could generate low levels of plasmin activity from existing plasminogen (Tsirka et al., 1997a). This low level of protease activity might facilitate matrix remodeling during activity-dependent modulation of synaptic function, and it might therefore be beneficial. This possibility awaits more information about the structure of the ECM in the CNS and how it might change during normal activity.

Finally, it is intriguing to consider the possible therapeutic implications of these results. tPA is approved for treatment of thrombotic stroke due to its fibrinolytic activity (Marler et al., 1995). However, based on their involvement in neuronal degeneration, tPA/plasmin use for stroke might have deleterious consequences. Our results suggest that it might be possible to dissociate the fibrinolytic properties of this system, which are required to restore blood flow, from the undesired neurotoxic properties. If a tPA/plasmin-based therapy could be devised that was effective at degrading fibrin but had lost its ability to cleave laminin, then leakage of this protease into the CNS would have little effect. Given the magnitude of stroke as a neurological disorder, it would seem worthwhile to pursue this goal.

Experimental Procedures

Intrahippocampal Injections

Adult male mice, weighing approximately 25 g, were injected intraperitoneally with atropine (0.6 mg/kg of body weight) and then were anesthetized deeply with 2.5% avertin (0.02 ml/g of body weight). They were placed in a stereotaxic apparatus and injected unilaterally with 1.5 nmol or 0.33 nmol kainate or 15 nmol of NMDA in 0.3 μl PBS into the hippocampus (Andersson et al., 1991; Tsirka et al., 1995). In control mice, 0.3 μl of PBS was injected. The coordinates of the injection were bregma −2.5 mm, medial-lateral 1.7 mm, and dorsoventral 1.6 mm. The excitotoxin was delivered over 30 s. After kainate was delivered, the injection needle remained at the above coordinates for another 2 min to prevent reflux of fluid. After variable lengths of time (indicated in each experiment), the animals were anesthetized and perfused through the heart with PBS, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were removed, postfixed in the same fixative overnight at 4°C, and then left in 30% sucrose in PBS for 48 hr at 4°C. Coronal brain sections (30 μm) were cut on a microtome, collected in PBS, and then processed for cresyl violet staining and immunohistochemistry. For cresyl violet staining, the sections were mounted onto slides, dehydrated, and then stained with cresyl violet, which stains ribosomal RNA in neuronal cell bodies.

Immunohistochemistry

Immunohistochemistry procedures were based on the methods of Jucker et al. (1992). Brain sections of mice, manipulated as described above, were incubated with (1) affinity-purified rabbit anti-mouse laminin polyclonal antibody (Sigma) at 1:1000 dilution; (2) rabbit anti-mouse collagen type IV polyclonal antibody (Chemicon) at 1:250 dilution; or (3) rabbit anti-mouse fibronectin polyclonal antibody (Chemicon) at 1:250 dilution. As a control, the sections were incubated with laminin antigen (Sigma) preabsorbed antibody. 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 (Tsirka et al., 1995). Similar results for laminin were obtained with monoclonal antibodies against the laminin $\beta 1$ chain or $\gamma 1$ chain (Chemicon).

Intrahippocampal Infusions

Adult wild-type or tPA$^{-/-}$ (Carmeliet et al., 1994) mice were anesthetized as above, placed in a stereotaxic apparatus, and a microosmotic pump (Alzet) containing 100 μl of PBS, 50% normal rabbit serum in PBS, 1% BSA in PBS, α_2-antiplasmin in PBS (1 mg/ml; Sigma), affinity-purified rabbit anti-laminin antibodies (0.25 mg/ml in 1% BSA /PBS, Sigma) was placed subcutaneously in the back of the animals. To demonstrate the specificity of laminin antibody effects, the antibodies were preabsorbed with either mouse laminin–coated plates or protein A-Agarose, and the efficiency of depletion of anti-laminin IgG was checked by immunohistochemistry; 100 ml of each depleted antibody solution was also used for control animals. For the infusions, a brain infusion cannula connected to the pump was positioned at the following coordinates: bregma −2.5 mm, medial-lateral −0.5 mm, and dorsoventral 1.6 mm to deliver the compound near the midline. The infusion rate was 0.5 μl/hr. The pump was allowed to infuse the designated solution for 2 days, and the kainate was injected as described above. The mice were sacrificed 2 hr or 2 days after the kainate injection, and their brains were examined for neuronal survival and processed for immunohistochemistry.

tPA-deficiency not only protects neurons from excitotoxic damage but also reduces the severity of seizures induced by kainate (Tsirka et al., 1995). The correlation between neuronal degeneration and seizure, although not studied in detail, was also observed in the experiments reported here: α_2-antiplasmin infusion into wild-type mice protected neurons and reduced seizure, whereas anti-laminin antibody infusion into $tPA^{-/-}$ mice caused increased neuronal death and induced more seizure activity.

Western Blot Analysis of Laminin Expression

Four adult wild-type mice were treated as follows: one side was injected with kainate as above except the solution contained 0.1 mg/ml bromophenol blue, and the other side was injected with PBS with the same dye concentration. The dye allowed direct visualization of the delivery of the injected solution. Four hours later, the hippocampal regions showing maximal dye penetration on the kainate and PBS sides were dissected out separately and frozen until homogenization in 0.3 ml lysis buffer (2% Triton X-100, 0.5 M NaCl, 10 mM Tris-HCl [pH 7.4], 1 mM PMSF, 10 mM EDTA, 1 μg/ml aprotinin). The homogenates were then treated as follows (Murtomäki et al., 1992): they were incubated on ice for 30 min, centrifuged at 14,000 g for 20 min at 4°C, and the supernatant was transferred to a new tube. The protein concentration was determined using a BCA kit (Pierce), and 120 μg protein from each side was loaded on a 4%-20% gradient SDS-polyacrylamide gel electrophoresis under reducing conditions. Equal protein loading of the two samples was documented by Coomassie blue staining of parallel lanes. Partial digestion of laminin (Chemicon) was achieved by incubating 20 μg purified mouse laminin (Chemicon) with 0.5 μg plasmin (Sigma) in 50 μl 20 mM Tris-HCl (pH 7.5), 150 mM NaCl at 37°C for 2 hr. One microgram of purified mouse laminin and 1 μg plasmin-digested laminin were applied to the same gel under reducing conditions. After electrophoresis, the blots were transferred to nitrocellulose membrane, blocked with 5% nonfat milk, 5% goat serum in TBS-T (20 mM Tris-HCl [pH 7.5], 50 mM NaCl, 0.05% Tween 20) at room temperature for 3 hr, then incubated with laminin antibody diluted 1:1000 in TBS-T buffer containing 5% goat serum for 12 hr at 4°C. The blot was washed three times in TBS-T, incubated with biotinylated second antibody (Vector Laboratories) for 2 hr at room temperature, and the avidin-biotin-peroxidase complex (ABC reaction) was visualized with diaminobenzidine and hydrogen peroxide (Vector Laboratories) as described in Immunohistochemistry.

Acknowledgments

We are very grateful to Stella Tsirka for advice and suggestions and to Fernando Sallés for comments on the manuscript. Z.-L. C. thanks all the members of the Strickland laboratory for their support, and S. S. is grateful to Mark Ginsburg for helpful discussions. This work was supported by a Human Frontier Science Program Fellowship (LT0486) to Z.-L. C. and grants from the National Institute of Health (NS-35704) and the American Cancer Society (CB205) to S. S.

Received July 15, 1997; revised November 11, 1997.

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