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LAMININ CHAIN EXPRESSION SUGGESTS THAT LAMININ-10 IS A MAJOR ISOFORM IN THE MOUSE HIPPOCAMPUS AND IS DEGRADED BY THE TISSUE PLASMINOGEN ACTIVATOR/PLASMIN PROTEASE CASCADE DURING EXCITOTOXIC INJURY

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Abstract—Laminins are important components of the extracellular matrix, and participate in neuronal development, survival and regeneration. The tissue plasminogen activator/plasmin extracellular protease cascade and downstream laminin degradation are implicated in excitotoxin-induced neuronal degeneration. To determine which specific laminin chains are involved, we investigated the expression of laminins in the hippocampus, and the cell types expressing them. Reverse transcription-PCR demonstrated that the messenger RNAs for all laminin chains could be detected in the hippocampus. To determine the localization of laminin chain expression, immunostaining was used. This method showed that α5, β1 and γ1 are most highly expressed in the neuronal cell layers. Immunoblotting confirmed the hippocampal expression of the chains α5, β1 and γ1, and RNA *in situ* hybridization showed a neuronal expression pattern of α5, β1 and γ1. At early time points following intrahippocampal injection of kainate, α5, β1 and γ1 chain immunoreactivities were lost. In addition, tissue plasminogen activator-deficient mice, which are resistant to kainate-induced neuronal death, show no significant change in laminins α5, β1 and γ1 after intrahippocampal kainate injection.

Taken together, these results suggest that laminin-10 (α5-β1-γ1) comprises a major neuronal laminin in the mouse hippocampus, and is degraded before neuronal death during excitotoxic injury by the tissue plasminogen activator/plasmin protease cascade. By identifying a neuronal laminin (laminin-10) that participates in neuronal degeneration after excitotoxic injury, this study clarifies the molecular definition of the extracellular matrix in the hippocampus and further defines a pathway for mechanisms of neuronal death.

Key words: neurodegeneration, kainate, proteolysis, pyramidal neuron, extracellular matrix proteins.

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Abbreviations: AP, alkaline phosphatase, BCIP, 5-bromo-4-chloro-3-indolyl phosphate toluidine salt; DIG, digoxigenin, ECM, extracellular matrix; MMP, matrix metalloproteinase, mRNA, messenger RNA; NBT, nitro blue tetrazolium chloride, PAGE, polyacrylamide gel electrophoresis; RT-PCR, reverse transcription-polymerase chain reaction, PBS, phosphate-buffered saline; PVDF, poly(vinylidene difluoride), SDS, sodium dodecyl sulfate; TE, tris-EDTA buffer, tPA, tissue plasminogen activator.

The extracellular matrix (ECM) provides a critical surface for proper cell function and survival (Werb, 1997; Lukashev and Werb, 1998). One important component of the ECM in many tissues, including the nervous system, is the laminin family of proteins. Laminins are large heterotrimeric glycoproteins, consisting of an α chain, β chain, and γ chain, that participate in many aspects of nervous system physiology. These activities include neurite outgrowth and migration (Luckenbill-Edds, 1997), synapse function and stability (Patton et al., 1998), neuronal adhesion and growth (Powell and Kleinman, 1997), regeneration (Luckenbill-Edds, 1997); and neuronal viability (Edgar et al., 1985; Ernsberger et al., 1989; Chen and Strickland, 1997). To date, five α chains, three β chains, and three γ chains have been identified, from which 15 laminin heterotrimers have been characterized *in vivo* (Burgeson et al., 1994; Libby et al., 2000). Several of the laminin chains are essential for development, since genetic knockouts of laminin α2 (Xu et al., 1994; Miyagoe et al., 1997), α3 (Ryan et al., 1999), α5 (Miner et al., 1998), β2 (Noakes et al., 1995) and γ1 (Smyth et al., 1999) all produce lethality early in life. One exception is the α4 chain, in which knockouts are viable but show defects in synapse formation (Patton et al., 2001).

Degradation of laminin can affect neuronal survival. One experimental paradigm for studying neurodegeneration is excitotoxin (e.g. kainate) injection into the mouse hippocampus (Coyle et al., 1978). Following kainate injection, a serine protease cascade involving tissue plasminogen activator (tPA) and plasminogen promotes hippocampal neuronal death through the degradation of laminin (Tsirka et al., 1995, 1996, 1997; Chen and Strickland, 1997). Interruption of this cascade, either by genetically knocking out tPA or plasmin(ogen), or by pharmacologically inhibiting tPA or plasmin activity, prevents laminin degradation and neuronal cell death (Tsirka et al., 1995, 1996, 1997; Chen and Strickland, 1997). Furthermore, the loss of laminin immunostaining precedes neuronal death after excitotoxin injection into the mouse hippocampus, and is spatially coincident with regions that subsequently show a loss of neurons (Chen and Strickland, 1997). A working model proposes that tPA/plasmin-catalyzed deg-

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Table 1. Mouse laminin chain specific primers used in this study

Name	Primers	Sequences	Position
α1	Sense	5'-GCGCGTAAAGATTTCCAGCC-3'	nucleotides 5113–5133
	Antisense	5'-GTCTCTGTCCAAAGCTCCTG-3'	nucleotides 5716–5736
	Reference	Sasaki et al., 1988	
α2	Sense	5'-GGGTCGGATCAATCATGCTG-3'	nucleotides 8457–8477
	Antisense	5'-CGTGGAACATAAGCTTCTCG-3'	nucleotides 9023–9043
	Reference	Bernier et al., 1995	
α3	Sense	5'-AAAGGTGCACCTGGTGGTGG-3'	nucleotides 6944–6964
	Antisense	5'-TTCGGTGGGAAGGAAAGCTG-3'	nucleotides 7508–7528
	Reference	Galiano et al., 1995	
α4	Sense	5'-CATGGGATCCTATTGGCCTG-3'	(nucleotides 4675–4694)
	Antisense	5'-CACATAGCCGCCTTCTGTGG-3'	(nucleotides 5317–5336)
	Reference	Liu and Mayne, 1996	
α5	Sense	5'-ACGGCTCAGAAGGTTTCCCG-3'	(nucleotides 9673–9693)
	Antisense	5'-CTTCAGACAGCCGCTGAACC-3'	(nucleotide 10201–10221)
	Reference	Miner et al., 1995	
β1	Sense	5'-GCTGGATCCGCTTGCAGCAGAGTGCAGCTGA-3'	(nucleotides 4858–4889)
	Antisense	5'-CGCGAATTCGCTAAGCAGGTGCTGTAAACCG-3'	(nucleotides 5504–5535)
	Reference	Sasaki et al., 1987	
β2	Sense	5'-CTGCAGCGGGTATGACTTCC-3'	(nucleotides 4475–4495)
	Antisense	5'-GGCTTGTGCAGCCAGAACAC-3'	(nucleotides 5002–5022)
	Reference	Durkin et al., 1996	
β3	Sense	5'-CAGGTAGATGATGTGGTCGG-3'	(nucleotides 3079–3099)
	Antisense	5'-ACTGCGGATCTGCTCCACAC-3'	(nucleotides 3601–3621)
	Reference	Utani et al., 1995	
γ1	Sense	5'-GCGGGATCCCCAATGACATTCTCAACAAC-3'	(nucleotides 4331–4351)
	Antisense	5'-GCAGATATCGGGCTTCTCGATAGACGGGG-3'	(nucleotides 5047–5067)
	Reference	Sasaki and Yamada, 1987	
γ2	Sense	5'-TATTAGCCAGAAGGTTGCGG-3'	(nucleotides 2973–2993)
	Antisense	5'-TAGTCTCCAGCAGATGGAGG-3'	(nucleotides 3500–3520)
	Reference	Sugiyama et al., 1995	
γ3	Sense	5'-TTTGGGGATCCTCTAGGGCT-3'	(nucleotides 2469–2488)
	Antisense	5'-CCAGTCACAGGGTTGCAGGT-3'	(nucleotides 2721–2740)
	Reference	Iivanainen et al., 1999	

radation of laminin causes an interruption in neuron-ECM interactions and predisposes neurons to die. This death may occur via a process called anoikis, a subset of apoptosis caused by loss of contact to the ECM (Frisch and Francis, 1994; Frisch and Ruoslahti, 1997). Consistent with this idea, tPA-deficient mouse hippocampal neurons can be rendered sensitive to kainate excitotoxicity by blocking neuron-laminin interaction with intracerebral infusions of laminin antibody (Chen and Strickland, 1997).

The aim of this study was to characterize the laminins present in the mouse hippocampus to define which chains are involved in excitotoxic neuronal death. For this purpose, we determined which laminin chains were present in the hippocampus and the cell types that expressed them. We show here that several laminin chains are expressed in the hippocampus, and that the components of laminin-10 (α5-β1-γ1) comprise a neuronal laminin isoform that is degraded early during acute excitotoxic injury.

EXPERIMENTAL PROCEDURES

Reverse transcription-polymerase chain reaction

Using chain-specific primers for the different mouse laminin genes (Niimi et al., 1997), reverse transcription-polymerase chain reaction (RT-PCR) was performed to detect expression of 11 different laminin chains. Hippocampi from approximately 10 adult C57BL6/J inbred mice (Taconic, Germantown, NY, USA, or Jackson Laboratories, Bar Harbor, ME, USA) were dissected and messenger RNA (mRNA) was isolated using a Poly(A)Pure kit (Ambion, Austin, TX, USA), and reverse transcribed using random hexanucleotide primers (Sigma, St. Louis, MO, USA). PCR was then performed using mouse laminin chain-specific primers according to Niimi et al. (1997), except for the α5-specific primers, which were re-designed. The details for the primers are shown in Table 1. In order to optimize the PCR reactions for each chain, a temperature gradient was performed using an Eppendorf Mastercycler Gradient thermal cycler (Brinkmann Instruments, Westbury, NY, USA), and the optimal chain-specific annealing temperature was selected. The PCR protocol: 94 °C 4 min, followed by 30 cycles of 94 °C 30 s, 1 min (at the following temperatures, in °C: α1, 64; α2, 64; α3, 64; α4, 62; α5, 58; β1, 68; β2, 64; β3, 62; γ1, 60; γ2, 58; γ3, 62; β-actin, 64), 72 °C 1 min, then 72 °C for 10 min. The PCR experiments were repeated several times and all gave similar results as shown in Fig. 1. RNA obtained from neonatal and adult kidney served as a positive control for optimization (data not shown). β-actin (sense: 5'-GTC CCT GTA TGC CTC TGG TC-3', antisense: 5'-TCG TAC TCC TGC TTG CTG AT-3'; Sanford et al., 1997) was used as control for confirming the absence of genomic DNA contamination; β-actin was also used as a normalization control.

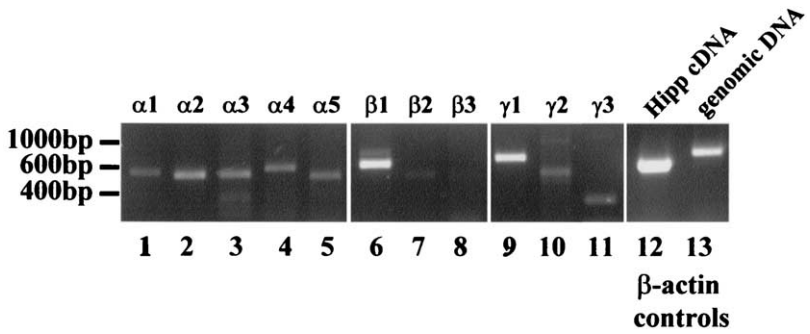

Fig. 1. RT-PCR for laminin chains in mouse hippocampus. Adult mouse hippocampal mRNA was reverse transcribed, and PCR was performed using mouse laminin chain-specific primers as described by Niimi et al. (1997), except for the α5 primers, which were redesigned (see Experimental Procedures). The details for the primers are shown in Table 1. Neonatal and adult kidney RT-PCR was used as a positive control for all laminin chains, and showed that the products from the hippocampus were the correct size (data not shown). Equal amounts of RT product were added to all reaction mixtures, and all reactions were performed in parallel. β-actin primers were used to determine possible genomic DNA contamination, and none was seen (compare last two lanes). Agarose gel electrophoresis was used to visualize the products. Molecular size markers are displayed on the left.

All experiments conformed to international guidelines on the ethical use of animals and all efforts were made to minimize the number of animals used and their suffering.

Antibodies

Table 2 summarizes the laminin antibodies used in this study. Laminin chain-specific antibodies were generously provided by Drs Jeff H. Miner and Josh R. Sanes (Washington University, St. Louis, MO, USA) and by Dr Lydia M. Sorokin (University of Erlangen-Nuremberg, Erlangen, Germany). Affinity purified rabbit anti mouse laminin-1 was obtained from Sigma. Anti-mouse laminin γ1 polyclonal antibody was obtained from Santa Cruz (Santa Cruz, CA, USA).

RNA *in situ* hybridization

To analyze laminin chain expression and cell type specificity, mRNA *in situ* hybridization was performed. Products from RT-PCR using mouse laminin chain-specific primers for α5, β1 and as described above were cloned into pBluescript II KS+ (Stratagene, La Jolla, CA, USA). Plasmid DNA was sequenced to certify correct insert sequence and orientation. BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/; National Center for Biotechnology Information) revealed that all the cloned laminin chain-specific cDNAs have no significant homology to other laminin chains, or to any other proteins. DNA clones for mouse laminin γ1 were obtained from Drs J. H. Miner and J. R. Sanes (Washington University). Digoxigenin (DIG)- labeled RNA probes (Boehringer Mannheim, Indianapolis, IN, USA) were synthesized from laminin chain-specific clones using T3- or T7-polymerase transcription according to manufacturer's instructions. Probes were purified by LiCl/EtOH precipitation, and correct probe size was verified by formaldehyde gel electrophoresis. *In situ* hybridizations were performed based on protocol described by Chen et al. (1995). Briefly, 14 μm-thick fresh frozen coronal cryostat sections of adult mouse brain on SuperFrost Plus slides (Fisher, Pittsburgh, PA, USA) were fixed in 4% paraformaldehyde in 10 mM phosphate-buffered saline (PBS), followed by washes in 0.1 M phosphate buffer, treatment with 10 μg/ml Proteinase K in 50 mM Tris–HCl, pH 7.5, 5 mM EDTA, and acetylation in 2.25% acetic anhydride in 15% triethanolamine. Sections were then dehydrated in serial ethanol solutions and chloroform baths. Following probe denaturation, sections were incubated overnight at 55 °C in hybridization solution containing RNA probe (sense or anti-sense), 10% dextran sulfate, 20 mM Tris–HCl, pH 8.0, 300 mM NaCl, 0.2% sarcosyl, 1×Denhardt's solution, 0.2 mg/ml salmon sperm DNA, 0.5 mg/ml yeast tRNA, 50% formamide and 1 μg probe. Post-hybridization washes at 65 °C consisted of 4×SSC and 50% formamide in 2×SSC, followed by 37 °C washes in 10 mM Tris–HCl, 1 mM EDTA, 500 mM NaCl. Sections were then treated with 2 μg/ml RNase A in RNase buffer [10 mM Tris–HCl, 1 mM EDTA, 500 mM NaCl] for 30 min at 37 °C, followed by washes in RNase buffer, 50% formamide in 2×SSC at 65 °C, and Buffer 1 [0.1 M Tris–HCl, pH 7.5, 150 mM NaCl]. Sections were then blocked with 1.5% Blocking Reagent (Boehringer Mannheim) in Buffer 1 and incubated overnight with anti-DIG antibody (1:500 anti-DIG Fab fragment-alkaline phosphatase (AP)-conjugated; Boehringer-Mannheim) and 1% Blocking Reagent in Buffer 1. Following several washes in 0.1 M Tris–HCl, pH 7.5, 150 mM NaCl, sections were washed in Buffer 3 [100 mM Tris–HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$] and color reaction performed in the dark using 75 mg/ml nitro blue tetrazolium chloride (NBT) and 50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate toluidine salt (BCIP) (Boehringer-Mannheim) in Buffer 3. Reaction was stopped by washes in Buffer 3 and tris-EDTA buffer (TE), and sections cover-slipped with TE/glycerol and photographed. Sense probes were used as negative controls in all experiments.

Table 2. Laminin antibodies used in this study

Specificity/Name[a]	Host	Reference/Supplier
LN-1 L-9393 (p)	Rabbit	Sigma
α1 8B3	Rat	Abrahamson et al., 1989
α2 hα2G	Rabbit	Vachon et al., 1996
α3 P3H9-2	Mouse	Chemicon
α4 377 (p)	Rabbit	Sixt et al., 2001
α5 8948 (p)	Rabbit	Miner et al., 1997
β1 5A2 (m)	Rat	Abrahamson et al., 1989
γ1 H-190 (p)	Rat	Santa Cruz

[a] p, polyclonal; m, monoclonal.

X-gal staining

Laminin γ1 gene trapping heterozygous mice (Mitchell et al., 2001), generously provided by Dr William C. Skarnes (University of California at Berkeley, Berkeley, CA, USA), were killed and their brains were removed. Fresh frozen cryostat brain sections were fixed in 0.4% paraformaldehyde in 0.1 M 1,4-piperazinediethanesulfonic acid buffer (pH 6.9), 2 mM $MgCl_2$, 5 mM EGTA on ice for 1 h followed by 3 washes in PBS. The sections were then rinsed in detergent buffer containing 0.1 M phosphate buffer (pH 7.3), 2 mM $MgCl_2$, 0.01% sodium deoxycholate, and 0.02% NP-40 for 10 min on ice. Finally the sections were stained in the dark at room

temperature in a staining solution containing 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 0.1 M phosphate buffer (pH 7.3), 2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% NP-40, 20 mM Tris (pH 7.3), and 1 mg/ml X-gal, and were visualized under light microscopy and photographed. X-gal stained sections were counterstained with Nuclear Fast Red for visualization under high magnification. Wild-type mouse brain sections were used as a negative control for X-gal staining (data not shown).

Intrahippocampal injection

C57BL6/J inbred mice, either wild-type (Taconic) or tPA-deficient$^{-/-}$ Carmeliet et al., 1994) of appropriate age or weight (older than 8 weeks and/or mass greater than 20 g) were anesthetized using Avertin (20 μl/g body weight of a 2.5% solution in saline) and atropine (1.5 μl/g body weight of a 0.54 mg/ml solution). Mice were placed in a stereotaxic apparatus (Stoelting, Wood Dale, IL, USA). Following an antero-posterior incision on the skull (approximately 1 cm), a small burr hole (approximately 1 mm diameter) was drilled for an injection at coordinates bregma −2.5 mm, mediolateral −1.7 mm, dorsoventral −1.6 mm. A total volume of 300 nl of kainate (Alexis, San Diego, CA, USA) for a dose of 0.25 nmol was delivered using a microinjector apparatus (Stoelting) via a 2.5 μl Hamilton syringe equipped with a 33-gauge needle, over the course of 60 s. After retracting the needle 0.1 mm, the needle was kept in place for 2 min to allow diffusion of the kainate, and then was completely removed. The area was then cleaned and the wound sutured. To control for mechanical damage, injections were performed with vehicle alone (PBS), and consistently showed no neuronal death in the hippocampus (data not shown).

Tissue preparation and immunohistochemistry

For fixed, free floating sections, mice were transcardially perfused with ice-cold PBS followed by cold 4% paraformaldehyde (approximately 25 ml each). Brains were removed, post-fixed overnight in 4% paraformaldehyde at 4 °C, then cryoprotected in cold 30% sucrose in phosphate buffer for 1–2 days. Brains were then frozen in powdered dry ice, embedded in OCT (Tissue-Tek), and cut into 30 μm sections either on a freezing microtome or cryostat and stored in 10 mM PBS with 0.2% sodium azide. For fresh frozen sections, brains were removed from PBS perfused mice and rapidly frozen in dry ice. Frozen cryostat sections (14 μm) were stored at −70 °C until use. Sections were blocked in 5–10% normal goat serum and 0.3% Triton X-100 in 10 mM PBS. Primary antibody incubations in 5–10% normal goat serum and 0.3% Triton X-100 in 10 mM PBS were performed either for 2 h at room temperature, or overnight at 4 °C, and were followed by washes in 10 mM PBS. Biotinylated secondary antibody (Vector Laboratories, Burlingame, CA, USA, or Jackson ImmunoResearch, West Grove, PA, USA) incubation was followed by 10 mM PBS washes, the Avidin-Biotin Reaction (VECTASTAIN Elite ABC; Vector Laboratories) and either diaminobenzidine reaction or Nova Red staining (Vector Laboratories). Free-floating sections were washed and mounted onto Superfrost Plus Slides (Fisher) overnight at 55 °C using mounting medium (2-mg/ml gelatin, 10% EtOH, 20 mM phosphate buffer). Controls for immunostaining either consisted of omission of primary antibody, substituting normal IgG/serum as the primary antibody reagent, and/or preabsorbing the antibody with purified laminin-1 or bacterial extracts containing antigen (for α5), and showed no significant background staining (data not shown). For α5 immunohistochemistry, expression vector (Novagen, Madison, WI, USA), containing an α5-chain fragment (Miner et al., 1997) was used to prepare induced and uninduced protein extracts for preabsorbing the primary antibody.

Fluoro Jade B staining

Fluoro Jade B can sensitively and selectively detect degenerating neurons (Schmued et al., 2000), and has been shown to correlate strongly with the number of TUNEL-positive cells following intrastriatal injection of quinolinic acid in mice (Hansson et al., 1999), and was performed as described (Schmued et al., 2000). Briefly, free floating sections were mounted onto slides, dried overnight, and incubated at room temperature in the following baths: 1% NaOH in 80% ethanol, 70% ethanol, H_2O, 0.06% potassium permanganate, H_2O, 0.0004% Fluoro Jade B (Histo-Chem, Jefferson, AR, USA), and three H_2O washes. Slides were then dried, immersed in xylene and cover-slipped using DPX mounting medium (Aldrich, St. Louis, MO, USA). Fluorescence was visualized using a fluorescein isothiocyanate filter on a Nikon Eclipse 600 microscope with PCM2000 laser scanning confocal system.

Western blot analysis

Tissue extracts from hippocampus, lung and kidney were prepared by homogenization of freshly PBS-perfused mice in Sewry buffer [50 mM Tris, pH 6.8, 4 M urea, 1% sodium dodecyl sulfate (SDS), 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid, 0.1% Nonidet P-40, 0.1% Triton X-100, and 1:100 mammalian protease inhibitor cocktail (Sigma)] (Sewry et al., 1998) using a Dounce homogenizer on ice, incubated for 30 min, and centrifuged. Purified mouse hippocampal extracts were run either on a reducing 4–20% SDS-polyacrylamide gel electrophoresis (PAGE) or 4–12% bis-Tris SDS-PAGE (Invitrogen, Carlsbad, CA, USA), blotted to poly(vinylidene difluoride) (PVDF) membrane (Immobilon-P; Millipore, Bedford, MA, USA), incubated using primary antibody; α5, 1:1000 (Miner et al., 1997); β1, 1:500 (Abrahamson et al., 1989); γ1, 1:1000 (Santa Cruz), horse-radish peroxidase-labeled secondary antibody (Vector Laboratories or Jackson ImmunoResearch), and stained using True Blue or TMB reagent (KPL, Gaithersburg, MD, USA). Membranes were stained with Ponceau S (Sigma) for verification of equal loading. Because of the large size of these chains, we used purified laminin-1 to confirm molecular size, with known sizes of approximately 400 kDa (for the α1 chain) and approximately 200 kDa (for the β1 and γ1 chains).

RESULTS

Laminin RNA expression in the mouse hippocampus

Previous work has shown that after excitotoxin injection in the CNS, laminin is degraded via a plasmin-mediated mechanism, in a manner that correlates spatially and temporally with neuronal death (Chen and Strickland, 1997). Since those experiments were performed using a pan-laminin-1 antibody, the data only supported the involvement of the laminin chains that form laminin-1, namely either α1, β1 or γ1. In order to specifically determine which laminin chains are degraded in this process, we analyzed all the chains expressed.

Using RT-PCR, mRNA from adult mouse hippocampus was analyzed with laminin chain-specific primers (Fig. 1). Among the α chains, all five were present, and the intensity of the bands were comparable (lanes 1–5). Among the β chains, β1 clearly gave the strongest product, and β2 and β3 were almost non-detectable (lanes 6–8). Among the γ chains, γ1 gave the strongest signal, although γ2 and γ3 chains were detected (lanes 9–11). RT-PCR using β-actin primers that distinguish cDNA from genomic DNA indicated no genomic DNA contam-

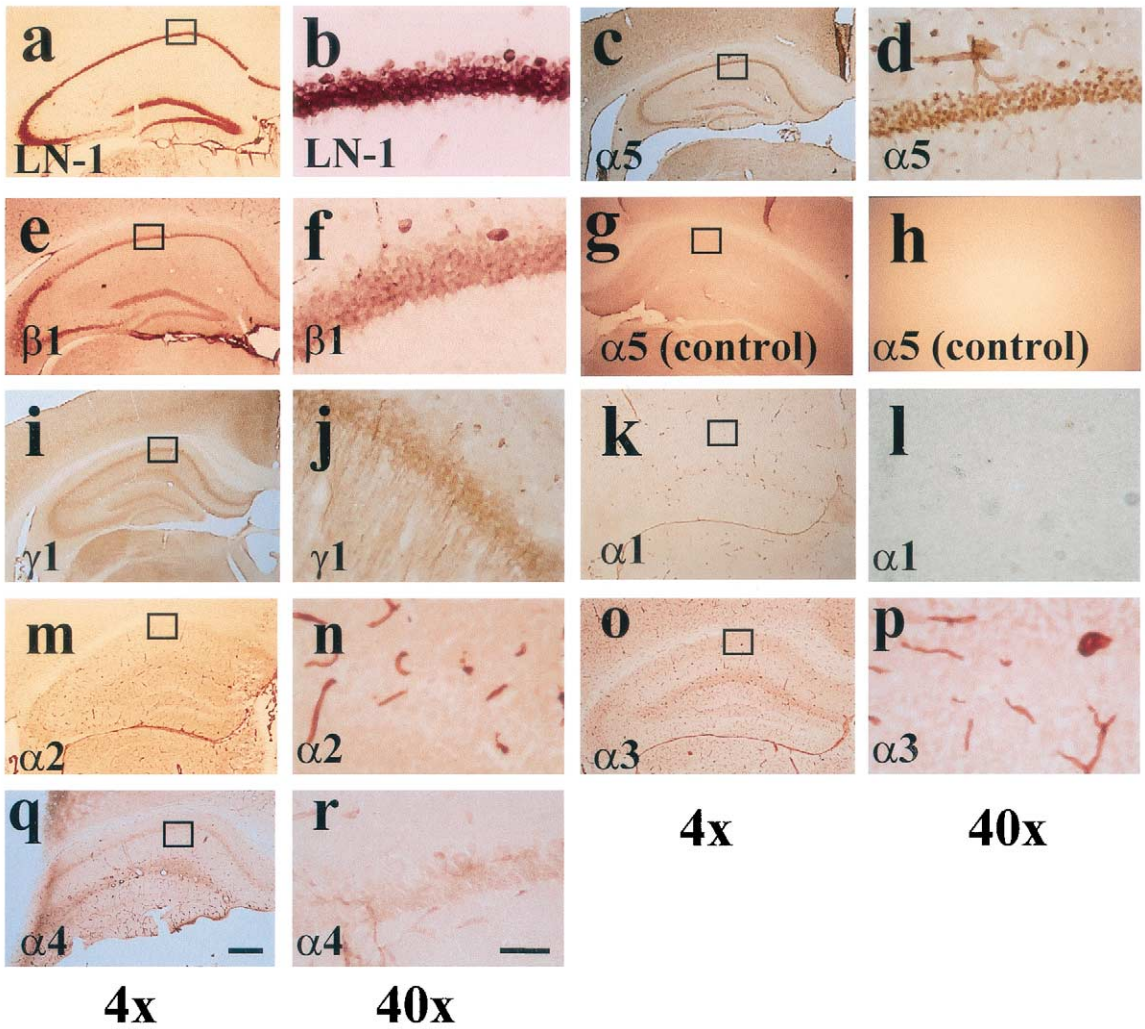

Fig. 2. Immunohistochemistry for laminin chains. Coronal sections of mouse brain were stained using multiple laminin antibodies, including laminin-1 (a and b,), α5 (c and d), β1 (e and f), γ1 (i and j), α1 (k and l), α2 (m and n), α3 (o and p), and α4 (q and r). Under low magnification of the entire hippocampus (panels a, c, e, i, k, m, o, q), several chains show clear strong staining for the complete CA layer and the dentate granule cell layer, specifically α5, β1, and γ1. Upon higher magnification of the CA1 region (see boxes), more defined neuronal-like staining is apparent. Note dendrite staining for γ1 (j). Laminin chains α1, α2, α3, and α4 showed a vascular pattern and some subpopulations of neurons or glia cells as shown by some punctuate staining. Staining with control antibodies showed no signal (example for α5 shown in g and h). Scale bar in q=400 μm and applies to a, c, e, g, i, k, m, o, and q. Scale bar in r=50 μm and applies to b, d, f, h, j, l, n, p, and r.

ination (lanes 12 and 13). In addition, negative controls using the RT reaction only or RT-PCR without DNA added showed no product (data not shown). Taken together, the RT-PCR results showed that the strongest signals came from β1 and γ1. Although this method is non-quantitative, it does suggest that a principal hippocampal laminin trimer(s) might contain the structure: α_x-β1-γ1 (where α_x denotes one of the α chains). These results are consistent with those seen previously (Chen and Strickland, 1997) and also when the RT-PCR laminin panel was performed on RNAs isolated from cultured hippocampal neurons (JA Indyk, FJ Sallés and S Strickland, unpublished results). In summary, all laminin chain mRNAs are detected in mouse hippocampal tissue, with strong RT-PCR products for β1 and γ1 chains.

Laminin protein expression

To examine for the presence of laminin protein in the hippocampus, immunohistochemistry and Western blotting were performed. The presence of laminin chains in blood vessels has been demonstrated in the rodent brain, including α2, α4, α5, β1, and γ1 (Hagg et al., 1997, Tian et al., 1997, Sixt et al., 2001), and the γ1 chain (Hager et al., 1998; Grimpe et al., 2002) and α2-like antigens (Tian et al., 1997) have been reported in hippocampal neurons. A rabbit polyclonal pan-laminin-1 antibody strongly stained neurons throughout the hippocampus (Fig. 2a, b). Higher magnification shows consistent staining of pyramidal neurons in the CA1 (Fig. 2b) and CA3 (data not shown), in addition to staining the granule neurons of the dentate gyrus (data not shown). All neurons in these regions appear to show

immunoreactivity. Although laminin staining has been previously shown in rodent (Yamamoto et al., 1988; Hagg et al., 1989; Tian et al., 1997; Chen and Strickland, 1997; Hager et al., 1998; Grimpe et al., 2002) and human brain (Liesi et al., 2001), we obtained laminin chain-specific antibodies and tested for individual laminin chains to determine their expression pattern in the hippocampus. Of the chains tested, three gave positive staining in the neuronal cell layers of the hippocampus: α5, β1 and γ1 (Fig. 2). The rabbit polyclonal anti-mouse α5 antibody 8948 (Miner et al., 1997) gave strong staining throughout the pyramidal cell layers of the hippocampus and in the dentate gyrus (Fig. 2c, d); this staining appeared to be perinuclear, the significance of which is unknown. On higher magnification, neuronal as well as vascular staining can be observed (Fig. 2d). The rat anti-mouse β1 monoclonal antibody 5A2 (Abrahamson et al., 1989) showed strong staining throughout the neuronal cell layers in CA1, CA3 and dentate gyrus, with all neurons showing immunoreactivity (Fig. 2e, f). A γ1 chain polyclonal antibody (Santa Cruz H-190) also prominently stained neurons throughout the hippocampus (Fig. 2i, j). Interestingly, dendritic staining is also visible as it extends from the cell bodies into the stratum radiatum (Fig. 2j). A vascular pattern could also be observed for the α5 (Fig. 2d), β1 and γ1 chains (data not shown), and was more often observed with less tissue fixation. Immunostaining for α1, α2, α3 and α4 chains was also performed, and resulted in a seemingly vascular pattern, with no detectable hippocampal pyramidal neuronal cell layer-associated staining (Fig. 2k–r). The reason why we do not see neuronal staining for α2 laminin as previously reported (Tian et al., 1997) is not clear, but may be due to differences in reagents or protocols. Higher magnification clearly showed the characteristic structure of blood vessels. The extent of vasculature staining varied among these α chains, as the α1 chain (Fig. 2l) appeared to have more limited vascular staining than the other α chains. Some punctuate staining was also notable, which may represent some laminin expression in some sub-populations of neurons or glial cells. Staining of hippocampal sections with control antibody showed no signal for any of the chains (example for α5 shown in Fig. 2g, h). In summary, immunostaining for the laminin chains demonstrates that α5, β1, and γ1 chains show a consistent strong neuronal cell staining pattern, whereas a vascular pattern was observed for the α1, α2, α3 and α4 chains. A summary of all the expression results is shown in Table 3.

Western blotting was performed for laminin α5, β1, and γ1 chains on hippocampal protein extracts to confirm the presence of these laminin chains using biochemical methods, and is shown in Fig. 3. We observed the presence of the laminin chains α5, β1, and γ1 in the mouse hippocampus (Fig. 3, lanes 2, 5, and 8), using lung and kidney protein extracts as positive controls for these chains. α5 antibody does not cross-react with any of the chains of the laminin-1 protein (data not shown), nor does the omission of primary antibody incubation yield any non-specific color reaction (data not shown). In addition, western blotting using these laminin chain-specific antibodies helped to confirm the specificity of the antibodies as used for immunohistochemistry.

Table 3. Summary of laminin chain expression in hippocampus

Laminin chain	RT-PCR	IHC	Localization
α1	+	+	V
α2	+	+	V
α3	+	+	V
α4	+	+	V
α5	+	+	N, V
β1	+	+	N, V
β2	±	ND	
β3	±	ND	
γ1	+	+	N, V
γ2	±	ND	
γ3	±	ND	

IHC, immunohistochemistry; N, neuronal pattern; ND, not detected; V, vascular pattern; +, detected; ± weak/non-detectable.

Cellular localization of laminin α5, β1, and γ1 chain mRNA in the mouse hippocampus

The above results showed that laminin α5, β1 and γ1 are associated with neurons in the mouse hippocampus. In order to determine the cells that synthesize these proteins, we performed RNA *in situ* hybridization for these three chains. Using digoxigenin-labeled RNA probes to the different laminin chains, we observed significant staining within the stratum pyramidal of the hippocampus for these three chains (Fig. 4a, d, g). On

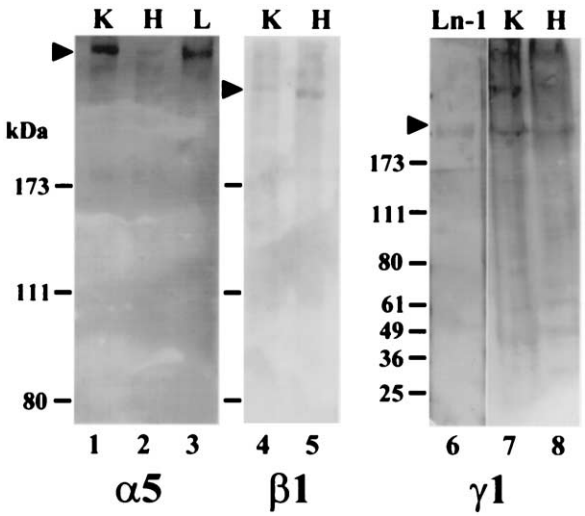

Fig. 3. Western blotting for laminin chains. Tissue extracts (100 μg) from hippocampus (H), lung (L) and kidney (K) were run by SDS-PAGE, blotted onto PVDF or nitrocellulose membranes and stained with anti-laminin α5 (lanes 1–3), β1 (lanes 4–5), γ1 (lanes 6–8) antibodies, respectively, peroxidase-labeled secondary antibody, and chromogenic detection as described in Experimental Procedures. Purified laminin-1 (Ln-1) was run in parallel to confirm correct molecular weight. Bands of the expected sizes are detected in the hippocampal extracts (arrowheads) for α5 (~400 kDa), β1 (~200 kDa), and γ1 (~200 kDa), with lung and kidney extracts serving as positive controls. Molecular weight markers are indicated.

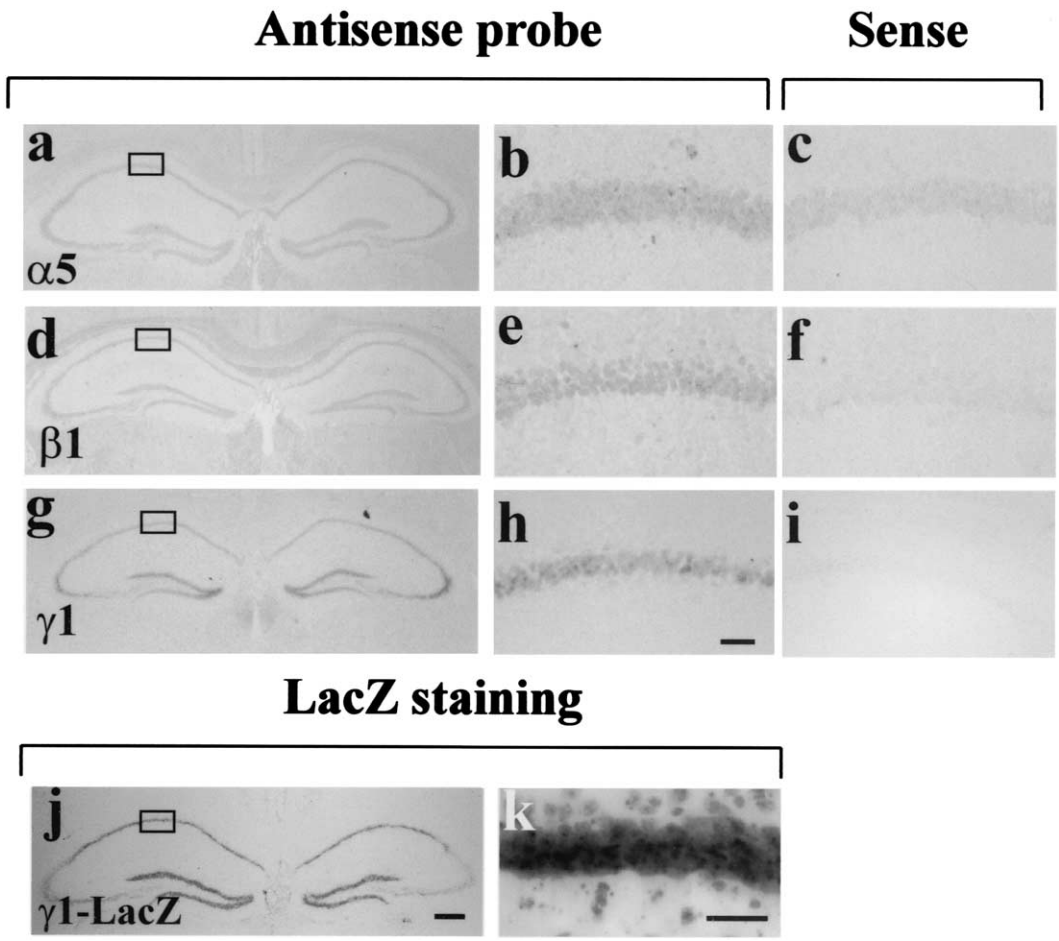

Fig. 4. mRNA localization of laminin α5, β1 and γ1 chains in the mouse hippocampus. Sense and antisense probes were synthesized from cloned mouse laminin α5, β1, and γ1 chain-specific RT-PCR products, using T3 and T7 *in vitro* transcription with DIG-labeled nucleotides. Fresh frozen cryostat coronal sections from mouse hippocampus were then used for *in situ* hybridization, and detected using AP-conjugated anti-DIG antibody and NBT/BCIP. Panels a, d, and g show hippocampal staining on coronal sections using antisense probe; panels b, e, and h show higher magnification of the CA1 region with antisense probe (see boxes), showing neuronal expression of α5 (panels a and b), β1 (panels d and e) and γ1 (panels g and h) chains. Sense probes showed little or no signal (c, f, and i). LacZ-γ1 gene trap mouse brain sections were stained using conventional β-gal staining methods to visualize the expression pattern of the γ1 chain (panels j and k), with counterstaining by nuclear fast red. Upon higher magnification of CA1 (panel k), a strong staining pattern is seen to correlate with the pyramidal cell layer (the β-gal staining is shown by the dark punctate staining; the other staining is the cells stained with fast red). Scale bar in j=400 μm and applies to a, d, g, j; scale bar in h=50 μm and applies to b, c, e, f, h, i. Scale bar in k=50 μm.

higher magnification of the CA1 region, the pattern of the staining was coincident with the neuronal cell layer (Fig. 4b, e, h). All sense probes showed no significant staining (Fig. 4c, f, i).

The expression pattern for the γ1 chain was further confirmed using a LacZ-γ1 fusion gene trap mouse (Mitchell et al., 2001). This mouse contains an insertion into a γ1 gene intron that produces LacZ protein fused to a fragment of the γ1 chain. The homozygous insertion is lethal, but heterozygotes are viable and healthy, and in these mice γ1 expression can be easily analyzed by using β-galactosidase histological staining methods. We observed an expression pattern identical to that seen with *in situ* hybridization, namely, strong staining throughout the hippocampal formation, in the CA and dentate gyrus neuronal layers (Fig. 4j, k). The punctate pattern is often seen with positive β-gal staining, and as the fusion protein is not secreted (it mislocalizes to endoplasmic reticulum and lysosomes), the staining pattern is reflective of the RNA message (Kelley et al., 1994). We also noted that in the *in situ* hybridization for these laminin chains, there are some cellular hybridization signals outside the pyramidal neuronal cell layers (Fig. 4b, e, h), which may represent expression from sub-populations of neurons or glia.

In summary, *in situ* hybridization indicates a neuronal expression pattern for the α5, β1, and γ1 laminin chains.

Early loss of laminin chains α5, β1 and γ1 during excitotoxicity

Intrahippocampal injection of kainate was used to investigate which neuronal laminin chains are affected at early timepoints following an acute excitotoxic injury. We exam-

ined neuronal survival at several timepoints following kainate injection using Cresyl Violet (Nissl) (Fig. 5a–c) and Fluoro Jade B staining (Fig. 5d–f). By Cresyl Violet staining, no significant neuronal loss is observed at 2 h (Fig. 5b) following injection. Significant degeneration of the CA1 pyramidal cell layer was detected by 24 h after kainate injection (Fig. 5c), as assessed by the shrunken pycnotic nuclei along the previously highly populated CA1, and is complete by 48 h (data not shown). The uninjected contralateral control hippocampus (Fig. 5a) is shown for comparison (Fig. 5a). As a more sensitive assay for neuronal degeneration, we utilized Fluoro Jade B, a stable, nontoxic anionic fluorescent derivative that can sensitively detect degenerating neurons by staining cell bodies, axons and dendrites (Schmued et al., 2000). We observed no discernible cellular staining at 2 h after kainate injection (Fig. 5e) while significant staining was visible at 24 h (Fig. 5f). The positively stained degenerating neurons and their processes are also noticeable in the CA3 (data not shown), and in the hilar region of the dentate gyrus (data not shown). The uninjected contralateral side showed no significant staining at all timepoints tested (representative section shown in Fig. 5d). Interestingly, although there is no clear cellular-like staining at times before 16 h (data not shown), a diffuse hazy staining is visible in the CA1 region, where the most degeneration will eventually occur, as is visible at 2 h after injection (Fig. 5e).

Laminin immunoreactivity was then performed for laminin-1 and the laminin chains $\alpha5$, $\beta1$, and $\gamma1$, and we observed very early loss of laminin immunoreactivity, usually within one to 2 h (Fig. 5g–r). Staining with the pan-laminin-1 antibody shows early loss of laminin-1 immunoreactivity (see Chen and Strickland, 1997), with significant loss in the CA1 hippocampal region by 2 h after excitotoxin injection (Fig. 5h) when compared with CA1 regions in the contralateral sides (Fig. 5g). Some loss is already detected by 1 h after kainate injection (data not shown). We then looked for the presence of $\alpha5$ chain, which shows a similar loss of immunoreactivity before detectable neuronal death (Fig. 5j–l). By 2 h after kainate injection, very little $\alpha5$ immunoreactivity is visible, compared with the contralateral sides (compare Fig. 5j and k). $\beta1$ chain immunohistochemistry was also examined following kainate injection (Fig. 5m–o). Compared with the normal staining in the hippocampal CA1 region (Fig. 5m), immunoreactivity for laminin $\beta1$ was lost by 2 h (Fig. 5n), and remained absent through the 48 h timepoint (Fig. 5o). Laminin $\gamma1$ immunoreactivity was also lost very early after kainate injection. By the 2 h timepoint we detected diminished neuronal staining for $\gamma1$ chain (Fig. 5q), as compared with uninjected control hippocampus (Fig. 5p), and the $\gamma1$ chain was completely absent at 48 h after kainate injection (Fig. 5r). Our data indicate that loss of the laminin chains $\alpha5$, $\beta1$, and $\gamma1$ occurs before neuronal degeneration, and correlates spatially and temporally with subsequent neuronal death after excitotoxic injury. In contrast, vascular laminin immunoreactivity is not decreased following excitotoxic damage.

Following intrahippocampal kainate injection, a tPA/plasmin cascade is upregulated and promotes laminin degradation and neuronal death. To confirm that our observations of specific laminin degradation are consistent with the pathway of tPA and plasmin(ogen), we performed similar analyses on tPA-deficient mice, and our results are shown in Fig. 6. Consistent with previous reports, tPA-deficient neurons were resistant to intrahippocampal kainate injection, as assessed by Cresyl Violet (Fig. 6a, b, e, f) and Fluoro Jade B staining (data not shown). We then tested the same panel of neuronal laminin antibodies as above, and found no significant loss of laminin immunoreactivity at 24 h following kainate injection (Fig. 6d, h, j, l). Compared with the contralateral uninjected controls, no detectable laminin degradation was observed for laminin-1 (Fig. 6i, j), $\alpha5$ (Fig. 6c, d), $\beta1$ (Fig. 6g, h), and $\gamma1$ (Fig. 6k, l). Thus, tPA is required for the degradation of laminin chains $\alpha5$, $\beta1$, and $\gamma1$ during kainate induced excitotoxicity.

DISCUSSION

Whereas most tissues, such as the kidney and intestine, possess a very organized basement membrane, the organization of the ECM in the CNS is unusual (Ruoslahti, 1996). Laminins appear to be important components of the ECM in the CNS, and are critical for neuronal survival, as loss of a laminin foundation can predispose neurons to excitotoxic death (Chen and Strickland, 1997). Here we present evidence that multiple laminin chains are expressed in the mouse hippocampus. Although by RT-PCR most of the laminin chains are detectable in the mouse hippocampus, by immunostaining only $\alpha5$, $\beta1$, and $\gamma1$ are associated with neurons. *In situ* hybridization confirmed that mRNAs for $\alpha5$, $\beta1$, and $\gamma1$ are present in neurons, indicating that the neurons synthesize these chains. The other laminin chains detected by RT-PCR, but not present in the neuronal layers by immunostaining or *in situ* hybridization, may represent vascular expression. Combining all our evidence, we suggest that laminin-10 ($\alpha5$-$\beta1$-$\gamma1$) is formed by hippocampal neurons.

To establish a temporal correlation between laminin degradation and subsequent neuronal loss in excitotoxicity, we investigated laminin expression following kainate injection. We found that after excitotoxic injury, the loss of immunoreactivity of multiple laminin chains precedes neuronal death and is spatially coincident with regions that will eventually show neurodegeneration. We observe a loss of immunoreactivity for $\alpha5$, $\beta1$ and $\gamma1$, at very early time points following an injection of kainate into the hippocampus. Our data suggest that laminin-10 ($\alpha5$-$\beta1$-$\gamma1$) is degraded during kainate-induced excitotoxicity.

Laminin-10 possesses interesting properties. It has recently been found that laminin-10 prevents apoptosis induced by serum depletion through activation of protein kinase B/Akt (Gu et al., 2002). It has also been shown that laminin-10 along the endothelial cell basement membrane poses a barrier to T cell infiltration in experimental autoimmune encephalomyelitis (Sixt et al., 2001). In addition, laminin-10 can promote cell migration, mediated intracellularly by Rac, a member of the Rho family of GTPases (Gu et al., 2001). Laminin-10 appears to have a strong

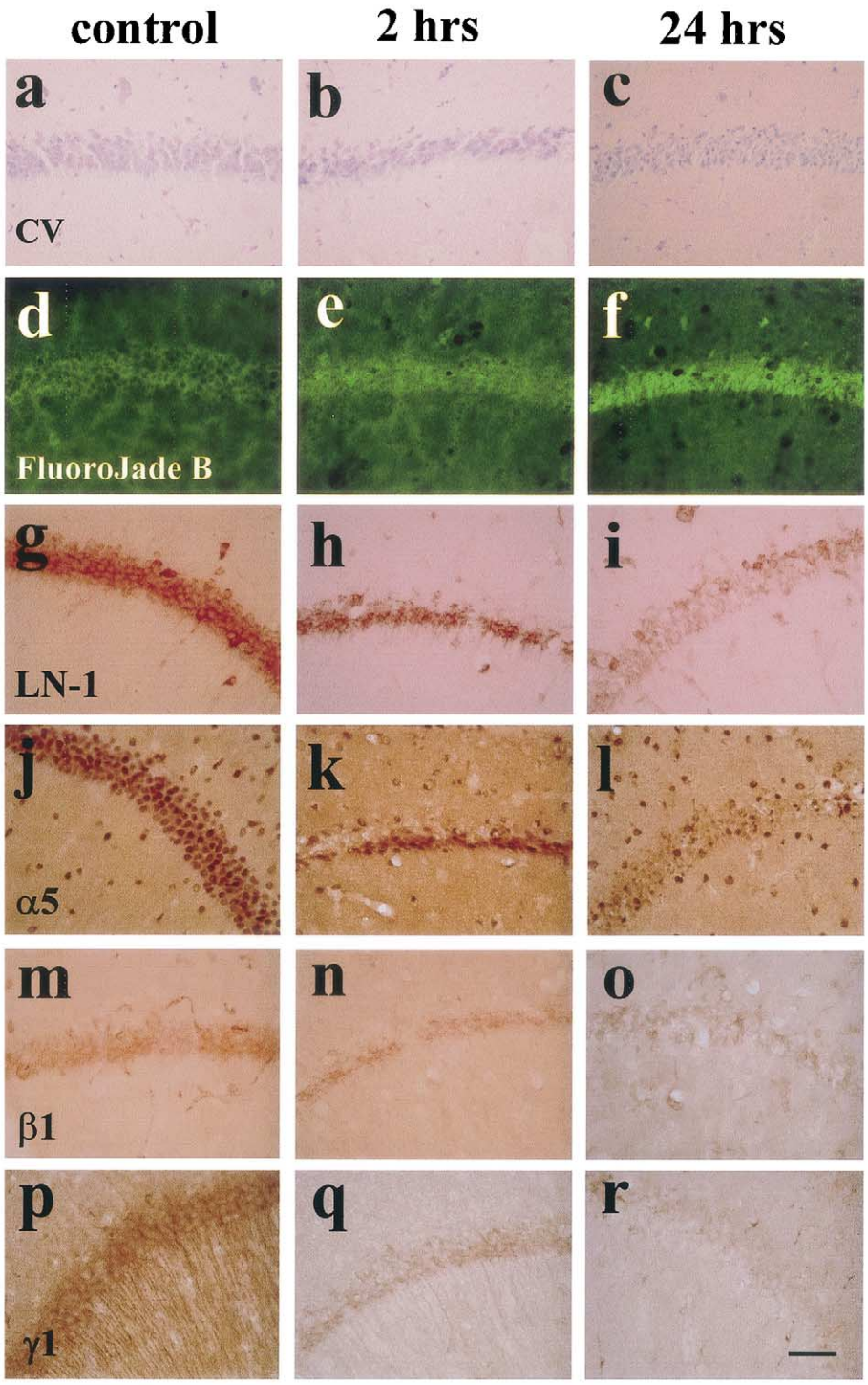

Fig. 5. Degradation of laminin α5, β1 and γ1 chains prior to neuronal degeneration following kainate injection. Stereotaxic injection of kainate was performed and at various time points, mice were killed, and fixed coronal brain sections were processed for staining using Cresyl Violet (Nissl) (panels a–c), the fluorescein derivative Fluoro Jade B, which sensitively detects degenerating neurons and their processes (panels d–f), or laminin immunostaining using affinity purified pan-laminin-1 (g–i), polyclonal α5 (j–l), monoclonal β1 (m–o), and polyclonal γ1 (p–r) antibodies. Sections are shown from contralateral sides of the hippocampus (left-side panels, a, d, g, j, m, p), and kainate-injected sides of the hippocampus at 2 h (middle panels, b, e, h, k, n, q) and 24 h following kainate injection (right-side panels, c, f, i, l, o, r). With Cresyl Violet staining, no significant neuronal death is observed until 24 h following intrahippocampal kainate injection (c), compared with the contralateral sides of the hippocampus (a). Fluoro Jade B staining shows no clear neuronal-like staining until 24 h after kainate injection (f). Significant loss of laminin-like immunostaining is seen for laminin-1 (h), and the laminin chains α5 (k), β1 (n), and γ1 (q) by 2 h after hippocampal kainate injection, compared with control. CV, Cresyl Violet. Scale bar=50 μm.

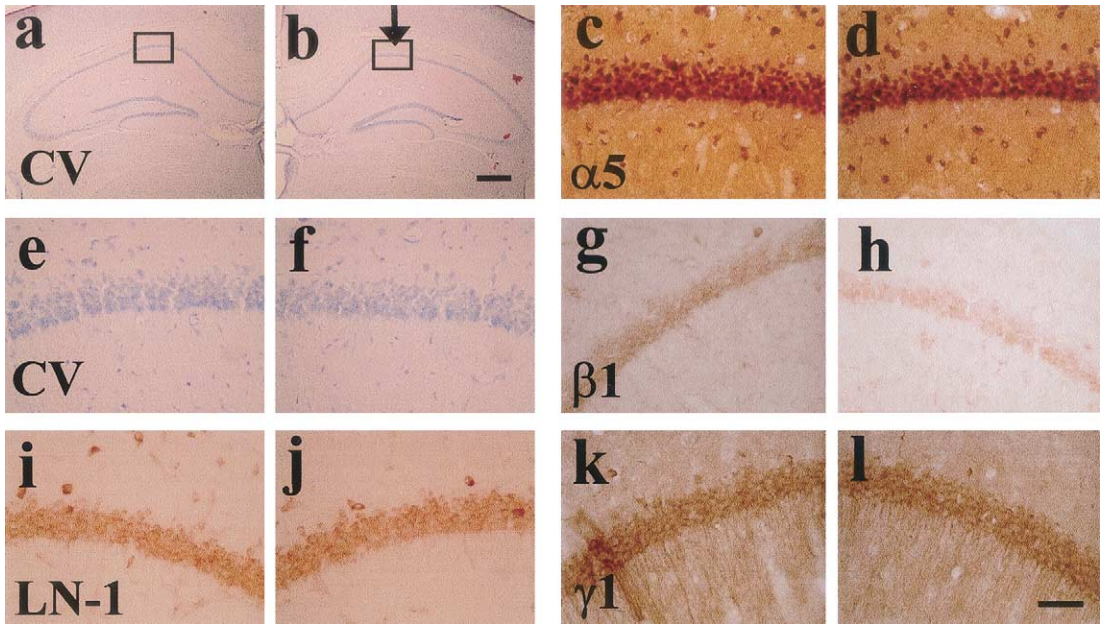

Fig. 6. Laminins α5, β1 and γ1 chains are not degraded in tPA-deficient mice following kainate injection. Twenty-four hours after intrahippocampal kainate injection, tPA-deficient mice were killed. Fixed coronal sections were prepared and stained using Cresyl Violet (a, b, e, f), affinity purified pan-laminin-1 (i and j), polyclonal α5 (c and d), monoclonal β1 (g and h), and polyclonal γ1 (k and l) antibodies. Compared with the contralateral side of the hippocampus (a, c, e, g, i, k), kainate injected sections (24 h; panels b, d, f, h, j, l) show no significant neuronal loss by Cresyl Violet staining (high magnification of boxed CA1 region in panels a and b is shown in e and f), and no significant loss of immunoreactivity to laminin-1, and laminin chains α5, β1 and γ1 chains. CV, Cresyl Violet; arrow, kainate injection site. Scale bar in b=400 μm and applies to a, b. Scale bar in l=50 μm and applies to c–l.

capacity as a barrier and a stratum for cell motility, depending on cell type. An important question for the future is to identify the laminin receptors that take part in initiating the cell death process following loss of laminin-neuron interaction. Several integrins and non-integrin class molecules have been identified to be laminin receptors, and the integrin expression profile has been characterized in the hippocampus (Pinkstaff et al., 1999). Laminin-10 has been shown to bind to several integrins, including α3β1 and α6β1 (Kikkawa et al., 2000), of which α3β1 is expressed in the hippocampal pyramidal cell layers (Pinkstaff et al., 1999), and is one candidate for the neuronal cell surface receptor for laminin.

Laminin proteolysis has been shown to occur in multiple contexts, both physiological and pathological (Ghosh and Stack, 2000). There are many examples of laminin proteolytic modification affecting cell function, particularly by plasmin or by the matrix metalloproteinase (MMP) family of extracellular proteases. For example, cleavage of the laminin γ2 chain by MMP-2 leads to an increase in keratinocyte migration (Giannelli et al., 1997), and plasmin-mediated cleavage of laminin α3 affects hemidesmosome expression and alters cell adhesion (Goldfinger et al., 1998).

The process observed following kainate injection, however, is different in that it appears to be complete degradation. We observe no obvious degradation intermediates in Western blot analyses of kainate-injected samples (Chen and Strickland, 1997; JA Indyk and S Strickland, unpublished results). In addition, immunostaining results for the laminin chains show complete loss of immunoreactivity in the affected areas of the hippocampal CA regions. One possibility is that the degradation intermediates could be further degraded by other proteases, which are also increased after brain injury (Yong et al., 2001). We have observed that purified laminin-1 could be digested to several small fragments with plasmin. However by western blotting, only one fragment could be detected by affinity-purified anti-laminin-1 polyclonal antibody (Sigma) (ZL Chen and S Strickland, unpublished results). Nonetheless, an alternative explanation is that the laminin degradation products are undetectable by our methods, and that once laminin cleavage occurs, the antibodies cannot recognize the resulting fragments. This hypothesis leaves open the possibility that cryptic laminin peptides may be released during excitotoxic injury.

It is intriguing to speculate that the effects shown here after excitotoxic injury are merely a pathological amplification of that seen in normal physiological processes in the hippocampus, such as learning and memory. Indeed, this idea is consistent with the results seen using an experimental model for learning and memory, where plasmin-mediated laminin cleavage regulates the maintenance of long term potentiation (Nakagami et al., 2000). Although laminin cleavage occurs, it is not severe enough to affect neuronal attachment to the ECM and cause the subsequent neuronal loss. Intrahippocampal kainate injection, however, may be inducing a greater magnitude of depolarization, and subsequent loss of laminin-10 does lead to neurodegeneration. In accordance with these results, we suggest that in order to cause neuronal death, two events

are necessary: a depolarization phenomenon of sufficient severity, and a loss of cell-matrix attachment. Neither alone is sufficient to cause neurodegeneration, since kainate injection is not sufficient for neuronal death in the absence of tPA or plasmin(ogen) (Tsirka et al., 1996, 1997). In addition, incubation of hippocampal slice cultures with active plasmin (even to the extent that laminin immunoreactivity is completely lost) does not lead to neuronal death (Nakagami et al., 2000). Furthermore, without a depolarization event of sufficient magnitude (e.g. kainate injection), overexpression of tPA is not toxic to hippocampal neurons (Madani et al., 1999), nor is infusion of polyclonal laminin antibodies (Chen and Strickland, 1997).

A better understanding of the degradation process and how it affects neurons is also important. Since laminins contain multiple biologically active domains and sequences (Yamada, 1991; Aumailley and Smyth, 1998; Ghosh and Stack, 2000), laminin cleavage may liberate peptide domains that possess unique activities but are not exposed in the intact laminin trimer. Indeed, a cryptic sequence present in cleaved laminin α1 chain (but not intact laminin) promotes protein kinase C-dependent activation of MAP kinase in macrophages (Khan et al., 2000). Biologically active sequences or domains are contained within the laminin α5, β1 and/or γ1 chains, and these may affect neuronal survival if altered or removed via proteolysis, and this possibility merits further investigation. Moreover, the tPA/plasmin cascade can activate MMPs, many of which are upregulated during brain injury, and are known to degrade laminin (Yong et al., 2001). Our initial results, however, show no significant difference in neuronal death following kainate injection in MMP-7 or MMP-9 deficient mice, which suggests that these MMPs do not participate in excitotoxic neuronal degeneration (JA Indyk, Z-L Chen and S Strickland, unpublished results). Still, other MMPs deserve a closer examination, as these proteases have shown potential roles in CNS pathology, such as Alzheimer's disease, multiple sclerosis, ischemic stroke and malignant glioma (reviewed by Yong et al., 2001).

By identifying the specific laminin chains and isoforms expressed in the mouse brain, we can better define the molecular composition of the ECM and understand how the ECM influences neuronal function. In addition, the continued study of the extracellular events and the molecular pathways that occur during neurodegeneration will help lead to new developments in the quest for better clinical outcomes in the treatment of neurological disease (Sanes et al., 1990; Sorokin et al., 1997).

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REFERENCES

Abrahamson DR, Irwin MH, St John PL, Perry EW, Accavitti MA, Heck LW, Couchman JR (1989) Selective immunoreactivities of kidney basement membranes to monoclonal antibodies against laminin: localization of the end of the long arm and the short arms to discrete microdomains. J Cell Biol 109:3477–3491.

Aumailley M, Smyth N (1998) The role of laminins in basement membrane function. J Anat 193 Pt 1:1–21.

Bernier S, Utani A, Sugiyama S, Doi T, Polistina C, Yamada Y (1995) Cloning and expression of laminin α2 chain (M-chain) in the mouse. Matrix Biol 14:447–455.

Burgeson RE, Chiquet M, Deutzmann R, Ekblom P, Engel J, Kleinman H, Martin GR, Meneguzzi G, Paulsson M, Sanes J, et al. (1994) A new nomenclature for the laminins. Matrix Biol 14:209–211.

Carmeliet P, Schoonjans L, Kieckens L, Ream B, Degen J, Bronson R, De Vos R, van den Oord JJ, Collen D, Mulligan RC (1994) Physiological consequences of loss of plasminogen activator gene function in mice. Nature 368:419–424.

Chen ZL, Strickland S (1997) Neuronal death in the hippocampus is promoted by plasmin-catalyzed degradation of laminin. Cell 91: 917–925.

Chen ZL, Yoshida S, Kato K, Momota Y, Suzuki J, Tanaka T, Ito J, Nishino H, Aimoto S, Kiyama H, et al. (1995) Expression and activity-dependent changes of a novel limbic-serine protease gene in the hippocampus. J Neurosci 15:5088–5097.

Coyle JT, Molliver ME, Kuhar MJ (1978) In situ injection of kainic acid: a new method for selectively lesioning neural cell bodies while sparing axons of passage. J Comp Neurol 180:301–323.

Durkin M, Gautam M, Loechel F, Sanes JR, Merlie JP, Albrechtsen R, Wewer UM (1996) Structural organization of the human and mouse laminin B2 chain genes, and alternative splicing at the 5' end of the human transcript. J Biol Chem 271:13407–13416.

Edgar D (1985) Nerve growth factors and molecules of the extracellular matrix in neuronal development. J Cell Sci Suppl 3:107–113.

Ernsberger U, Edgar D, Rohrer H (1989) The survival of early chick sympathetic neurons in vitro is dependent on a suitable substrate but independent of NGF. Dev Biol 135:250–262.

Frisch SM, Francis H (1994) Disruption of epithelial cell-matrix interactions induces apoptosis. J Cell Biol 124:619–626.

Frisch SM, Ruoslahti E (1997) Integrins and anoikis. Curr Opin Cell Biol 9:701–706.

Galliano M, Aberdam D, Aguzzi A, Ortonne JP, Meneguzzi G (1995) Cloning and complete primary structure of the mouse laminin α3 chain. J Biol Chem 270:21820–21826.

Ghosh S, Stack MS (2000) Proteolytic modification of laminins: functional consequences. Microsc Res Tech 51:238–246.

Giannelli G, Falk-Marzillier J, Schiraldi O, Stetler-Stevenson WG, Quaranta V (1997) Induction of cell migration by matrix metalloprotease-2 cleavage of laminin-5. Science 277:225–228.

Goldfinger LE, Stack MS, Jones JC (1998) Processing of laminin-5 and its functional consequences: Role of plasmin and tissue-type plasminogen activator. J Cell Biol 141:255–265.

Grimpe B, Dong S, Doller C, Temple K, Malouf AT, Silver J (2002) The critical role of basement membrane-independent laminin gamma 1 chain during axon regeneration in the CNS. J Neurosci 22:3144–3160.

Gu J, Fujibayashi A, Sumida KM, Sekiguchi K, Gu J, Fujibayashi A, Sumida KM, Sekiguchi K (2002) Laminin-10/11 and fibronectin differentially prevent apoptosis induced by serum removal via PI 3-kinase/Akt- and MEK1/ERK-dependent pathways. J Biol Chem 277:19922–19928.

Gu J, Sumida Y, Sanzen N, Sekiguchi K (2001) Laminin-10/11 and fibronectin differentially regulate integrin-dependent Rho and Rac activation via p130(Cas)-CrkII-DOCK180 pathway. J Biol Chem 276:27090–27097.

Hager G, Pawelzik H, Kreutzberg GW, Zieglgansberger W (1998) A peptide derived from a neurite outgrowth-promoting domain on the

gamma 1 chain of laminin modulates the electrical properties of neocortical neurons. Neuroscience 86:1145–1154.

Hagg T, Muir D, Engvall E, Varon S, Manthorpe M (1989) Laminin-like antigen in rat CNS neurons: Distribution and changes upon brain injury and nerve growth factor treatment. Neuron 3:721–732.

Hagg T, Portera-Cailliau C, Jucker M, Engvall E (1997) Laminins of the adult mammalian CNS; laminin-alpha2 (merosin M-) chain immunoreactivity is associated with neuronal processes. Brain Res 764:17–27.

Hansson O, Petersén A, Leist M, Nicotera P, Castilho RF, Brundin P (1999) Transgenic mice expressing a Huntington's disease mutation are resistant to quinolinic acid-induced striatal excitotoxicity. Proc Natl Acad Sci USA 96:82727–88732.

Iivanainen A, Morita T, Tryggvason K (1999) Molecular cloning and tissue-specific expression of a novel murine laminin gamma3 chain. J Biol Chem 274:14107–14111.

Kelley KA, Friedrich VL Jr, Sonshine A, Hu Y, Lax J, Li J, Drinkwater D, Dressler H, Herrup K (1994) Expression of Thy-1/lacZ fusion genes in the CNS of transgenic mice. Brain Res Mol Brain Res 24:261–274.

Khan KM, Falcone DJ (2000) Selective activation of MAPK(erk1/2) by laminin-1 peptide alpha1: Ser(2091)-Arg(2108) regulates macrophage degradative phenotype. J Biol Chem 275:4492–4498.

Kikkawa Y, Sanzen N, Fujiwara H, Sonnenberg A, Sekiguchi K (2000) Integrin binding specificity of laminin-10/11: Laminin-10/11 are recognized by alpha 3 beta 1, alpha 6 beta 1 and alpha 6 beta 4 integrins. J Cell Sci 113 Pt 5:869–876.

Libby RT, Champliaud MF, Claudepierre T, Xu Y, Gibbons EP, Koch M, Burgeson RE, Hunter DD, Brunken WJ (2000) Laminin expression in adult and developing retinae: Evidence of two novel CNS laminins. J Neurosci 20:6517–6528.

Liesi P, Fried G, Stewart RR (2001) Neurons and glial cells of the embryonic human brain and spinal cord express multiple and distinct isoforms of laminin. J Neurosci Res 64:144–167.

Liu J, Mayne R (1996) The complete cDNA coding sequence and tissue-specific expression of the mouse laminin alpha 4 chain. Matrix Biol 15:433–437.

Luckenbill-Edds L (1997) Laminin and the mechanism of neuronal outgrowth. Brain Res Rev 23:1–27.

Lukashev ME, Werb Z (1998) ECM signalling: Orchestrating cell behaviour and misbehaviour. Trends Cell Biol 8:437–441.

Madani R, Hulo S, Toni N, Madani H, Steimer T, Muller D, Vassalli J-D (1999) Enhanced hippocampal long-term potentiation and learning by increased neuronal expression of tissue-type plasminogen activator in transgenic mice. EMBO J 18:3007–3012.

Miner J, Lewis RM, Sanes JR (1995) Molecular cloning of a novel laminin chain, α5, and widespread expression in adult mouse tissues. J Biol Chem 270:28523–28526.

Miner JH, Cunningham J, Sanes JR (1998) Roles for laminin in embryogenesis: Exencephaly, syndactyly, and placentopathy in mice lacking the laminin alpha5 chain. J Cell Biol 143:1713–1723.

Miner JH, Patton BL, Lentz SI, Gilbert DJ, Snider WD, Jenkins NA, Copeland NG, Sanes JR (1997) The laminin α chains: Expression, developmental transitions, and chromosomal locations of α1-5, identification of heterotrimeric laminins 8-11, and cloning of a novel α3 isoform. J Cell Biol 137:685–701.

Mitchell KJ, Pinson KI, Kelly OG, Brennan J, Zupicich J, Scherz P, Leighton PA, Goodrich LV, Lu X, Avery BJ, Tate P, Dill K, Pangilinan E, Wakenight P, Tessier-Lavigne M, Skarnes WC (2001) Functional analysis of secreted and transmembrane proteins critical to mouse development. Nat Genet 28:241–249.

Miyagoe Y, Hanaoka K, Nonaka I, Hayasaka M, Nabeshima Y, Arahata K, Takeda S (1997) Laminin alpha2 chain-null mutant mice by targeted disruption of the Lama2 gene: A new model of merosin (laminin 2)-deficient congenital muscular dystrophy. FEBS Lett 415: 33–39.

Nakagami Y, Abe K, Nishiyama N, Matsuki N (2000) Laminin degradation by plasmin regulates long-term potentiation. J Neurosci 20: 2003–2010.

Niimi T, Kumagai C, Okano M, Kitagawa Y (1997) Differentiation-dependent expression of laminin-8 (α4β1γ1) mRNAs in mouse 3T3-L1 adipocytes. Matrix Biol 16:223–230.

Noakes PG, Gautam M, Mudd J, Sanes JR, Merlie JP (1995) Aberrant differentiation of neuromuscular junctions in mice lacking s-laminin/laminin beta 2. Nature 374:258–262.

Patton BL, Chiu AY, Sanes JR (1998) Synaptic laminin prevents glial entry into the synaptic cleft. Nature 393:698–701.

Patton BL, Cunningham JM, Thyboll J, Kortesmaa J, Westerblad H, Edstrom L, Tryggvason K, Sanes JR (2001) Properly formed but improperly localized synaptic specializations in the absence of laminin alpha-4. Nat Neurosci 4:597–604.

Pinkstaff JK, Detterich J, Lynch G, Gall C (1999) Integrin subunit gene expression is regionally differentiated in adult brain. J Neurosci 19:1541–1556.

Powell SK, Kleinman HK (1997) Neuronal laminins and their cellular receptors. Int J Biochem Cell Biol 29:401–414.

Ruoslahti E (1996) Brain extracellular matrix. Glycobiology 6:489–492.

Ryan MC, Lee K, Miyashita Y, Carter WG (1999) Targeted disruption of the LAMA3 gene in mice reveals abnormalities in survival and late stage differentiation of epithelial cells. J Cell Biol 145:1309–1323.

Sanes JR, Engvall E, Butkowski R, Hunter DD (1990) Molecular heterogeneity of basal laminae: Isoforms of laminin and collagen IV at the neuromuscular junction and elsewhere. J Cell Biol 111:1685–1699.

Sanford LP, Ormsby I, Gittenberger-de Groot AC, Sariola H, Friedman R, Boivin GP, Cardell EL, Doetschman T (1997) TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes. Development 124:2659–2670.

Sasaki M, Kato S, Kohno K, Martin GR, Yamada Y (1987a) Sequence of the cDNA encoding the laminin B1 chain reveals a multidomain protein containing cysteine-rich repeats. Proc Natl Acad Sci USA 84:935–939.

Sasaki M, Yamada Y (1987b) The laminin B2 chain has a multi domain homologous to the B1 chain. J Biol Chem 262:17111–17117.

Sasaki M, Kleinman HK, Huber H, Deutzmann R, Yamada Y (1988) Laminin, a multi-domain protein: The A chain has a unique domain and homology with the basement membrane proteoglycan and laminin B chains. J Biol Chem 263:16536–16544.

Schmued LC, Hopkins KJ (2000) Fluoro-Jade B: A high affinity fluorescent marker for the localization of neuronal degeneration. Brain Res 874:123–130.

Sewry CA, Uziyel Y, Torelli S, Buchanan S, Sorokin L, Cohen J, Watt DJ (1998) Differential labelling of laminin alpha 2 in muscle and neural tissue of dy/dy mice: Are there isoforms of the laminin alpha 2 chain? Neuropathol Appl Neurobiol 24:66–72.

Sixt M, Engelhardt B, Pausch F, Hallmann R, Wendler O, Sorokin LM (2001) Endothelial cell laminin isoforms, laminins 8 and 10, play decisive roles in T cell recruitment across the blood-brain barrier in experimental autoimmune encephalomyelitis. J Cell Biol 153:933–946.

Smyth N, Vatansever HS, Murray P, Meyer M, Frie C, Paulsson M, Edgar D (1999) Absence of basement membranes after targeting the LAMC1 gene results in embryonic lethality due to failure of endoderm differentiation. J Cell Biol 144:151–160.

Sorokin LM, Pausch F, Frieser M, Kroger S, Ohage E, Deutzmann R (1997) Developmental regulation of the laminin alpha5 chain suggests a role in epithelial and endothelial cell maturation. Dev Biol 189:285–300.

Sugiyama S, Utani A, Yamada S, Kozak CA, Yamada Y (1995) Cloning and expression of the mouse laminin gamma 2 (B2t) chain, a subunit of epithelial cell laminin. Eur J Biochem 228:120–128.

Tian M, Hagg T, Denisova N, Knusel B, Engvall E, Jucker M (1997) Laminin-alpha2 chain-like antigens in CNS dendritic spines. Brain Res 764:28–38.

Tsirka SE, Gualandris A, Amaral DG, Strickland S (1995) Excitotoxin-induced neuronal degeneration and seizure are mediated by tissue plasminogen activator. Nature 377:340–344.

Tsirka SE, Rogove AD, Bugge TH, Degen JL, Strickland S (1997) An extracellular proteolytic cascade promotes neuronal degeneration in the mouse hippocampus. J Neurosci 17:543–552.

Tsirka SE, Rogove AD, Strickland S (1996) Neuronal cell death and tPA. Nature 384:123–124.

Utani A, Kopp JB, Kozak CA, Matsuki Y, Amizuka N, Sugiyama S, Yamada Y (1995) Mouse kalinin B1 (laminin β3 chain): cloning and tissue distribution. Lab Invest 72:300–310.

Werb Z (1997) ECM and cell surface proteolysis: Regulating cellular ecology. Cell 91:439–442.

Xu H, Wu X-R, Wewer WM, Engvall E (1994) Murine muscular dystrophy caused by a mutation in the laminin α2 (Lama2) gene. Nat Genet 8:297–301.

Yamada KM (1991) Adhesive recognition sequences. J Biol Chem 266:12809–12812.

Yamamoto T, Iwasaki Y, Yamamoto H, Konno H, Isemura M (1988) Intraneuronal laminin-like molecule in the central nervous system: Demonstration of its unique differential distribution. J Neurol Sci 84:1–13.

Yong VW, Power C, Forsyth P, Edwards DR (2001) Metalloproteinases in biology and pathology of the nervous system. Nat Rev Neurosci 2:502–511.

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