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CNS injury, γ1 laminin, and its KDI peptide

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ABBREVIATIONS

AD:	Alzheimer's disease
AIF:	Apoptosis-inducing factor
ALS:	Amyotrophic lateral sclerosis
AMPA:	α-3-amino-hydroxy-5-methyl-4-isoxalolepropionate
ATP:	Adenosine triphosphate
BBB:	Blood-brain barrier
BDNF:	Brain derived neurotrophic factor
BM:	Basement membrane
CCA:	Common carotid artery
CNS:	Central nervous system
CNTF:	Ciliary neurotrophic factor
CSPG:	Chondroitin sulphate proteoglycan
DG:	Dentate gyrus
DMEM:	Dulbecco's modified Eagle's medium
DRG:	Dorsal root ganglia
ECM:	Extracellular matrix
EGF:	Epithelial growth factor
FGF:	Fibroblast growth factor
GAG:	Glucosaminoglycan
GFAP:	Glial fibrillary acidic protein
HEK:	Human embryonic kidney
IGF-1:	Insulin growth factor-1
KA:	Kainic acid
KDI:	Lysine, aspartic acid, isoleucine (Lys-Asp-Ile)
LIF:	Leukemia inhibitory factor
MCAO:	Middle cerebral artery occlusion
MS:	Multiple sclerosis
NGF:	Neural growth factor
NMDA:	N-methyl-D-aspartate
NO:	Nitric oxide
NT-3:	Neurotrophin-3
NT-4/5:	Neurotrophin-4/5
NT-6:	Neurotrophin-6
NT-7:	Neurotrophin-7
OPC:	Oligodendrocyte precursor cells
PD:	Parkinson's disease
PNS:	Peripheral nervous system
RT:	Room temperature
SC:	Spinal cord
SCI:	Spinal cord injury
TGF:	Transforming growth factor
TNF:	Tumor necrosis factor
TNFR:	Tumor necrosis factor receptor
tPA:	tissue plasminogen activator

LIST OF ORIGINAL PUBLICATIONS

I

Liebkind, R., Laatikainen, T., and Liesi, P. Is the Soluble KDI-domain of γ1 Laminin a Regeneration Factor for the Adult Mammalian CNS? J Neurosci Res. 73: 637-43, 2003.

II

Wiksten, M., Väänänen, A., Liebkind, R., Rauhala, P. and Liesi, P. The Soluble KDI-domain of γ1 Laminin Protects Adult Hippocampus from Excitotoxicity of Kainic Acid. J Neurosci Res. 78: 411-9, 2004.

III

Möykkynen, T., Liebkind, R., Sjöberg, J., Korpi, ER. and Liesi, P. The Neuroprotective KDI domain of γ1-Laminin is a Universal and Potent Inhibitor of Ionotropic Glutamate Receptors. J Neurosci Res. 81: 797-804, 2005.

IV

Liebkind, R., Tatlisumak, E., Wiksten, M., and Tatlisumak, T. Temporal and Spatial Expression Patterns of Laminins in Ischemic Brain Damage of the Adult Rat. 2008 (Manuscript).

ABSTRACT

The regeneration capacity of the adult mammalian central nervous system (CNS) is limited. Following an insult to the CNS, many environmental factors inhibiting or stimulating neuronal regrowth are activated. Several molecules including laminin have stimulating effects on CNS regeneration. Laminins are essential molecular constituents of all basement membranes of the body. In the CNS, laminins are involved in developmental events, such as neuronal migration and axon guidance, while in the adult CNS they participate in the formation and maintenance of the blood-brain-barrier (BBB) and are involved in trauma reactions of the CNS. Laminin-1 is a large glycoprotein and is composed from three disulfide-bonded subunits, α1, β1, and the γ1 chain. Many neurons express γ1 laminin in the mammalian adult brain; it is critical for hippocampal neuronal survival and is expressed by glial cells after CNS injury. The KDI peptide is derived from γ1 laminin as a neurotrophic peptide.

In this thesis study, we analyzed the role of γ1 laminin and its KDI peptide in different settings of CNS injury. An experimental cell-culture model mimicking a damaged CNS environment showed that the KDI peptide has a stimulating effect on neuronal survival and neurite outgrowth. We analyzed neurons from human embryonic spinal cord, retina, and neocortex, leading to findings that both neuronal survival and neurite outgrowth were stimulated by the soluble KDI peptide in all of these tissues.

A stereotaxic injection of kainic acid into rat hippocampus was done for testing the possible protective effect of the KDI peptide. A preceding injection of the KDI peptide protected both hippocampal and neocortical areas of excitotoxic brain damage caused by kainic acid. Kainic acid alone caused serious tissue damage to the hippocampal and neocortical injection sites.

The KDI peptide showed inhibition of glutamate excitotoxicity by inhibiting kainate, AMPA, and NMDA subclasses of glutamate receptors in patch clamp recordings. Neurons were cultured from human embryonic neocortex and from HEK 293 cells expressing recombinant glutamate subunits. These studies indicate that the KDI peptide of γ1 laminin enhances neuronal survival and protects against glutamate excitotoxicity at least partially through inhibition of various glutamate receptors.

Following middle cerebral artery occlusion leading to focal brain ischemia in rats, various laminins were mapped both spatially and temporally. Laminin-1 was increased in basement membranes and scar-forming extracellular matrix; γ1 laminin was induced earlier than other laminins and expressed by reactive astrocytes. Similarly, the KDI peptide of γ1 laminin was expressed by reactive astrocytes surrounding the ischemic region but detected along major neuronal pathways as well. The different patterns of laminin expression suggest involvement of different laminins in different functions in the pathophysiology of experimental brain ischemia. In general, these studies present novel data on the expression of laminins in the healthy and damaged brain, demonstrate protective effects of the KDI tripeptide of γ1 laminin in cell cultures and in vivo in the brain, and give clues as to the mechanisms of KDI peptide neuroprotection.

INTRODUCTION

THE VULNERABLE CENTRAL NERVOUS SYSTEM

During the past two decades neuroscience has developed greatly. One of the major challenges has been regeneration of the central nervous system (CNS). In the peripheral nervous system (PNS) injured axons often regenerate, but in the CNS, injury is usually permanent. This injury often leads to serious functional deficits and traumatizes the individual both physically and mentally. Current therapies in human CNS injury are primarily based on rehabilitation (Tsai and Tator, 2005), although several molecular and cellular strategies are being tested on animals (Thuret et al., 2006), and there is a desperate need for new treatments.

Until the beginning of the 1980s, it was unclear whether the lack of regeneration capacity in the CNS was from an intrinsic neuronal inability or lack of growth factors. Experiments testing differences in growth environments between the PNS and CNS show that peripheral nerves do not grow in a CNS glial environment (Aguayo et al., 1981), in opposition to CNS neurites that grow through transplanted PNS grafts (David and Aguayo, 1981). These findings show that neurons of the CNS can succeed in regenerating, and the influence of the environmental factors plays a central role. The major physical and molecular structures of inhibition in the spinal cord (SC) at cellular level seem to be the glial scar and myelin. Several molecules have been shown to interact in these inhibitory systems and are further specified later in the text. Focusing on inhibitory and stimulating factors of nerve regeneration after injury has produced many promising cellular and molecular strategies being tested in animal experiments on enhancing neurite outgrowth.

Injury in the CNS results from either trauma or disease. Disease can be of both an acute and chronic nature whereas trauma is always acute. Depending on the nature of damage to the CNS, the mechanisms activated on a cellular and molecular level differ. Acute cerebral ischemia causes early excitotoxicity, inflammation, blood-brain-barrier (BBB) disruption, and brain edema, leading to both programmed apoptosis and passive neuronal necrosis (Dirnagl et al., 1999, Ayata and Ropper, 2002). Spinal cord injury (SCI) can result from various types of injury: in the acute phase, via actual physical trauma, oxi-

dative stress, excitotoxicity, edema, and hemorrhagic toxicity, all leading to functional loss. Secondary processes such as apoptosis, demyelinization, and inflammation further affect and cause deterioration in the SCI (Crowe et al., 1997, Jones et al., 2005). The most common diseases that cause neuronal degeneration in their own specific manner are Alzheimer's disease (AD), stroke, Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), and various bacterial and viral infections. Toxic damage to the CNS can be induced by drugs, alcohol, chemical compounds, or diseases such as acute liver failure. A variety of animal models allow testing of human CNS injuries and diseases (Tatlisumak and Fisher, 2006). Interestingly, various major CNS diseases involve similar injury and repair mechanisms.

Some spontaneous repair and collateral sprouting are evident after SC damage even though restoration of functional loss is poor (Stichel and Muller, 1998). Similarly, new neurons are created in the human CNS, although selectively and mainly in the olfactory bulb, subventricular zone, and hippocampus (Falk and Frisen, 2005). Since these adaptive changes in the CNS are insufficient, CNS damage often leads to permanent and severe functional loss. All mechanisms and interventions that may enhance regeneration in the brain and hence attenuate functional loss are therefore a target of intense research.

REVIEW OF THE LITERATURE

1. EXTRACELLULAR MATRIX AND THE BASEMENT MEMBRANE

The extracellular matrix (ECM), which fills the pericellular space, has been defined as a composition of mainly different glycoproteins and proteoglycans. The basement membrane (BM) is a thin structure considered a specialized ECM and mainly comprises type IV collagens, laminins, perlecan, nidogens, and other molecules (Colognato and Yurchenco, 2000, Yurchenco et al., 2004). The BM separates different cells from their underlying tissues, it provides mechanical stability, and it takes part in cellular functions such as migration, proliferation, and cell survival (Timpl and Brown, 1996). The many functional properties are often linked with molecular interaction of the BM (Yurchenco et al., 2004). Many ECM molecules are thus considered to be BM-associated molecules but are not constituents of the actual BM structure. The term "basement membrane" was used after its appearance in light microscopy, and with electron microscopy its structure can be further distinguished into layers known as the lamina rara externa (or lamina lucida), lamina densa, lamina rara interna, and lamina fibroreticularis (Laurie et al., 1982, Leblond and Inoue, 1989). The term "basal lamina" can serve to describe the first three layers above, but according to today's practice the term BM includes all four layers. Because the basal lamina connects to the lamina fibroreticularis, containing collagen fibrils, it connects to the underlying tissue (Merker, 1994)(Figure 1).

The molecular composition of the ECM can be diverse and therefore provide the BM with many functional abilities. Since the ECM molecules can bind and send signals to surrounding cells, they reflect the functions of the tissues they surround. The ECM takes part in tissue maintenance and is also a dynamic medium for molecular signalling (Yurchenco and Schittny, 1990).

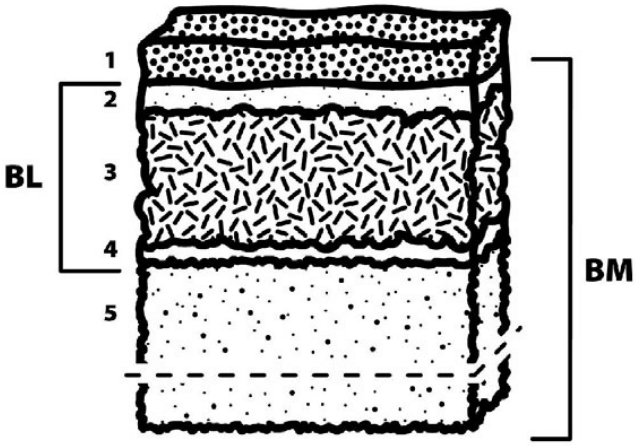

Figure 1. A simplified scheme of basement membrane (BM) structure. 1. Epithelium. 2. Lamina rara externa (or lamina lucida). 3. Lamina densa. 4. Lamina rara interna. 5. Lamina fibroreticularis. Three layers can be designated basal lamina (BL).

2. LAMININS

2.1. Nomenclature

Several classifications allow for classification of laminins and the composition of their chains. The first laminin chains identified were called A, B1, and B2 chains, later renamed as α, β, and γ. A nomenclature was created around the Greek vocabulary to identify the laminin isoforms composed of the α, β, and γ chains (Burgeson et al., 1994). A heterotrimeric isoform of laminin is formed by one chain from each class. Arabic numbers are combined with the Greek letters that describe the chain so that each isoform can be classified in the order of its discovery (Burgeson et al., 1994). For example, laminin-1 would be described as α1β1γ1 and laminin-2 as α2β1γ1, often also with their chain assembly in parenthesis (Table 1).

In 2005 came a new nomenclature, no longer using Greek letters for the composition of the different chains but three Arabic letters representing each chain (Aumailley et al., 2005), and the first number representing the α chain, the second the β chain, and the third number the γ chain. For example, laminin-1 would be called laminin-111, because its chain composition is **α1β1γ1**; laminin-2 is called laminin-211 (Aumailley et al., 2005),

since it is the first laminin described with a variant form of the α chain **α2β1γ1**. According to this system the list goes on. This thesis, however uses the nomenclature created earlier (Burgeson et al., 1994), since the publications presented in this study have used both Greek and Arabic letters to describe laminin chains.

2.2. Structure

Laminin-1 (α1β1γ1) is a cross-shaped glycoprotein with three short arms and one long arm (Engel et al., 1981). It is composed of three polypeptides that consist of several different domains, α1 with nine domains (Sasaki et al., 1988), β1 with seven (Sasaki et al., 1987), and γ1 with six (Sasaki and Yamada, 1987). Laminin-1 was originally purified from the Engelbreth-Holm-Swarm tumor (Timpl et al., 1979), and the structures of the human and mouse laminin-1 (Pikkarainen et al., 1987, Sasaki et al., 1987, Sasaki and Yamada, 1987, Pikkarainen et al., 1988, Nissinen et al., 1991) were found to be compatible with the purified protein (Sasaki et al., 1988). The N-terminus of laminin-1 is composed of the three short arms of the α, β, and γ chains. The N-termini of these chains are closely related and consist of domains III toVI. The long arm of laminin-1, consisting of domains I and II, has a rod-like structure in which the C-terminal of the three chains are combined. A globular G-domain extends to the α1 chain in the C-terminal. Almost in the center of the laminin cross in domain II, and in domain I near the globular C-terminal part, the three polypeptide chains are linked by disulfide bonds (Engel et al., 1981, Beck et al., 1993) (Figure 2).

The three monomeric polypeptide chains forming laminin-1 vary in molecular weight depending on tissue and species as well as on their degree of glycosylation (Engel et al., 1981, Beck et al., 1990, Beck et al., 1993, Burgeson et al., 1994). Laminin-1 is a highly glycosylated protein, meaning that 25 to 30% of its molecular weight is formed by carbohydrate (Knibbs et al., 1989). Laminin-1 also contains many N-glycosylation sites—74 potential sites (Beck et al., 1990)—reported to play a functional role in multiple cellular properties such as neurite outgrowth, tumor cell adhesion, cell migration, and molecular interactions (Dennis et al., 1984, Dean et al., 1990, Chammas et al., 1991).

The short arms in the N-terminal of laminin-1 contain cysteine-rich residues. These residues are closely related to the epidermal growth factor (EGF) (Engel, 1989). In

the β1 and γ1 polypeptide chains two cysteine-rich EGF-like repeats occur in domains III and V (Sasaki et al., 1987, Sasaki and Yamada, 1987). Similarly, in the α1 chain, three cystein-rich chains occur in domains IIIa, IIIb, and V (Sasaki et al., 1988) (Figure 2).

The long arms of the polypeptide chains of laminin-1 form double- and triple-stranded coiled-coil structures and require a short sequence at the C-terminal of each chain (Utani et al., 1994, Utani et al., 1995). There is a low sequence homology of the polypeptide long arms, but they all include repeating sequences of seven residues that allow them to coil around each other (Beck et al., 1990, Beck et al., 1993). The β and γ chains first form a dimeric structure and then follow a trimeric formation with the α chain (Peters et al., 1985, Utani et al., 1994, Utani et al., 1995, Nomizu et al., 1996).

Table 1. Laminin trimers, nomenclature, and chains

Former name	chains	new name
Laminin-1	α1β1γ1	Laminin-111
Laminin-2	α2β1γ1	Laminin-211
Laminin-3	α1β2γ1	Laminin-121
Laminin-4	α2β2γ1	Laminin-221
Laminin-5a	α3Aβ3γ2	Laminin-332
Laminin-5b	α3Bβ3γ2	Laminin-3B32
Laminin-6	α3β1γ1	Laminin-311
Laminin-7	α3β2γ1	Laminin-321
Laminin-8	α4β1γ1	Laminin-411
Laminin-9	α4β2γ1	Laminin-421
Laminin-10	α5β1γ1	Laminin-511
Laminin-11	α5β2γ1	Laminin-521
Laminin-12	α2β1γ3	Laminin-213
Laminin-14	α4β2γ3	Laminin-423
Laminin-15	α5β2γ3	Laminin-523

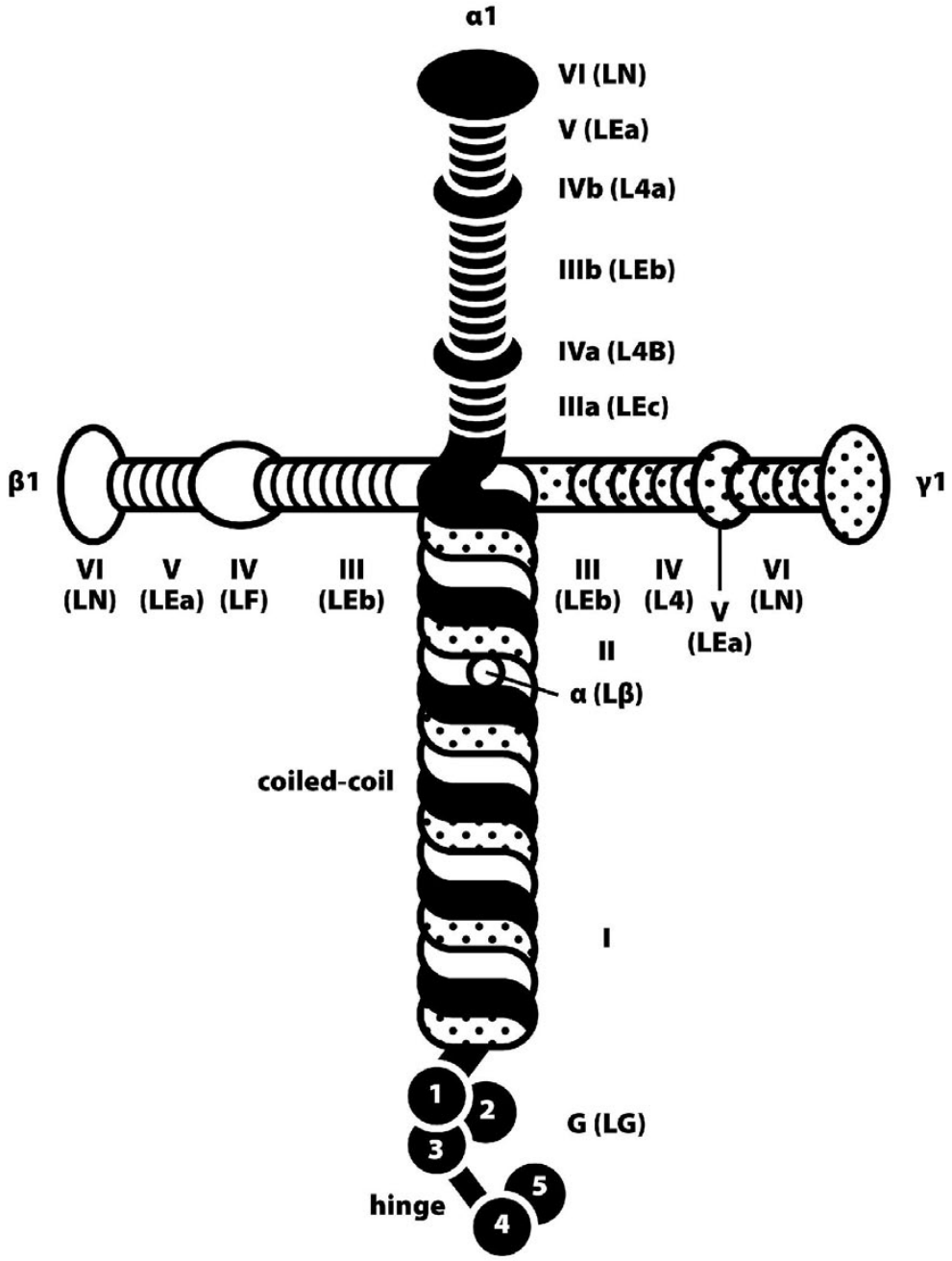

Figure 2. Laminin-1 structure, α1β1γ1 trimer, including domain structure indicated with Roman numerals (I-IV, α) according to the earlier nomenclature (Burgeson et al., 1994) and within parenthesis newly proposed (laminin-111) nomenclature (coiled-coil, LN, LEa, LEb, L4a, L4b, LEc, L4, LF, Lβ)(Aumailley et al., 2005). A finger domain of α1 laminin contains five globular domains (G or (LG)). "Hinge" indicates an inter-domain bridge between marked globular domains.

Table 2. Laminin receptors

Integrins: α1β1, α2β1, α3β1, α6β1, α7β1, α6β4
67/68-kDa laminin receptor
α-dystroglycan

2.3. Role in CNS development

Laminin-1 is known for its central part in the architecture of the BMs in almost all mammalian tissues. Its fundamental role appears during development in both embryonic and extraembryonic BMs starting from embryonic implantation (Leivo et al., 1980, Dziadek and Timpl, 1985, Miner et al., 2004) and also taking part in organ development (Miner and Yurchenco, 2004). The γ1 chain of laminin-1 is required for endodermal differentiation and formation of the BM (Halfter et al., 2002).

In Drosophila, laminin is essential for CNS function and proper development of the visual system (Garcia-Alonso et al., 1996), and in Xenopus it also seems to play a central role during CNS development (Lallier et al., 1996). In Drosophilia and C. elegans, two laminins are relevant for viability and are essential even in intervertebrate developmental functions (Huang et al., 2003).

Mutations in mammalian laminin genes representing the heterotrimers laminin-1 and -10 are fatal to the embryo and lead to defects in gastrulation, in neural tube closure, and in placentation (Miner et al., 1998, Smyth et al., 1999, Miner et al., 2004). Other reports on mutations in laminin genes show a more organ-specific defect less fundamental to early embryonal development, such as in the kidney (Noakes et al., 1995, Miner and Li, 2000), lungs (Nguyen et al., 2002), placenta (Miner et al., 1998), retina (Libby et al., 1999), PNS (Chen and Strickland, 2003), pancreas (Miner et al., 2004), and in skin/epithelial disruption (McLean et al., 2003) and CNS myelinization (Chun et al., 2003). Laminin receptor mutations also show the importance of molecular interaction in the developing brain: in conditional knockouts affecting dystroglycan and in integrin β1, resulting in disruption of the pial membrane (glia limitans) (Graus-Porta et al., 2001, Moore et al., 2002). Mutation of the nidogen binding site of γ1 laminin leads to a disruption of their interaction and also leads to discontinuites in the pial membrane together with neuronal migration errors (Moore et al., 2002). For major laminin receptors see Table 2.

2.4.CNS distribution

In the healthy CNS, laminin depositions appear mainly in the vascular- and pial BMs and the choroid plexus (Martin and Timpl, 1987, Miner and Yurchenco, 2004). In hippocampal cell layers, laminin-1 can be detected in neurons of both the developing and adult rat brain (Hagg et al., 1989). Laminin chain α5, β1, and γ1 expression in the hippocampal neuronal layers (CA1, CA5, and dentate gyrus) seem to be the highest (Indyk et al., 2003). Additionally, neuronal expression of laminin-1 occurs in the neocortex of the adult rat, and its γ1 chain actively participates in modulating neuronal activity (Hager et al., 1998).

In the adult mammalian CNS, α2 laminin is associated with neuronal tracts of the limbic system, and the γ1 chain appears throughout the brain in neuronal cell bodies (Hagg et al., 1997). The γ3 laminin expression has been characterized not only in BM structures but also in different structures of the brain (Koch et al., 1999).

Neurons expressing genes of the laminin subtypes α1, α5, β1, and γ1 were studied by use of a β-geo marker in gene trapping methodology (Yin et al., 2003). Expression of β1 and γ1 laminin immunoreactivity was evident but no α chain in adult mouse neurons in the cerebral cortex, hippocampus, and retina. A co-trapping method was also used to show that γ1 laminin and β1 laminin can form a protein complex intracellularly, and since no α chains were expressed here, the secretion of a laminin dimer was possible (Yin et al., 2003). In the same experimental assembly, α5 chains occurred in the choroid plexus and vascular basement membrane. Lack of the α1 subunit, but expression of β1 and γ1 laminin, are possible in both mouse brain and astrocyte cultures from rat cortex and olfactory bulb, where a dimeric laminin-1 was a possibility (Liesi and Risteli, 1989). Many studies show laminin subunit expression in the CNS in astrocytes and neurons.

Many of these studies show laminin subunit expression, but whether all subunits can be independently secreted to the extracellular space still remains unclear since some propose that the α1 chain drives the secretion of β1 and γ1 laminins (Yurchenco et al., 1997, Miner and Yurchenco, 2004).

Rat brain astrocytes in primary culture express laminin-1; the younger the source, the longer the expression. The same astrocytes, positively double immunostained by glial fibrillary acidic protein (GFAP) and laminin-1, were cultured from embryonic,

newly born, and 5-day-old rat brain (Liesi et al., 1983). A neurotoxic injury to the rat brain induces laminin-1 in reactive astrocytes (Liesi et al., 1984). In both normal and pathological CNS, laminins are expressed in neuroectodermal cells (Bernstein et al., 1985). In AD, laminin-1, α1, and γ1 laminin occur in reactive astrocytes and the γ1 chain could be localized to the senile plaques typical for AD pathology (Palu and Liesi, 2002). After SCI in adult rats, α1 and γ1 laminin can also be detected in reactive astrocytes (Liesi and Kauppila, 2002, Wiksten et al., 2004).

2.5. Migration and axonal growth

Through its neurotropic properties—participating in directing neurite growth—laminin plays an important role in the developmental process which involves migration and formation of neuronal pathways. In vitro, laminin-1 promotes the axonal growth activity of central neurons (Manthorpe et al., 1983), and in the developing rat brain, in vivo expression of laminin-1 coincides with neuronal migration (Liesi, 1985). Laminin-1 is expressed throughout the embryonic and early developing CNS, often correlating with neuronal pathways and migration (Cohen et al., 1987, Liesi and Silver, 1988, Morissette and Carbonetto, 1995). Both neurons and glial cells synthesize α, β, and γ chains of laminins in the developing mammalian CNS (Libby et al., 2000, Liesi et al., 2001, Wiksten et al., 2003). A different expression pattern of the laminin isoforms in human embryonic SC and brain has also been reported (Liesi et al., 2001). Laminin isoforms α1, β1, β3, and γ1 are associated with axonal guidance and are also localized in the floor plate of the developing embryonal human SC (Wiksten et al., 2003).

Retinal ganglion cells need laminin-1 during development for the neurite outgrowth and neuronal guidance in which they themselves actively participate. Interestingly, this response is lost during maturation (Cohen et al., 1986, Cohen et al., 1987). In the retina, β2 laminin expression in particular has been connected with synapse development and outer layer photoreceptor morphogenesis (Libby et al., 1996, Libby et al., 1997); other laminins such as α2, α3, α4, α5, β3, γ2, and γ3 can occur outside the retinal BM (Libby et al., 2000). Characterization of these subunits led to characterizing two new laminin heterotrimers (Libby et al., 2000). Laminin α2 is expressed when retinal pathways start to de-

velop within the brain (Morissette and Carbonetto, 1995). Laminin α2 has been identified in the CNS through dysfunctional states: A mutation in the α2 laminin has been associated with dysmyelinization, lack of development, and muscular dystrophy (Sunada et al., 1995, van der Knaap et al., 1997). The connection to demyelinization is through the oligodendrocyte laminin receptors (Colognato et al., 2007).

Laminin-1 expression is connected with neuronal survival in the hippocampus, and degradation of laminin makes them more susceptible to dying (Chen and Strickland, 1997); the γ1 chain seems to be of specific importance in this process (Chen et al., 2003). An axon regeneration model of the CNS, in lesioned hippocampal slices, shows that γ1 laminin plays a central role in neuronal regeneration, and that hippocampal neurons synthesize γ1 laminin (Grimpe et al., 2002). The hypothesis of γ1 laminin involvement as an axon growth-promoting laminin isoform was tested in culture studies using synthetically derived peptides. A decapeptide (RDIAEIIKDI) of γ1 laminin, from the C-terminal part, promoted neurite growth of both central and peripheral neurons (Liesi et al., 1989). The same decapeptide of γ1 laminin also occurs in the hippocampus (Matsuzawa et al., 1996). Furthermore, a tripeptide (KDI), derived from the same decapeptide, induces potassium currents in central neurons and mediates the neuronal outgrowth-stimulating properties of γ1 laminin (Liesi et al., 2001). Matrigel cultures show how neurites from spinal cord explants tend to grow towards and into matrigel if it has added KDI (Wiksten et al., 2003).

In the cerebellum, between the meningeal layer and the Purkinje cell layer lies an external granular layer (Goldowitz and Hamre, 1998, Wang and Zoghbi, 2001) in which granular precursor cells express laminin receptors of integrin β type, and the proliferating cells are in close association with laminin isoforms embedded within the BM (Blaess et al., 2004). Within this granular layer, laminin enhances the effects of sonic hedgehog (Ssh) and induces proliferation (Pons et al., 2001, Lewis et al., 2004).

2.6 Fragments, peptides, and their functions

Using enzymes to split laminin-1 into fragments could localize this molecule's different functions (Engel et al., 1981, Ott et al., 1982, Bruch et al., 1989). With proteolytic enzymes such as pepsin, elastase, cathepsin-G, and trypsin, functional studies could reveal

fragments active in cell attachment (Timpl et al., 1983), mitosis (Panayotou et al., 1989), and nidogen binding (Paulsson et al., 1987). Use of elastase in a similar manner allowed neuronal outgrowth capacity to be localized to the E8 fragment of laminin-1 (Edgar et al., 1984). In this fragment, the peptides RDIAEIIKDI and KDI may also be located. The fragments defined are in Figure 3.

Amino-acid sequencing of laminin-1 fragments has enabled further investigation and definition of the laminin-1 peptides and their biological functions. Eleven neuronally active laminin-1-derived peptides and their functional activities are presented in Table 3.

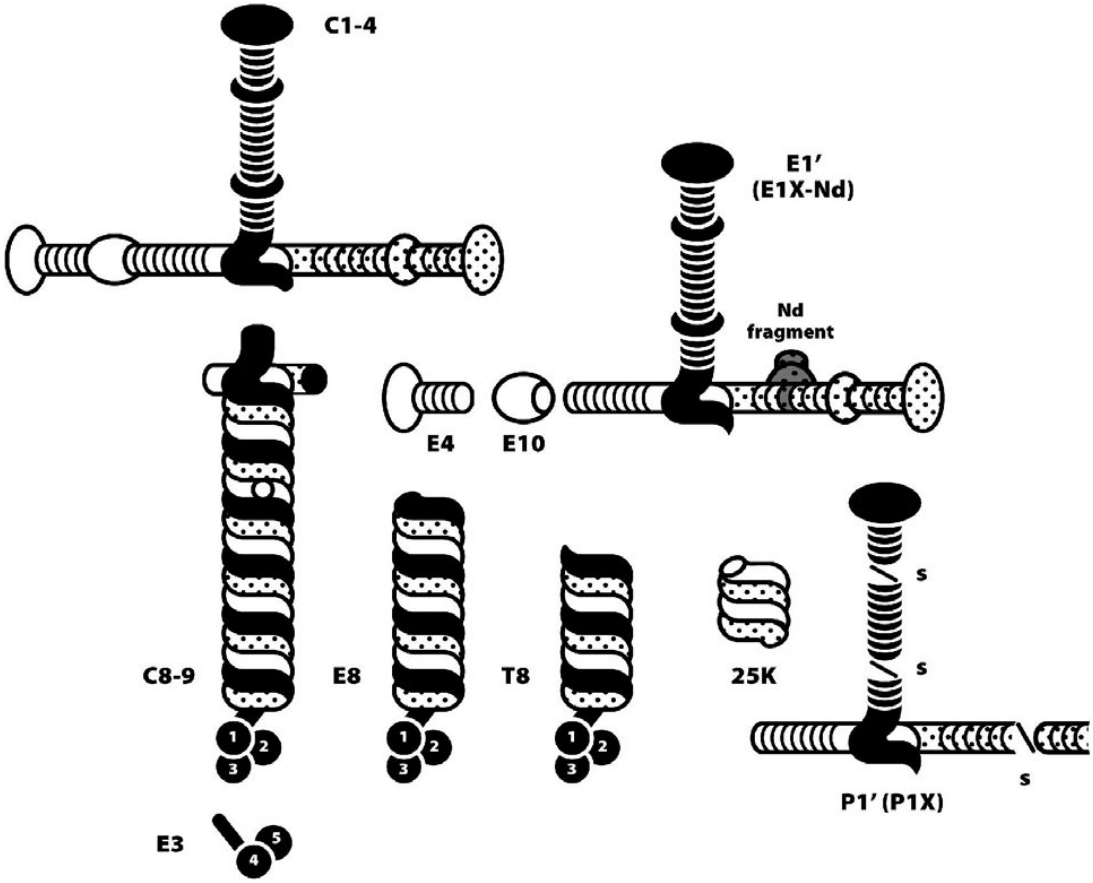

Figure 3. Proteolytic fragments of laminin-1. Enzymes used to cleave laminin-1 into experimental fragment *C* by cathepsin-G , *E* by elastase, *P* by pepsin, *T* by trypsin. The 25K fragment was generated by treating mouse tumor laminin-1 with clostripatin-containing collagenase and purifying its trypsin digests (Timpl et al., 1979). The P1' fragment is partially cleaved, but disulphide bridges hold it together (Aumailley et al., 2005). The globular domains of α1 laminin are presented here as the spheres 1 to 5.

Table 3. Laminin-1 derived peptides and their neuronal functions.

Location	Function	Peptide	Reference
α1 G	Neurite outgrowth	KATPMLKMRTSFHGCIK	(Skubitz et al., 1991)
α1 G	Neurite outgrowth	KEGYKVRDLNITLEFRTTSK	(Skubitz et al., 1991)
α1 G	Neurite outgrowth	KNLEISRSTFDLLRNSYGRK	(Skubitz et al., 1991)
α1 G	Neurite outgrowth	DGKWHTVKTEYIKRKAF	(Skubitz et al., 1991)
α4 G	Neurite outgrowth	LAIKNDNLVYVY	(Ichikawa et al., 2005)
α4 G	Neurite outgrowth	DVISLYNFKHIY	(Ichikawa et al., 2005)
α4 G	Neurite outgrowth	VIRDSNVVOLDV	(Ichikawa et al., 2005)
α4 G	Neurite outgrowth, peptide in cyclic form	CTLFLAHGRLVFX	(Ichikawa et al., 2005)
β2 I	Inhibition of neurite outgrowth	LRE	(Porter et al., 1995)
	Neuronal cell attachment		(Hunter et al., 1989)
	Promotion of motor axon growth		(Brandenberger et al., 1996)
γ1 I	Neurite outgrowth, neurotrophic and neurotoxic effect, activation of potassium currents	RDIAEIIKDI	(Liesi et al., 1989, Liesi et al., 2001)
	Neuronal migration and nuclear translocation		(Liesi et al., 1995)
	Axonal differentation and guidance		(Matsuzawa et al., 1996, Matsuzawa et al., 1998)
γ1 I	Guidance of neurite outgrowth	KDI	(Wiksten et al., 2003)
	Regeneration of SCI		(Wiksten et al., 2004)
	Neurite outgrowth and activation of potassium currents		(Liesi et al., 2001)
	Protection against 6-OHDA		(Väänänen et al., 2006)

Studies in Table 3 show results using primary neurons in their experimental setting. Amino acids: A, alanine; R, arginine; N, aspargine; D, aspartic acid; C, cysteine; Q, glutamine; E, glutamic acid; G, glycine; H, histidine; I, isoleucine; L, leucine; K, lysine; M, methionine; F, phenylalanine; P, proline; S, serine; T, threonine; W, tryptophan; Y, tyrosine; V, valine.

3. MECHANISMS OF INJURY

3.1 Excitotoxicity

In the CNS the major excitatory neurotransmitter is glutamate. Its release from neuronal synapses leads to neuronal glutamate receptor stimulation and excitatory synaptic transmission. Glutamate and its receptors are central communicative constituents for neurons; they support neuronal network activity and play an important role in maintaining both normal and pathological conditions in the CNS.

Excitotoxicity contributes in both acute and chronic CNS diseases to neuronal degeneration. Often a lack of oxygen or of glucose leads to depolarization of neurons which leads in turn to the release of glutamate into the extracellular space. Uptake of glutamate by astroglial cells is hampered during ischemia and hypoxia, resulting in even higher glutamate concentrations in the postsynaptic cleft. Excessive glutamate release can lead to a disruption of Ca^{2+} homeostasis (Choi, 1995). It is suggested that because glutamate toxicity is primarily dependent on Ca^{2+} influx, ionotrophic glutamate receptors may play a key role (Choi, 1987, Choi et al., 1987). The ionotrophic glutamate receptors have traditionally been classified into three subtypes, N-methyl-D-aspartate (NMDA), α-3-amino-hydroxy-5-methyl-4-isoxalolepropionate (AMPA), and kainate (KA) receptors. When they are activated, their ion channels open for influx especially of Ca^{2+} and Na^{+} ions into neurons. Activation of metabotrophic receptors leads to release of intracytoplasmic Ca^{2+} stores, further increasing the cytoplasmic calcium overload. The rise in intracellular calcium stimulates intracellular neurotoxic pathways and enzymes, neuronal mitochondrical respiration is impaired, and cessation or near cessation of adenosine triphosphate (ATP) production occurs. Furthermore, production of reactive oxygen radicals and activation of apoptotic signals extend CNS tissue damage. Prior to the Ca^{2+}-dependent excitotoxicity, the entry of Na^{+} and Cl^{-} ions into neurons leads to acute cellular swelling called cytotoxic edema (Dirnagl et al., 1999, Arundine and Tymianski, 2003, Arundine and Tymianski, 2004).

Glutamate receptors, which can be found throughout the brain, participate in the pathophysiology of many brain diseases and CNS trauma. Mediating excitotoxicity,

they may take part in ischemic brain damage (Hossmann, 1994), epilepsy (Swann et al., 2000), and brain injury caused by trauma (Siesjo et al., 1995). Neurodegenerative diseases such as AD (Smith-Swintosky and Mattson, 1994), PD (Beal, 1998), Huntington's disease (Ross et al., 1997), and ALS (Eisen and Weber, 2001) involve excessive glutamate receptor stimulation and thereby neuronal cell death.

3.2 Trauma

Traumatic CNS injury has several forms including mechanical, chemical, thermal, and electrical. Among these, mechanical trauma is overwhelmingly the most common and most disabling. A traumatic blow to the brain or SC results in both acute and secondary tissue damage to the CNS. In the acute reaction, direct cell death occurs at the damage site. Neurons, astrocytes, oligodendrocytes, and endothelial cells suffer from acute cell death, and lack of both oxygen and glucose further damages the tissue. Hemorrhage, vascular damage, inflammation, and edema follow and contribute to the acute phase of the injury (Hagg and Oudega, 2006).

Head trauma can be both focal and diffuse, depending on the mechanism of impact (Gennarelli et al., 1982) (Medana and Esiri, 2003). Since research on the molecular mechanisms of CNS trauma is more clearly defined regarding spinal cord injury, and most of the pathophysiological mechanisms in brain and SC trauma are similar, the following insights into secondary traumatic CNS tissue damage will be studied with focus on the SC. Acute cell death spreads through secondary mechanisms of injury in the white matter of the SC where extracellular glutamate also participates, through excitotoxicity to continuous neural damage (Park et al., 2004).The secondary processes continue to hamper the function of the already damaged SC through apoptosis of oligodendrocytes and loss of myelin (Crowe et al., 1997), and demyelinization can continue for months and possibly for years (Guest et al., 2005). During the first 3 to 7 days, many inflammatory cells such as monocytes, macrophages, microglia, and T-lymphocytes can be identified in the SCI area (Popovich et al., 1997). Many mechanisms contribute to necrotic cell damage: nitric oxide (NO) production, Ca^{2+} homeostasis disruption, membrane breakdown, and oxidative stress (Casha et al., 2001). During the secondary phase, a scar forms in the SC, also known as the

glial scar, its molecular constituents actively repelling axonal growth (Rhodes and Fawcett, 2004). The glial scar is formed around the lesion site and contains reactive astrocytes (McKeon et al., 1991). Moreover, meningeal fibroblasts and macrophages invade the lesion site, creating a more fibrous scar, adding to the mechanical obstacle against neurons' growing and re-forming networks (Stichel and Muller, 1998, Preston et al., 2001).

3.3 Ischemia

3.3.1 Focal brain ischemia

In treating patients with functional impairments caused by CNS damage, the vulnerability of the human CNS is always a challenge. One of the most common causes of CNS damage is brain ischemia caused by a blood perfusion deficit. Since the neurons are unable to store valuable resources of energy such as glucose or oxygen, their survival is dependent on a continuous supply through the blood circulation.

Focal brain ischemia is caused by lack or critical attenuation of blood flow to one region of the brain. Depending on the site of vascular occlusion, various parts of the brain can be affected and clinically recognized based on signs, symptoms, and imaging studies. Ischemic brain damage has a complex pathophysiology. Decreased blood flow leads to a lack of substrates for the cells that are dependent on both glucose and oxygen, causing depolarization of neurons and astroglia. Excessive amounts of glutamate are released to the extracellular space, and through receptor activation, intracellular calcium rises (see part 3.1). Due to the vascular occlusion and reduction in blood flow, disruption of the BBB follows, leading to brain edema and escalation of inflammatory processes. The cause of BBB breakdown is believed to be connected with proteases such as the matrix metalloproteases destroying the basal lamina, inflammatory molecules, and free radicals. The free radicals arise mainly due to a lack of substrate (glucose, oxygen), leading to anaerobic glycolysis and acidosis. They generate an increase in intracellular Ca^{2+}, Na^{+}, and Cl^{-} ions, thus leading to apoptosis and necrosis. Postischemic inflammation has many components, microglia and leukocytes being important ones (Stoll et al., 1998). Ischemia-

induced upregulation of adhesion molecules leads to recruitment of circulating leukocytes to the surface of endothelial cells (Lindsberg et al., 1996) and through pro-inflammatory cytokines infarct volume further increase (Relton et al., 1996).

3.3.2 Ischemic brain edema

On the cellular level, two types of edema after ischemia are usually recognized. Cytotoxic edema, described as cellular swelling, is mainly caused by sodium influx into cytoplasm, followed by water. Na^+ and Ca^{2+} ion influx is partly the result of neurotransmitter stimulation of ligand-gated receptors. The Ca^{2+} overload and the cellular swelling is thought to be cytotoxic for the injured cells (Choi, 1992, Neumann-Haefelin et al., 2000). Vasogenic edema, also described as the extracellular component of brain swelling, is caused mainly by a disrupted BBB (Ayata and Ropper, 2002). Contributing to the endothelial damage and vascular permeability, leading to a shift of water to the extracellular compartments, are inflammatory processes and free radicals. Further damaging the BBB and increasing its permeability are matrix metalloproteases that actively break down the matrix proteins in the basal lamina (Mun-Bryce and Rosenberg, 1998).

3.4 Kainic acid-induced neurotoxicity

Kainic acid (KA) is a neurotoxic glutamate agonist (Olney et al., 1974). A well-described model for studying neurodegeneration is the injection of kainic acid into the hippocampus (Ben-Ari et al., 1979). Kainic acid binds non-NMDA-type glutamate receptors (AMPA and kainate receptors) and takes part in excitotoxic cell death (Chen et al., 1995). Activation of kainate receptors raises intracellular calcium, the production of reactive oxygen species, and mitochondrial dysfunction, all leading to apoptotic cell death in neurons (Cheng and Sun, 1994, Milatovic et al., 2002). In the rat hippocampus, after KA injection, proliferation of astrocytes and microglia is evident (Wang et al., 2005). KA has been useful for studying neurological conditions such as epileptic seizures (Ben-Ari, 1985), Huntington's disease (Coyle et al., 1983), AD (Coyle et al., 1983), and trauma (Wang et al.,

2005). It has been applied both in vivo and in vitro to test pharmacological and neuroprotective compounds against excitotoxic events in the CNS. Intrahippocampal, intracerebroventricular, and systemic administration of KA has been a tool in various settings for study of neurodegeneration (Ben-Ari, 1985). Neurodegenerative changes, especially in the hippocampus, are of value in studying areas such as the CA1 and CA3, where pyramidal neurons as well as interneurons in the hilus of the dentate gyrus (DG) are particularly vulnerable to KA (Ben-Ari, 1985, Grooms et al., 2000). Interestingly, pyramidal cells in the hippocampal CA2 and glomerular cells of the DG seem to be more tolerant to excitotoxicity (Grooms et al., 2000, Oprica et al., 2003). The molecular basis for this difference may be the diverse expression of kainate and AMPA receptors, which thereby influence the sensitivity of Ca^{2+} permeability and the selective vulnerability of the specific region (Malva et al., 1998, Grooms et al., 2000, Weiss and Sensi, 2000). Excitotoxic cell death caused by KA may be inhibited by the AMPA receptor antagonist CNQX (6-cyano-7-nitroquinoxalone-2, 3-dione), but not by use of an NMDA or a membrane calcium antagonist; this fact shows the central position of the AMPA receptor in this model of injury (Li et al., 2003).

3.5 Inflammation

Immediately following lesioning in the CNS, inflammatory cells migrate to the site of injury. Macrophages, T-cells, and neutrophils migrate from the periphery towards the lesion and are joined by activated astrocytes and microglia (Popovich et al., 1997, Bethea and Dietrich, 2002). Maximal infiltration of the inflammatory cells can be seen 3 to 7 days post-injury (Popovich et al., 1997). Following injury, the invading leukocytes take part in activating the inflammatory response in macrophages. Macrophages can be actively localized at the injury site, and their number starts to decline 7 days post-injury; at the primary SC lesion, microglia also are present for up to 2 to 4 weeks (Popovich et al., 1997). Neutrophil activation to the injury is rapid and starts to decline 48 h after injury (Taoka et al., 1997). Neutrophils and macrophages also contribute to the secondary damage by taking part in tissue destruction (Taoka et al., 1997, Gris et al., 2004). Primarily produced by macrophages and microglia, pro-inflammatory chemokines and cytokines (tumor necrosis factor (TNF); interleukin-1, -6, -10, and interferon) further provoke the damage (Bartholdi

and Schwab, 1997, Klusman and Schwab, 1997). A vicious circle continues through induction of reactive oxygen, nitric oxide, and additional chemokines/cytokines (Bredt and Snyder, 1994, Xu et al., 2001). Over-expression of these toxic molecules further damages the SC, contributes to scar formation, and hampers axonal growth (Jones et al., 2002).

3.6 Apoptosis

Programmed cell death is a natural process in the developing CNS (Blomgren et al., 2007). Premature cell death, also known as pathological apoptosis, is associated with many neurodegenerative disorders such as AD, PD, ALS, stroke, and brain and spinal cord trauma (Heidenreich, 2003, Ekshyyan and Aw, 2004). When damage occurs in the CNS, some cells die from direct necrosis, and others are programmed towards apoptosis. In many cases the necrotic part of the lesion can be larger than the part affected by pathological apoptosis. Necrosis is the dominant cell death mechanism within the ischemic lesion's core, whereas the role of apoptotic cell death is clearly greater within the penumbral region (Li et al., 1998). The temporal profile also differs, with necrosis more instant, but apoptosis continuing for days, even for weeks, and also affecting projecting pathways (Schwab and Bartholdi, 1996). Post-injury, apoptosis is activated by various stimuli and appears over the same time-spectrum as do the inflammatory processes. Among the toxins produced by inflammation are oxygen free radicals, mediating apoptosis by mitochondrial and DNA damage (Mabuchi et al., 2000). Apoptotic signalling can be caspase-dependent or independent. Caspase-dependent apoptosis is mostly stimulated through "death receptors" such as tumor necrosis factor receptors (TNFR) and Fas/CD95. These receptors are up-regulated after SCI and traumatic brain injury (Casha et al., 2001, Keane et al., 2001), and FAS deficiency reduces post-traumatic apoptosis (Casha et al., 2005). This pathway towards apoptosis is also stimulated through mitochondrial damage and further activates caspase-3 (Springer et al., 1999). Apoptosis-inducing factor (AIF) is a caspase-independent inducer of cell death released from the mitochondrial intermembrane in response to death stimuli (Joza et al., 2001). AIF causes chromatine condensation and DNA fragmentation in the cell nucleus through large DNA strands (Lorenzo et al., 1999, Susin et al., 1999). NMDA neurotoxicity, neurodegenerative disorders, and stroke itself activate a

nuclear enzyme called poly(ADP-ribose) polymerase-1 (PARP-1) (Zhang et al., 1994, Eliasson et al., 1997, Endres et al., 1997) which mediates AIF signalling (Yu et al., 2002).

Antiapoptotic treatment is a intriguing target for neuroprotective research, with some promising molecules proposed as having therapeutic properties in ischemic brain damage. After cerebral ischemia, erythropoietin, which reduces ischemic neuronal damage and neuronal dysfunction in rodent models of stroke (Sadamoto et al., 1998), prevents neuronal apoptosis (Siren et al., 2001). Another interesting intervention with potential clinical implications is the inhibition or inactivation of PARP-1 which leads to prevention of apoptotic cell death (Graziani and Szabo, 2005).

4. REGENERATION

In contrast to the PNS, where axonal regeneration is possible and can lead to functional recovery, axonal regeneration in the CNS is minimal, and functional loss is permanent. In the PNS, following axiotomy, Wallerian degeneration—describing degeneration of the distal part of the damaged axon—takes place. Thereafter, Schwann cells catabolize myelin, and macrophages are recruited to the injury site, participating in the removal of myelin- and axonal debris. The remaining Schwann cells form guidance channels together with chemoattractive factors with which the degenerated axons can start a regenerative process through development of growth cones. Neuronal transformation into a regenerative mode involves many molecular steps such as gene activation, upregulation of cytoskeletal proteins, and outgrowth-stimulating factors (Johnson, 1993, Fu and Gordon, 1997).

CNS neurons do attempt to regenerate their damaged axons but usually end by degenerating; this failure to regenerate is now well-accepted as crucially influenced by inhibitory factors in their environment (Stichel and Muller, 1998).

4.1 Hampering factors

4.1.1 Glial scar

Axonal regeneration (sprouting) and networking may be hampered by scar tissue which forms a mechanical barrier. After CNS injury, the scar consists of two areas, the lesion core and the surrounding zone (Reier and Houle, 1988). The core of the lesion, often considered as permitting no regeneration, is populated by vascular endothelial cells, meningeal fibroblasts, and oligodendrocyte precursors (OPCs). Surrounding the lesion are reactive astrocytes, microglia, and OPCs (Fawcett and Asher, 1999). Post-traumatically, after a few days, astrocytes become hypertrophic and up-regulate GFAP (Latov et al., 1979, Mathewson and Berry, 1985) and later in the formation of the glial scar proliferate towards the core of the lesion (Fawcett and Asher, 1999). Today, many inhibitory molecules produced by astrocytes are identified, and different experimental settings have shown their effects in hampering CNS regeneration (McGraw et al., 2001, Busch and Silver, 2007). Such molecules as Semaphorin 3 (Pasterkamp et al., 2001), ephrin-B2 (Bundesen et al., 2003), tenascin (Brodkey et al., 1995), and chondroitin sulphate proteoglycans (CSPGs) (Jones et al., 2003) are among the molecules upregulated after injury. The core proteins of the CSPGs are versican, neurocan, brevican, phosphacan, biglycan, aggrecan, and NG2 (Fawcett and Asher, 1999). These CSPGs are considered an important molecular group for inhibition in the glial scar; axons growing from transplanted dorsal root ganglion (DRG) stop at the site where CSPGs are expressed (Davies et al., 1999). The CSPGs localize around several different CNS injury models, appear a few days after injury, and continue to be expressed for weeks (Levine, 1994, Geisert et al., 1996). Not surprisingly, CSPGs are in vitro a poor substrate for neurons to grow neurites (Snow et al., 1990). After brain or spinal cord injury, regeneration of corticospinal or nigrostriatal tract axons can be promoted by digesting CSPGs with chondroitinase ABC (Moon et al., 2001, Bradbury et al., 2002). Interestingly, the sulphated glucosaminoglycan (GAG) chain of the CSPGs can bind to growth-promoting molecules such as laminin and thereby inhibit further regeneration (Smith-Thomas et al., 1995). Removal of the GAG chains from laminin allows promotion of neurite outgrowth (McKeon et al., 1995).

4.1.2 Myelin inhibition

The inhibitory effect of oligodendrocytes, white matter, and myelin is due to their molecules that actively repel growing neurites (Schwab and Caroni, 1988). The myelin inhibitory molecules are considered the second major source of inhibitory molecules in CNS regeneration. When an injury occurs, myelin is disrupted locally and directly, since myelin-based inhibitory molecules are expressed by normal oligodendrocytes in the CNS. To identify an inhibitory myelin molecule, in rat SC, antibody IN-1 was isolated, and showed promise in regeneration in injured nerve fibers (Schnell and Schwab, 1990). IN-1 was later designated Nogo, with now at least three isoforms of Nogo known (Nogo-A, -B, -C), among which Nogo-A is the largest and is highly expressed in oligodendrocytes (Huber et al., 2002). Nogo-A has two main active parts, one specific for Nogo-A (Oertle et al., 2003) and another 66-amino-acid loop called Nogo-66 (Fournier et al., 2001) which is common to all Nogo proteins. Both these two inhibitory domains are exposed to the ECM (Oertle et al., 2003). The Nogo-66 domain inhibits neurite outgrowth through a receptor called NgR (Fournier et al., 2001). Other myelin-associated molecules that can inhibit axon growth, the myelin-associated glycoprotein and oligodendrocyte-myelin glycoprotein, also act through the NgR receptor (Domeniconi et al., 2002, Liu et al., 2002). Myelin-associated glycoprotein is a transmembrane glycoprotein with immunoglobulin-like domains first identified in CNS myelin (McKerracher et al., 1994) and is also present in PNS Schwann cells (Schachner and Bartsch, 2000). In embryonic mouse spinal cord, myelin-associated glycoprotein has also shown neurite outgrowth-promotive effects (Turnley and Bartlett, 1998). Oligodendrocyte-myelin glycoprotein is a membrane myelin protein expressed by oligodendrocytes in the CNS (Mikol et al., 1990).

4.2 Inducing factors

4.2.1 Neurotrophins

Neurotrophins are molecules often linked to neuronal survival, differentation, and development. They consist of neuronal growth factor (NGF), brain-derived growth factor

(BDNF), neurotrophin-3 (NT-3), neurothrophin-4/5 (NT-4/5), neurotrophin-6 (NT-6), and neurotrophin-7 (NT-7) (Lessmann et al., 2003). NGF, BDNF, NT-3, and NT4/5 have been characterized (Lu et al., 2005) in the mammalian brain as well as their receptors, the Trk family of receptor tyrosine kinases (TrkA, TrkB, TrkC) and the p75 neurotrophin receptor (p75NTR) (Dechant and Barde, 2002, Huang and Reichardt, 2003). Astrocytes in the CNS produce NGF, NT-3, and NT-4/5 (Yamakuni et al., 1987, Condorelli et al., 1995). After spinal cord injury in rats, the reactive astrocytes upregulate NGF and BDNF (Goto and Furukawa, 1995). These are important for axonal branching (McAllister et al., 1995, Gallo and Letourneau, 1998) and for establishing proper functional connections between neurons (McAllister et al., 1999). In the hippocampus, BDNF infusions enhance neurogenesis and formation of new granule cells (Scharfman et al., 2005).

4.2.2 Other growth factors

Fibroblast growth factor-2 (FGF-2) is actively expressed by astrocytes (Sensenbrenner, 1993) and can be localized in the hippocampus, cerebral cortex, and spinal cord (Riva and Mocchetti, 1991). FGF enhances neurite growth and reduces gliosis in spinal cord grafts (Giacobini et al., 1991). Mixing FGF into a fibrin graft and placing it into a rat SC lesion model leads to regeneration of the corticospinal tract with improved levels of function (Cheng et al., 1996). FGF also stimulates proliferation of glial cells after contusive SCI (Zai et al., 2005). The viability of oligodendrocytes is potentiated by cytokines such as ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF) (Kahn and De Vellis, 1994). In animal models, CNTF and LIF protect against demyelinisation and oligodendrocyte apoptosis in autoimmune encephalomyelitis (Butzkueven et al., 2002, Linker et al., 2002); they both are also detectable in CNS astrocytes (Stockli et al., 1991, Aloisi et al., 1994), and CNTF is upregulated after cortical lesions (Lee et al., 1997) and ischemia (Park et al., 2000). During remyelinization, insulin growth factor-1 (IGF-1) is upregulated in astrocytes (McMorris et al., 1993) and stimulates their proliferation (Tranque et al., 1992). In the developing optic nerve, IGF-1 acts as a survival factor for oligodendrocyte progenitor cells and oligodendrocytes (Barres et al., 1992) and is increased after ischemia (Gluckman et al., 1992).

The transforming growth factor-βs (TGF-βs) are a family of proteins with diversive CNS activities including neuroprotection (Grande, 1997). TGF-β1 activates a hypertrophic response in astrocytes, thereby upregulating GFAP (Laping et al., 1994). Astrocytic TGF-β1 increases as a response to other neurotrophins such as NGF, and is further autoinduced (Lindholm et al., 1990, da Cunha and Vitkovic, 1992, Morganti-Kossmann et al., 1992). TGF-β1 also induces the synthesis of ECM proteins such as type I collagen, fibronectin, and laminin (Baghdassarian et al., 1993).

4.2.3 Laminin

Fetal sensory neurons with NGF added to the culture medium show the neurite outgrowth capacity of laminin-1 as a substrate. Laminin-1 shows enhancement in neurite growth without NGF, and can be suppressed with laminin antibodies (Baron-Van Evercooren et al., 1982). The axonal regrowth with laminin-1 added to culture wells, when further tested on neurons removed from different parts of the PNS and CNS, shows sensitive stimulation of neurite outgrowth (Manthorpe et al., 1983). In the PNS, Schwann cells in contact with axons produce laminin-1 on their surfaces (Cornbrooks et al., 1983). In the treatment of PNS injuries, the neurite outgrowth properties of laminin-1 have been used in the form of grafts implanted into the injury sites. Gel including laminin-1 enhances axonal growth through the transected sciatic nerve (Madison et al., 1987).

In the CNS, early cultured astrocytes produce laminin-1 (Liesi et al., 1983). Neurite outgrowth and attachment capacity in neurons from the postnatal rat brain (Liesi et al., 1984) and hippocampus (Lein et al., 1992) are stimulated by laminin-1. In vivo, laminin-1 is expressed by astrocytes in the rat olfactory bulb—known for its regenerational capacity—and in the frog brain where sectioning of the optic tract enhances expression of laminin-1 during regeneration (Liesi, 1985). Following an optic nerve crush in goldfish, laminin-1 is upregulated by optic nerve glia, and stimulates regeneration on retinal explants (Hopkins et al., 1985). Moreover, in the developing chick optic pathway, laminin-1 is expressed before axon growth is initiated and contributes to the pathfinding of the axons (Cohen et al., 1987). Laminin-1 is upregulated in the rat brain by astrocytes after KA-induced injury (Liesi et al., 1984), and lesions to the brain (Stichel and Muller, 1994) and to the spinal cord (Bernstein et al., 1985). In culture, CNS-derived myelin inhibition on

neurons may be neutralized by laminin-1 (David et al., 1995). In the hippocampus, plasmin-catalyzed degradation of laminin-1 makes neurons more sensitive to excitotoxic cell death (Chen and Strickland, 1997).

In rats, expression of γ1 laminin in reactive astrocytes after spinal cord injury and surrounding the necrotic area after focal cerebral ischemia (Liesi and Kauppila, 2002) (IV) is interesting because of the theory of the regenerational capacity of γ1 laminin. Using a model to investigate laminin impact in hippocampal slice cultures, a lesion was made to the mossy fiber pathway, and results showed that a reduction in γ1 laminin at mRNA level in this region reduces the normal capacity of axonal regeneration (Grimpe et al., 2002). This study showed that the BM-independent γ1 laminin is crucial to regeneration of an axon tract in the mammalian CNS. In addition, γ1 laminin is proposed to maintain the critical role of the laminin layer in the hippocampus and protect neurons against excitotoxic cell death (Chen et al., 2003). In the PNS, as well, mutant mice with reduced expression of γ1 laminin and sciatic nerve damage show impaired peripheral axonal regeneration (Chen and Strickland, 2003).

4.2.4 KDI peptide

The neurite outgrowth functions of γ1 laminin have been proposed to be carried out by a decapeptide (RDIAEIIKDI). Nanomolar concentrations of this decapeptide, located in the C-terminal of the γ1 chain, promote neurite outgrowth in organotypic cultures on both peripheral and central neurons (Liesi et al., 1989). This RDIAEIIKDI peptide also directs axonal growth in rat embryonic hippocampal neurons in cell culture (Matsuzawa et al., 1998) and participates in neuronal differentation (Matsuzawa et al., 1996). Using slice preparations of adult rat neocortex, intracellular recordings demonstrate that the decapeptide of γ1 laminin raises both the input resistance of the neuronal membrane and the excitability of pyramidal neurons (Hager et al., 1998). The decapeptide of γ1 laminin induces potassium currents in primary cultured cerebellar neurons, and the smallest peptide derived from it that is capable of inducing the same electrophysiological properties and supporting neurite outgrowth is the KDI tripeptide (Liesi et al., 2001). Since the decapeptide is neurotoxic in high concentrations, and these effects can be inhibited in cell culture by addition of pertussis toxin, an inhibitor of G-protein function, it is possible that the decapeptide of γ1

laminin can function also through a G-protein receptor-mediated mechanism (Liesi et al., 2001). In the developing human SC, KDI peptide is localized in the floor plate region where comissural axon guidance occurs (Wiksten et al., 2003). In matrigel culture systems, KDI also attracts axonal growth from SC explants (Wiksten et al., 2003). Local infusion of KDI peptide, shown by use of osmotic mini pumps in transected SC in rats, has also shown enhancement of neuronal regeneration and has improved the functional recovery after SCI (Wiksten et al., 2004).

AIMS OF THE STUDY

The overall purpose of the project was to clarify the role of γ1 laminin and its KDI peptide in neuronal regeneration and further elucidate the role of laminins in the injured CNS. The specific aims were:

I To determine the possible regenerational effects of the KDI peptide on neuronal survival and neurite outgrowth in a culture system mimicking challenging enviromental factors known to inhibit CNS regeneration.

II To determine whether the KDI peptide can protect hippocampal neurons against excitotoxic cell death caused by kainic acid.

III To determine the hypothesis of KDI peptide function, through the glutamate system, at cellular level by investigating glutamate receptors and electrophysiological responses.

IV To map the temporal and spatial distribution of laminins in the CNS of adult rats with permanent focal cerebral ischemia.

MATERIALS AND METHODS

For more specific information on the experimental procedures please see the original publications (I), (II), (III), and (IV). All experiments were approved by the relevant authorities.

Human CNS tissues (I), (III)

The human CNS tissues were collected with the permission of the donors from aborted 8- to 10-week-old human fetuses in collaboration with the Maternity Hospital of the Helsinki University Central Hospital. Adult human spinal cord came from the Neurological Specimen Bank (Baltimore, MD, USA).

KDI peptide (I), (II), (III) and Kainic acid (II)

KDI peptide was synthetically manufactured as a C-terminal acid with a 95% purity level (Multiple Peptide Systems, La Jolla, CA, USA) and dissolved in deionized sterile water to a concentration of 10 mg/ml, from which it was further diluted to the concentration used. Kainic acid (Sigma, St. Louis, MO, USA) was diluted to 1mg/ml in 0.9% NaCl, and further diluted to the concentrations used.

Human embryonic SC glia cultures (I)

Primary glial cell cultures were obtained by mechanical dissociation of human embryonic SC and cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Paisley, Scotland) with 10% fetal calf serum (Hyclone, Logan, UT, USA) and antibiotics. The cell lines were identified by 100% TUJ1 (Sigma) immunostaining, indicating that the glial cells were precursors of astrocytes; 10 to 20% of these cells were also positive to GFAP (Sigma) immunostaining, and no oligodendrocyte-like cells were detected, thus indicating that most of the cells were precursors of astrocytes.

Experimental culture system I: Glial injury (I)

Human embryonic glial cells were replated on glass coverslips and cultured to confluent monolayers. The monolayers were injured with a 18-G needle, and the culture medium was changed to 10% normal adult human serum in DMEM (Gibco). KDI peptide (Multiple Peptide Systems) was added in different concentrations to the culture medium, and dissociated cells from human embryonic neocortex, retina, and SC were added to the coverslips. After 72 hrs, the cultures were fixed with 2% paraformaldehyde in PBS (Sigma) for 15 min. Survival of neurons and length of neurites on the injured glial cells was evaluated by counting TUJ1-positive neurons and their TUJ1-positive neurites. The number of neurons was counted on ten random fields on six different coverslips. The amount of long neurites from all neurons was counted from six coverslips in each experimental group. For statistical evaluation, one-way analysis of variance (ANOVA) was used.

Embryonal bodies of human CNS (I)

Embryonal SC and neocortex containing immature stem cells was mechanically dissociated and cultured in Neurobasal medium (Gibco) with B27 supplement (Gibco), glutamine, and antibiotics. Failing to attach to the petri dish surface, the embryonal bodies grew in aggregates, releasing new bodies and increasing in size.

Experimental culture system II: Myelin inhibition (I)

Adult human SC sections (10 μm) were cut on slides and placed in sterile Quadriperm plates (In Vitro Systems and Services, Göttingen, Germany) with DMEM culture medium (Gibco), 10% human serum, and antibiotics. Various concentrations of KDI peptide (Multiple Peptide Systems) were added to the culture medium. Embryonal bodies were placed on top of the white matter of the SC sections and cultured for 10 days before fixation. Immunostaining of neurofilament proteins and glial cells identified the neurites and cells of the embryonal bodies. Evaluation of the neurites growing out from the embryonal bodies on top of the white matter was done by counting neurites of each slide and was analyzed with a nonparametric Mann-Whitney test.

Antibodies for immunocytochemistry (I), (II), (III), (IV)

For immunohistochemical detection of antibodies, various isoforms of laminins were used: rabbit polyclonal antibodies to identify α1-5, β1-3, and γ1-2 laminins (II, IV). Laminin-1 was detected by use of polyclonal antibodies against biochemically purified EHS-tumor laminin (II, IV). To detect the neurite outgrowth-promoting peptide of γ1 laminin (KDI), a polyclonal rabbit antibody raised against KLH-coupled (keyhole limpet hemocyanin) 6 amino-acid peptide (EIIKDI) was used (IV). Antibodies pre-absorbed with their respective peptide antigens or pre-immune sera of each rabbit used for immunizing served as controls (II, IV). All control experiments were negative.

Mouse monoclonal antibodies against neuron-specific β tubulin (TUJ1, Sigma) served to detect neurons, dendrites, and axons (I). TUJ1 also served as a marker for early glial cells (I). Mouse monoclonal antibodies against the glial fibrillary acidic protein (GFAP, Sigma) served for detection of astrocytes and radial glial fibers (I, II, IV).

Rabbit polyclonal antibodies against different glutamate subunit proteins AMPA (GluR1, GluR2/3, GluR4), kainate (GluR5, GluR6/7, KA2), and NMDA (NR1, NR2A, NR2B) (Upstate, Lake Placid, NY, USA) showed the expression of these glutamate subunit receptors (III).

Immunocytochemistry on sectioned rat brain (II), (IV)

Brains from decapitated adult male Wistar rats were sectioned. For paraffin sections (II), the brains were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS, Sigma) for 12 hrs at +4°C. The brains were then washed in PBS for 48 hrs before being embeded in paraffin wax. The serial sections were cut in the coronal plane through the hippocampus and stained with hematoxylin-eosin (II).

For immunocytochemistry of laminins and glial fibrillary acidic protein (GFAP) in rat brain, the brains were frozen in powdered dry ice: 10 days after stereotaxic injections (II) or following the variable post-ischemia time periods (IV). The sections were fixed in 0.4% para-benzoquinine (Fluka, Buchs, Switzerland) in PBS for 15 min at +4°C. The sections were then rinsed in PBS before dehydration and rehydration in a series of alcohols. Fixed sections were incubated overnight in normal goat serum (1:30 dilution in PBS; Sigma). Antibodies against the various isoforms of laminins were applied, and incu-

bation was for 24 hrs at +4°C. The sections were then rinsed in PBS and incubated with fluorescein isothiocyanate-coupled goat antirabbit immunoglobulins (FITC; Cappel, Cochranville, PA, USA) for 1 hr at room temperature (RT). For double-staining, the sections were incubated further with mouse monoclonal antibodies overnight at +4°C. Mouse monoclonal antibodies against GFAP served to identify reactive astrocytes. The sections were rinsed and exposed to goat anti-mouse immunoglobulins coupled to tetramethylrhodamine isothiocyanate (TRITC; Cappel) for 1 hr at RT. The sections were rinsed in PBS and mounted in PBS:glycerol (1:1) for microscopic analysis.

Immunocytochemistry on cultured neurons (I), (III)

The neurons were fixed in 2% paraformaldehyde/PBS for 15 min at RT and permeabilized with cold (-20°C) methanol for 5 min. In experimental culture system I (I), immunoreactivity for β-tubulin (TUJ1) served to identify and visualize neurons along with their long neurites. The antibody was incubated for 1 hr at RT. After being rinsed with PBS, the cultures were exposed to goat anti-mouse immunoglobulins coupled to fluorescein isothiocyanate-coupled goat-antirabbit immunoglobulins (FITC; Cappel) for 30 min at RT. The coverslips were then rinsed and mounted in PBS:glycerol (1:1) (I).

Immunocytochemistry on human embryonic neocortical neurons cultured for 14 to 30 days on a poly-D-lysine substratum (10μg/ml; Collaborative Research, Bedford, MA, USA) was used to demonstrate the expression of glutamate receptors (IV). After fixation with 2% paraformaldehyde/PBS the cultures were treated with 0.05% Tween-20/PBS for 30 min at RT. Incubation with rabbit polyclonal antibodies against proteins representing different receptor subunits of AMPA (GluR1, GluR2/3, GluR4), kainate (GluR5, GluR6/7, KA2), and NMDA (NR1, NR2A, NR2B) (Upstate) was done for 1 hr at RT. After being rinsed with PBS, the cultures were exposed to goat anti-rabbit immunoglobulins coupled to FITC (Cappel) for 30 min at RT. The coverslips were then rinsed and mounted (IV).

Stereotaxic injections (II)

In adult male Wistar rats anesthetized with 3.5% chloral hydrate (350 mg/kg intraperitoneally), stereotaxic coordinates were used for the right side of the hippocampus (Paxinos and Watson 1986). First, an injection of the KDI solution (1µl at 100 or 500 µg/ml concentrations) or NaCl (1µl) was given over a time-period of 5 min and followed by a second injection of kainic acid (1µl at 1µg/µl, Sigma) or NaCl (1µl). To prevent backflow, a pause of 5 min occurred after the second injection. The tissue-spreading of the injected solutions was determined by injections of methylene blue. The animals were decapitated by guillotine and the brains removed. Tissue damage from injected NaCl + kainic acid, KDI + kainic acid, and NaCl + NaCl was determined at the injection site by light microscopy and evaluated by assigning lesion points depending on extent of damage.

Whole-cell patch-clamp recordings (III)

Human neocortical neuronal cultures were placed under a microscope for patch-clamp recordings. Neurons were continuously superfused with an external solution at RT. During the recordings, the cells were whole cell-voltage clamped at -60 mV. The drugs were diluted in the external solution and applied to the cells with a three-channel stepper motor-driven fast-application system (Warner Instrument, Hamden, CT, USA). The test drugs, laminin-1 and laminin peptides, were first preapplied to cells, followed by a coapplication of the agonists: L-glutamate, concanavalin A (Sigma), SYM 2081, NMDA (Tocris, Avonmouth, UK) and test drugs. The current recordings were done with an Axopatch 200B amplifier and analyzed with pClamp 8.0 software (Axon Instruments Inc., Foster City, CA, USA).

Human embryonic kidney cells (HEK 293 cells) were cultured and transfected with recombinant GluR4 (AMPA) and GluR6 (kainate) receptor subunit cDNA clones in order to improve kainate current recordings and to compare the AMPA currents measured in neocortical neurons.

Focal cerebral ischemia (IV)

All rats were adult male Wistar rats anesthetized with ketamine hydrochloride and medetomidine hydrochloride. Based on the suture occlusion model (Takano et al., 1997), permanent focal cerebral damage was induced. Through a ventral midline incision, the right common carotid artery (CCA) and the right external carotid artery were uncovered, and a 4-0 nylon monofilament suture (Ethilon Nylon Suture, Ethicon Inc., Somerville, NJ, USA) with a coated silicone tip (Bayer, Leverkusen, Germany) was inserted into the right CCA via an arteriectomy approximately 3 mm below the right carotid bifurcation. Advancing the suture along the internal carotid artery approximately 17 mm above the carotid bifurcation allowed it to be lodged into the anterior cerebral artery, occluding the orifice of the middle cerebral artery and the posterior communicating artery. The control animals (sham operated) were exposed to the same procedures, except that the suture was inserted only 10 mm above the carotid bifurcation and withdrawn one minute later. The animals with permanent middle cerebral artery occlusion (MCAO) were divided into 1-, 2-, 3-, 7-, 14-, and 28-day groups according to their post-ischemic time-periods before cardiac perfusion. Following the various post-ischemic periods, the rats received 120 mg of sodium pentobarbital (Mebunat, Orion Company, Finland), and cardiovascular perfusion was executed with 200 ml of ice-cold 0.9% saline (Liu et al., 2001). The brains were removed, frozen in fine dry ice, and stored at -70°C for further investigation.

RESULTS

1. Effects of KDI peptide in experimental culture systems (Study I)

An experimental culture system (I) was designed to test effects of the KDI peptide on neurons in an environment with damaged glial cells. It was tested on neurons from human embryonic retina, spinal cord (SC), and neocortex, all plated on top of injured SC glial cells. Neurons plated without KDI peptide on the damaged glial cells showed poor neurite outgrowth. Adding KDI peptide to the culture medium in low concentrations of 0.035 to 1.0 µg/ml improved both neurite outgrowth and neuron survival. Neurons of SC origin preferred lower concentrations of KDI peptide (0.035-0.1 µg/ml) in terms of long neurites and number of viable neurons in comparison to neocortical neurons requiring higher KDI concentrations (0.1-1.0 µg/ml). Long neurites and survival of retinal neurons showed the best effect at a KDI concentration 0.1 µg/ml.

An experimental culture system (II) was designed to test the effect of KDI peptide on axonal growth on damaged myelin sheets. Its effect was tested by using embryonal bodies planted on top of sectioned SC. The embryonal bodies were placed on the white matter of the spinal cord and were thereby in direct contact with the underlying cross-sectioned myelin sheets known to prevent neurite regeneration. Neurites were visible growing inside the embryonal bodies but were unable to grow on top of the CNS myelin. Addition of KDI peptide (5-10 µg/ml) to the culture medium enabled outgrowth of neurites on top of the SC white matter, with long neurites extending from the embryonal bodies visible.

2. Protection against KA-induced hippocampal damage (Study II)

Hippocampal damage in rats resulted from stereotaxic injections of KA, and the effects of pre-injected KDI peptide on this damage showed that the unilateral injection of KA caused severe tissue damage visualized in hematoxylin-eosin (HE)-stained brain sections. The tissue damage left cavities around the injection site of the hippocampus and neocortex, and

sections stained for GFAP showed gliosis around the areas of destruction. Animals treated with pre-injections of KDI peptide (100 µg/ml) showed, however, less tissue damage around the hippocampal areas than in KA-injected animals but was unable to protect the neocortical areas. A higher dose of KDI peptide (500 µg/ml) was able to protect the hippocampal and neocortical areas, so that destroyed tissue appeared only around the immediate area of injection. Reactive gliosis was visible in the ipsilateral hippocampal and neocortical areas in animals treated with the higher dose of KDI before KA-induced injury. Injections of NaCl + NaCl resulted in increased gliosis around the injection track. Sections stained with GFAP showed more gliosis in KDI peptide (500 µg/ml) + KA-treated animals than did KDI peptide (500 µg/ml) + NaCl or NaCl + NaCl-injected animals. Tissue damage was quantified by lesion points at the injection site by visual scoring of the extent of the injury, and showed that preinjection of KDI peptide (500 µg/ml) was able to protect both hippocampal and neocortical tissue from KA-induced damage, even though the tissue at the immediate injection site showed signs of injury.

Kainic acid injections caused neocortical and hippocampal tissue degradation also on the contralateral side of the brain sections. Expression of γ1 laminin on the contralateral side of the hippocampus 10 days after NaCl + kainic acid injections showed reactive astrocytes in the CA1-layer but no immunoreactive neurons. Animals injected with KDI peptide (500 µg/ml) + KA into the CA1-layer of the ipsilateral hippocampus showed γ1 laminin-immunopositive neurons protecting them from KA damage. At the very site of the injection, the animals treated with KDI peptide (500 µg/ml) had a gap area lacking γ1 laminin-positive neurons and showed only immunoreactive glial fibers. Similarly, a negative gap in immunoreactivity appeared in the expression of laminin-1 in the CA1 layer of the hippocampus. Laminin-1 expression showed an increase at the injection area of the neocortex, and accumulation of laminin-1 punctate deposits were visible, indicating laminin-1 degradation. These laminin-1 deposits were absent from the KDI-treated brain sections.

3. Glutamate receptor inhibition by KDI peptide (Study III)

KA takes part in excitotoxic cell death through glutamate receptors. Since KDI peptide injection was able to protect against KA-induced tissue damage in rat brain (II), we studied the possible interactions of KDI with glutamate receptor function by using immunocytochemistry and patch clamp recording.

Immunocytochemistry on human embryonic neocortical neurons showed expression of several different glutamate receptor proteins. AMPA receptor subunit GluR1 expression was strongest in cell bodies; GluR2/3 expression occurred along neurites and cell bodies; and GluR4 immunoreactivity was homogeneous along both neurites and cell bodies. Kainate subunit immunoreactivity was expressed in GluR5 as punctate deposits along the neurites; GluR6/7 was detected along the more mature-looking pyramidal neurons; and KA2 receptor immunoreactivity along both neurites and cell bodies. NMDA receptor subunit immunoreactivity was seen in NR1 as a patchy expression along neurites, and both NR2A and NR2B showed weaker expression, mainly in the cell bodies of the neurons. Therefore, NMDA heterodimeric receptor proteins NR1/NR2A and NR1/NR2B were expressed by the cultured neurons.

Application of glutamate to the cultured neocortical neurons under conditions that inhibit NMDA receptors produced a fast desensitizing current that was mediated primarily through AMPA receptors, since application of a selective kainate receptor agonist (SYM 2081 together with concavalin A) produced only a small, insignificant current. Immunocytochemically, kainate-receptor subunits were evident, but here, neocortical neurons failed to express functioning kainate receptor currents. Inhibition of the AMPA current by pre- and co-application of KDI peptide (0.1-10 µg/ml) was detected as dose-dependent, washable, and reproducible. In comparison to laminin-1, and the decapeptide of γ1 laminin (RDIAEIIKDI), which also showed effective AMPA receptor inhibition, the KDI peptide was more efficient and showed a higher precentage of inhibition. Peptides from β1 laminin (CDPGYIGSR) and β2 laminin (LRE) did not affect the AMPA receptor currents. KDI peptide inhibition of AMPA receptor currents was seen as dose-dependent, and a concentration of 0.1 µg/ml showed 50% inhibition (IC50). Half the maximal effective concentration (EC50) of glutamate yielded values of 2.5 and 1.9 mM in the absence and presence of KDI peptide, indicating the noncompetitive nature of this inhibition. The KDI peptide pre-

application time needed for maximal inhibition was measured as short (62 ± 22 msec), and coapplication with glutamate produced lower inhibition of AMPA currents.

NMDA receptor currents measured from neocortical cell cultures were also inhibited by KDI peptide (0.1-10 µg/ml) by 25 to 50%.

Kainate receptor currents were undetectable in human neocortical neurons, although immunocytochemistry showed their expression. Human embryonic kidney cells (HEK 293 cells) transfected with the recombinant GluR6 receptor subunit were used to study the effects of the KDI peptide on kainate receptor currents. The kainate currents were sensitive to KDI peptide inhibition, showing a IC50 at 0.1 µg/ml and almost a total inhibition at 10 µg/ml. HEK cells transfected with GluR4 AMPA receptors and human neocortical neurons were equally sensitive to the KDI peptide.

4. Distribution of laminins in focal cerebral ischemia (Study IV)

Permanent MCAO was performed in adult rats in order to map the temporal and spatial expression of laminins associated with ischemic brain damage. All animals with right MCAO had left-sided hemiparesis after the operation in comparison to sham-operated rats, which showed no neurologic deficits.

Laminin-1 was expressed in sham-operated rats in vascular basement membrane structures symmetrically on both sides of the brain hemispheres; 24 h after ischemia laminin-1 was expressed in neurons and vascular basement membrane structures of the ischemic region, and 2 to 3 days after ischemia the vascular basement membranes expressed laminin-1 but with no immunoreactive neurons visible. Seven days after MCAO, however, a large necrosis area was detectable, with laminin-1-positive basement structures and scar-forming extracellular matrix (ECM). And 14 and 28 days after ischemic brain damage, the laminin-1-positive necrotic area was clearly visible as immunoreactive matrix and punctate deposition.

In the ventrolateral parts of the brain, γ1 laminin was already expressed 24 h after MCAO in reactive astrocytes surrounding the ischemic area, and at 2 to 3 days a γ1 laminin-positive belt of glial cells formed. At 24 h, immunoreactive astrocytes appeared inside the ischemic regions, and also γ1 laminin-positive neuronal cell bodies and dendrites of pyramidal neurons were detectable. At 7, 14, and 28 days, reactive astrocytes

heavily expressing γ1 laminin surrounded the necrotic area, separating it from the rest of the brain. Here, no neuronal γ1 laminin immunoreactivity was detectable. In the contralateral intact brain hemisphere, normal, mainly neuronal expression of γ1 laminin was evident and was similar to that in the sham-operated rats.

KDI peptide was expressed, in a similar manner to that of γ1 laminin, intensely in glial cells between the intact brain and the ischemic regions. Additionally, KDI immunoreactivity was visible along the major neuronal pathways such as the corpus callosum and in the fiber areas close to the ischemic regions.

Laminins α1, α2, α5, and γ2 were induced in reactive astrocytes surrounding the ischemic infarct areas, differing in their expression patterns and from the expression of γ1 laminin. Three days after infarction, expression of laminins α1 and α5 was induced in dendrites of the pyramidal cells inside the ischemic area in comparison to the contralateral side, which lacked any such immunoreactivity.

DISCUSSION

Functional properties of the KDI peptide

The exact molecular mechanisms of the neuroprotective effects of KDI peptide remain unknown even though many studies elucidate its neuroprotective effects, and it seems fair to claim that the KDI peptide probably has several mechanisms of action. Its neuroprotective potential against CNS trauma, SC injury (Wiksten et al., 2004), and injuries induced by neurotoxins (II) (Väänänen et al., 2006), in concert with its promotion of neurite outgrowth and neuronal survival (I), indicates different functional pathways. Evidence that the KDI peptide protects hippocampal tissue from KA, that it takes part in excitotoxic cell death through glutamate receptors, points toward a possible interaction between the glutamate system and the KDI peptide (II). Indeed, patch clamp recordings show that the KDI peptide is capable of inhibiting the ionotrophic glutamate receptors NMDA, AMPA, and kainate in a dose-dependent and non-competitive manner, with a 50% inhibition of the NMDA receptors and almost a complete inhibition of kainate and AMPA receptors (III).

Experiments defining the shortest active sequence of the decapeptide RDIAEIIKDI showed, by patch clamp recordings, that the KDI peptide induces potassium currents through a G-protein coupled mechanism in rat cerebellar neurons (Liesi et al., 2001). The outward rectifying currents may enhance neuronal survival by inhibiting repeated depolarizations and reducing Ca^{2+} influx into the neurons. The decapeptide RDIAEIIKDI has also been shown to have a dual neurotoxic/neurotrophic role: When it is applied in high amounts to neurons, a neurotoxic effect results, a neurotoxic effect that can be attenuated by pertussis toxin, which inhibits the potassium current caused by the decapeptide (Liesi et al., 2001). When the concentration of the KDI peptide increases from the optimal concentration inducing neuronal survival and neurite outgrowth, interestingly, a reduction in these neuroregenerative functions also is noticeable (I).

Through integrin β1 signalling and activation of the PI3-kinase/Akt pathway, laminin enhances resistance to glutamate-induced apoptosis in embryonic hippocampal neurons (Gary and Mattson, 2001). Intriguingly, a peptide (EIKLLIS) derived from the α chain of laminin also protects hippocampal neurons from apoptosis induced by glutamate,

and enhances Akt activity in a similar manner (Gary et al., 2003). A recent report suggests that intracellular pathways stimulating neuronal survival and neurite outgrowth in DRG neurons (PI3-kinase/Akt- and MEK/MAPK-pathway) are induced by simultaneous stimulation of both laminin and NGF, suggesting that collaboration of integrin and neurothrophin signalling is essential in DRG neuron regeneration (Tucker et al., 2008). Concerning KDI function, when discussing integrin signalling and laminin binding, what should be noted is that even though the KDI peptide shows laminin-like effects on neurite outgrowth, laminins are not known to bind integrins via the C terminus of γ1 laminin. It is therefore possible that KDI peptide function may also involve laminin receptor stimulation. More importantly, due to the size and specific functions of the KDI peptide and other laminin-derived peptides, they should be investigated according to their own specific functions and not be considered identical in function to the large laminin-1 molecule. In contrast, regarding functions of the laminin protein, the active peptides derived from laminin-1 provide important information.

These findings supporting the neurotrophic properties of the KDI peptide indicate involvement of a more classical receptor stimulation. Laminin receptors, 67kDa, α-dystroglycan, and integrins may be potential targets for activation involving secondary messenger systems and thereby may take part in axonal outgrowth and neural regeneration.

Laminin and the KDI peptide in neural regeneration

Laminin-1 is known to be a promoter of neuritic regeneration (Manthorpe et al., 1983). Its regenerative function has been mapped in the C-terminus of γ1 laminin to the decapeptide RDIAEIIKDI (Liesi et al., 1989) and from there, more specifically to the KDI tripeptide (Liesi et al., 2001). It has been proposed that γ1 laminin plays an important role in the guidance of commissural axons in the developing human spinal cord (Wiksten et al., 2003). In the lesioned postnatal rat, the hippocampal mossy fiber, an inhibition of γ1 laminin, mRNA translation leads to a clear reduction in regeneration, and when inhibition ceases, regeneration capacity is restored (Grimpe et al., 2002). Matrigel culture experiments show that axons from dorsal embryonic spinal cord prefer to grow axons towards the KDI peptide-containing matrix (Wiksten et al., 2003) indicating that the KDI peptide may serve as a chemoattractant for these neurons. Since laminin-1 overrides myelin-

derived inhibitory effects on neurite outgrowth (David et al., 1995), we tested the effects of the KDI peptide in a environment hostile toward neuronal survival and neurite outgrowth.

Environmental factors that prevent spinal cord regeneration after injury are mainly the formation of a glial scar and myelin-derived inhibition (see 4.1.1 and 4.1.2) and therefore serve as an example in the experimental culture settings (I).

In two different experimental culture systems mimicking spinal cord injury, the KDI peptide attenuated the glia-derived and myelin-derived inhibitory signals and provided a better substrate for neuronal survival and neurite outgrowth. Both viability and neuronal outgrowth were improved by addition of KDI peptide to the culture medium, regardless of the CNS origin of the cultured neurons used. Neurons derived from human embryonic spinal cord, neocortex, and retina reacted somewhat differently to the KDI peptide applied. Spinal cord neurons preferred a lower concentration of KDI peptide than did neocortical neurons for optimal neurite outgrowth and survival (I). This fact indicates that application of KDI in vitro supports neurons to overcome inhibition and grow axons on adverse surfaces such as injured glial cells and the white matter of the spinal cord (I).

The in vitro results supporting the KDI peptide as a regenerative factor in CNS injury led to in vivo experiments on spinal cord injury and kainic-acid induced injury in rats. Following complete transection of rat spinal cord, KDI peptide was applied directly to the area of injury through osmotic mini-pumps. The KDI-treated animals showed improvement in motor-scoring and were able to support their weight using their hind legs, in comparison to the paralyzed control animals. Immunohistochemistry showed improvement in regeneration of the damaged neural tracts (Wiksten et al., 2004). Aside from its possible stimulating effects on axonal regeneration, the KDI peptide may enhance regeneration by affecting inhibitory enviromental factors and thereby supporting neuronal survival and axonal growth. Interestingly, in KDI-treated spinal cord injury, both scar- and cyst-formation was reduced (Wiksten et al., 2004), possibly promoting the ability of axons to overcome inhibition and giving neurons a more beneficial environment in which to regenerate.

Study 1 used an in vitro model to mimic the major inhibitory signals against regeneration caused by SCI. Since no established in vitro model for this purpose was available, we developed a novel model of our own, where myelin inhibitory molecules primarily produced by oligodendrocytes were represented in one experimental setting and the astroglial glial scar in another. When planning in vitro experiments aiming to test SCI, many aspects should be taken into consideration since the cellular and molecular incidents

following CNS damage in vivo are numerous and could hardly all be tested simultaneously in one single experimental setting. Here in Study 1, our goal was to test the effect of the KDI peptide added to the culture media as the external source, and we designed the in vitro experiment to test its effect on neuronal viability and axonal growth on damaged astroglia and myelin sheets.

Regeneration might also be stimulated by reducing the molecular constituents of the glial scar, as seen in Study II. Reactive gliosis (II) and cyst formation (Wiksten et al., 2004) was reduced by KDI peptide, but this claim should be considered speculative since our experiments do not provide any such direct evidence.

The KDI peptide and CNS protection

The protection provided by the KDI peptide against hippocampal injections of kainic acid is interesting. Since KA, a glutamate analogue, is known to mediate excitotoxicity through glutamate receptors, the protective effects of KDI against this neurotoxin indicates a link between the laminins and the glutamate system. Hippocampal neurons from the CA1-CA3 region exert a high concentration of ionotropic glutamate receptors and are particularly vulnerable to KA (Ben-Ari, 1985). KA has also been associated with neurodegenerative disorders (Fahn and Sulzer, 2004), CNS trauma (Arundine and Tymianski, 2004), and neuronal death (Ben-Ari and Cossart, 2000).

A pre-injection of KDI peptide (500 µg/ml) was able to protect the hippocampal tissue against KA injections, leaving only a small destroyed area around the exact point of the injection. Animals pre-treated with NaCl injections showed severe destruction of hippocampal and neocortical tissue due to KA neurotoxic injections (II). Intact hippocampal neurons express γ1 laminin (Hager et al., 1998), and this expression is reduced by KA (Indyk et al., 2003). Similarly, in our experiments following NaCl + KA injections, expression of γ1 laminin in the contralateral hippocampus showed reactive astrocytes in the CA1-layer but no immunoreactive neurons. At the very site of injection, the animals treated with KDI peptide (500 µg/ml) had a gap area lacking γ1 laminin-positive neurons, and showing only immunoreactive glial fibers (II).

Tissue plasminogen activator (tPA) and its substrate plasminogen are associated with excitotoxic neuronal degeneration in the hippocampus. Deficiency of tPA or

plasminogen induces resistance against neuronal death caused by KA (Tsirka et al., 1997). Extracellular plasmin generation that could promote neuronal death is interesting to examine from a substrate point of view, since laminin cleavage may promote neuronal degeneration. Interestingly, laminin is normally expressed in the hippocampus but cannot be detected after KA injections, and its disappearance occurs just before neuronal cell death occurs (Chen and Strickland, 1997). Neuronal degeneration can be inhibited by tPA-deficiency or infusion of a plasmin inhibitor that also inhibits laminin degradation; in contrast, excitotoxic sensitivity can be restored in tPA-deficient mice by infusion of anti-laminin antibodies (Chen and Strickland, 1997). Disruption of the laminin hippocampal matrix by infusion of soluble laminin-1 or anti-γ1 laminin antibodies sensitizes neurons to KA-induced cell death (Chen et al., 2003). These results further establish the importance of the laminin matrix and γ1 laminin in regard to excitotoxic cell death, degradation of laminin, and neuronal survival. Fragments of degraded laminin, due to plasmin induction after KA injections, may have functional effects on surrounding cells. Naturally, of interest here would be a hypothetical KDI peptide that could be detected as a peptide sequence following degradation of laminin. Since KA injection induces laminin degradation and neuronal death, the protective effect of the KDI peptide is not liberated, possibly because of either excessive proteolytic degradation or of incomplete fragmentation.

Following injections of the neurotoxin 6-hydroxy-dopamine (6-OHDA) to the rat substantia nigra (SN), the KDI peptide has also shown protective effects on dopaminergic neurons (Väänänen et al., 2006). 6-OHDA is a dopamine analogue that forms reactive oxygen species inhibiting the mitochondrial electron transfer chain that leads to dopaminergic neuronal death and neuronal tissue degeneration (Schober, 2004). This neurotoxin is used to induce PD in rodents (Schober, 2004), but through its mechanism of action, it can also be viewed as an inductor of oxidative stress. Therefore, the protective effect of the KDI peptide against 6-OHDA-induced neurotoxicity and oxidative stress provides a new perspective on KDI neuronal protection that still needs to be further elucidated. One possible mechanism of protection may be through KDI inhibition of the glutamate receptors (III), since simultaneous glutamate excitation and 6-OHDA dopamine receptor activation may lead to the neuronal overexcitation, causing neuronal death.

In Study II, the damage caused by KA is dramatic and if applied in lower amounts the neurotoxic solution could test other more neurotropic effects of laminins and KDI because of the less damaging environment. Since other possible molecular pathways

of the KDI peptide remain to be revealed, a less damaging environment could be of value here. However, we preferred to use high doses in order to reach the "proof of principle" point. The ability to protect neurons against oxidative stress caused by 6-OHDA is shared with neurotrophins, and the mechanism remains unclear. Moreover, the resistance against KA-induced neurotoxicity in tPA-deficient mice and their unaffected laminin degradation calls for further studies that could elucidate other molecular mechanisms that stimulate neuronal viability.

The neuroprotective potential of the KDI peptide is strengthened by findings regarding its ability to inhibit ionotrophic glutamate receptors. The application of KDI peptide to neocortical neurons has shown in patch clamp recordings almost a complete inhibition of AMPA and kainate receptors and a 50% inhibition of NMDA receptors (III), providing evidence for a direct interaction between the KDI peptide and the glutamate system. The receptor inhibition was non-competitive and dose-dependent. Both laminin-1 and the decapeptide RDAEIIKDI of γ1 laminin also showed an inhibition of AMPA receptor currents but were less efficient than the KDI peptide (III). The acid form of the decapeptide of γ1 laminin and of KDI peptide is each a more efficient antagonist of glutamate receptors then is the amide form of each (III), indicating that they would be effective even if cleaved by proteolysis from the laminin-1 molecule.

These results indicate that a matrix protein or its fragments cannot only mediate its functions indirectly through cell-matrix signaling cascades but can interact directly with neurons through glutamate receptors. To the best of our knowledge, no previous studies have shown such direct electrophysiological receptor interaction, even though laminin has been shown to play a role in inwardly rectifying potassium channel aggregation in hippocampal cultures (Guadagno and Moukhles, 2004) and in direct binding to voltage-gated calcium channels in mouse motor neurons (Nishimune et al., 2004).

The induced glial cell expression of laminin-1 (Liesi et al., 1984, Bernstein et al., 1985) and γ1 laminin in pathological conditions such as SC trauma (Liesi and Kauppila, 2002), ischemic stroke (IV), or AD (Palu and Liesi, 2002) may have a protective effect on their surrounding neurons even though insufficient for neuronal tissue survival.

Laminins in ischemic stroke

Study of laminins in an experimental stroke model revealed that laminin-1, γ1 laminin, and its KDI tripeptide were expressed in reactive astrocytes in the transitional zone between the ischemic regions of necrosis and the rest of the brain. The reactive astrocytes also expressed α1, α2, α5, β1, β3, and γ2 laminin, but only γ1 laminin appeared visible in these cells specifically isolating the ischemic region as early as 24 h after stroke. Persistent in the actual infarct area, only laminin-1 was expressed 28 days after ischemic insult, whereas the other laminin subtypes were detected then mainly in glial cells in the vicinity of the infarction. Due to their differing spatial and temporal expression patterns, laminin-1 and γ1 laminin are interesting when discussing the involvement of laminins in the pathophysiology of stroke.

Expression of γ1 laminin and the KDI peptide by reactive astrocytes at the boundary of the ischemic brain region may be a protective measure or an epiphenomenon. However, since laminin-1 and γ1 laminin induction in glial cells occurs following various mechanical and chemical insults to the brain (Liesi et al., 1984)(II)(Wiksten et al., 2004), the protective hypothesis is the primary option. In this sense, what is noticeable is that in regenerating CNS tissues such as the mammalian olfactory bulb, astrocytes express laminin-1 (Liesi, 1985). The possible protective expression of γ1 laminin and KDI peptide following ischemic brain damage is supported by evidence that in rats the KDI peptide protects against mechanical SCI (Wiksten et al., 2004) and reduces neurotoxic damage caused by the glutamate analogue KA (II). Since the KDI peptide is an inhibitor of glutamate receptors and non-competitively inhibits the AMPA receptor, it may improve neuronal survival in many different CNS injury types (III). Excitotoxic brain damage is the major common pathway of neuronal damage in many types of CNS injury and a major mechanism of neuronal degeneration following ischemic stroke. By improving neuronal regeneration and supporting neuronal survival following excitotoxic insults, the inductive expression of γ1 laminin and its KDI peptide may strengthen repair mechanisms in the brain after cerebral ischemic damage. A study by Yepes and colleagues showed that after focal ischemic brain damage, application of neuroserpin for preservation of laminin, primarily in neurons within the CNS, reduced infarct volume and apoptosis. That immunohistochemistry revealed inhibition of proteolysis of laminin in basement membranes supports the hypothesis of the protective role of laminin in a stroke model (Yepes et al., 2000). In

contrast, inhibition of the expression of laminins can hamper the brain's regenerative capacity and should be avoided after cerebral injuries. A study by Gu and colleagues showed, in a mouse focal cerebral ischemia model, that matrix metalloproteinase-9 degrades laminin, leading to induction of neuronal apoptosis. Blocking of matrix metalloproteinase-9 activity with a specific thiarine gelatinase inhibitor SB-3CT, and thereby blocking laminin cleavage, reduces neuronal apoptosis (Gu et al., 2005), suggesting that laminins may possess anti-apoptotic properties.

SUMMARY AND CONCLUSIONS

The KDI peptide is a potentially neuroregenerative and neuroprotective peptide derived from laminin-1. The purpose of this study was to clarify the role of γ1 laminin and its KDI peptide in neuronal regeneration and further elucidate the role of laminins in the injured CNS. The in vitro studies presented here suggest that the KDI peptide stimulates neurite outgrowth and neuronal survival. When KDI peptide is added to their culture media, human embryonal SC, neocortical, and retinal neurons cultured in a hostile environment benefit in viability and neurite outgrowth. Neurons cultured on injured glial cells and on the white matter of sectioned SC are able to grow long neurites in the presence of KDI peptide. That the KDI peptide protects against hippocampal tissue damage caused by local injections of KA in vivo supports the in vitro results and indicates that the KDI peptide can protect against excitotoxic insults. To expand this hypothesis, our electrophysiological experiments interestingly show that the KDI peptide inhibits glutamate receptors in a non-competitive and dose-dependent manner with almost a complete inhibition of AMPA and kainate currents and a partial inhibition of NMDA receptor currents. Finally, in an experimental ischemic stroke model, we showed that laminin-1, γ1-laminin, and its KDI peptide are expressed in reactive astrocytes surrounding the ischemic regions, suggesting their involvement in the pathophysiology of this devastating disease.

Further experiments testing the protective effects of the KDI peptide in other neurodegenerative disorders would clarify its possible therapeutic potential. For example, testing the hypothesis of its potential involvement in cerebral stroke requires more extensive studies involving application of KDI peptide.

Exciting questions should be elucidated in the future involving the role of the laminins in maintaining the BBB: Do laminins stimulate neurogenesis in the dentate gyrus and the subventricular zone? How do laminins co-localize with newly formed neurons, and do they participate in forming pathways for neurons to migrate to regions of destruction?

Therapeutically, the KDI peptide may provide a new tool for neuronal regeneration and support the viability of neurons in degenerative CNS diseases. Our animal and cellular experiments show that if used at therapeutic concentrations, the KDI peptide is not only effective in regenerating and protecting the CNS but is also unlikely to produce overt adverse effects. A better understanding of KDI as a clinical therapy in treating CNS dis-

eases calls for more research. In addition, investigating the mechanisms at cellular and molecular levels through which the KDI peptide may support neuronal viability and protect against injury is vital for its development towards a therapeutic tool. In today's research, many successful biochemical strategies against neuronal death are emerging, and application of the KDI peptide together with other therapies could in fact aid in the struggle for neuroprotection and neuronal regeneration.

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