THE ROLE OF LAMININ IN DEVELOPMENT, REGENERATION AND INJURIES OF THE NERVOUS SYSTEM

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PREFACE

The experiments of the present study were carried out at the Institute of Biotechnology, University of Helsinki, and the Institute of Biomedicine, Department of Anatomy, University of Helsinki during the years 1992-2000.

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ABBREVIATIONS

Aβ	amyloid β-peptide
ACT	α-antichymotrypsin
AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
AMOG	adhesion molecule on glia
APP	β-amyloid precursor protein
BL	basal laminae
BMP	bone morphogenetic protein
CAM	cell adhesion molecule
CMD	congenital muscular dystrophy
CNS	central nervous system
CO	cytochrome c oxidase
CS	chondroitin sulfate
dbcAMP	dibutyryl cyclic adenosine mono phosphate
DCC	deleted in colorectal cancer
GAG	glycosaminoglycan
GFAP	glial acidic fibrillary protein
GIRK	G-protein coupled inwardly rectifying K^+ channel gene
DS	dermatan sulfate
ECM	extracellular matrix
EGF	epidermal growth factor
EHS	Engelbreth-Holm-Swarm
FAD	familial Alzheimer's disease
FGF	fibroblast growth factor
HC	hippocampus
HS	heparan sulfate
HSPG	heparan sulfate proteoglycans
KS	keratan sulfate
LSB-	Laemmli sample buffer without β-mercaptoethanol
Ng-CAM	neuron-glia cell adhesion molecule
N-CAM	neural cell adhesion molecules
NF	neurofilament
NMDA	N-methyl-D-asparate
PGs	proteoglycans
PNS	peripheral nervous system
PS-1	presenilin-1
PS-2	presenilin-2
RA	retinoic acid
RGC	retinal ganglion cell
RMP	resting membrane potential
RPE	retinal pigment epithelial
SAP	serum amyloid P
Shh	Sonic hedgehog
SN	substantia nigra
TGF beta 1	transforming growth factor beta 1
tPA	tissue plasminogen activator
TUJI	neuron specific β-tubulin III isoform

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original papers, referred to in the text by Roman numerals I-IV

I   S. Murtomäki, J. Risteli, L. Risteli, U.-M. Koivisto, S. Johansson and P. Liesi.: Laminin and its neurite outgrowth-promoting domain in the brain in Alzheimer's disease and Down's syndrome patients. *J. Neurosci. Res.* 32:261-273, 1992.

II.   S. Murtomäki, E. Trenkner, J.M. Wright, O. Saksela and P. Liesi: Increased proteolytic activity of the granule neurons may contribute to neuronal death in the weaver mouse cerebellum. *Dev. Biol.* 168, 635-648, 1995.

III.   T. Kauppila, E. Jyväsjärvi, S. Murtomäki, H. Mansikka, A. Pertovaara, I. Virtanen and P. Liesi: Use of paper for treatment of a peripheral nerve trauma in the rat. *Neuroreport.*, 8, 3151-3155, 1997.

IV   S. Murtomäki, I. Virtanen and P. Liesi: Neurofilament proteins are constitutively expressed in F9 teratocarcinoma cells. *Int. J. Dev. Neurosci*, 17, 829-838, 1999.

INTRODUCTION

Mechanisms of neuronal migration, neurite outgrowth, neuronal degeneration and neuronal regeneration have been the main focus of neurobiological research for the past hundred years. Even though several major principles have emerged, molecular mechanisms of brain development and neuronal injuries are still largely unknown. In recent years, investigators have concentrated on identification of molecules involved in cell-to-cell interactions in the nervous system. Laminin-1, originally isolated from a mouse tumor (so called EHS sarcoma), rich in basement membrane proteins, has been shown to be one of the key molecules in nervous system development and response to trauma. Laminin-1 promotes neurite outgrowth, guides neuronal migration, promotes neuronal regeneration and is involved in neuronal differentiation as well as death mechanisms.

As soon as these nervous system related general functions of laminin-1 were identified, attempts began to 1) determine specific domains of laminin-1 that mediate various functions of this protein and 2) understand the molecular interactions that mediate the different functions of laminin-1. Thusfar, five neurite outgrowth promoting domains have been identified in the laminin-1 molecule: One in domain I of the γ1-chain, one in the α1-chain close to the G-domain and three in the G-domain of the α1-chain. The most studied of these neurite outgrowth domains is a decapeptide derived from the carboxy terminal part of the γ-1-chain of laminin-1. This domain is involved in neuronal migration and axon guidance, and has a dual neurotrophic/neurotoxic role. Synthetic peptides derived from this domain have a neurotoxic effect when applied to neurons at high (micromolar) concentrations whereas low (nanomolar) concentrations promote neurite outgrowth and neuronal survival in both soluble and substrate-bound forms.

After the neurite outgrowth domain of the γ1-chain of laminin-1 was identified, the Aβ peptide, a major constituent of plaques in Alzheimer's disease, was also shown to have a concentration dependent dual neurotrophic/neurotoxic effect. This effect was thought to result in neuronal degeneration and/or sprouting of neurites detected in the plaques. Thus, it became relevant to investigate the distribution of laminin-1 and its γ1-chain peptide in Alzheimer's disease. Neurological mutants with known defects in neuronal survival and migration, such as the weaver mutant mouse, offer suitable model systems to study the molecular mechanism of neuronal death and migration defects. We used both normal and

weaver mutant mice to elucidate the possible roles of laminin-1, its γ1-chain and tissue plasminogen activator in the migratory failure of the homozygous weaver granule neurons, and in death of the weaver cerebellar granule neurons.

Few attempts have been made to use laminin-1 or its neurite outgrowth promoting γ1-chain decapeptide in repair of CNS injuries. In PNS injuries, a number of different laminin-1 grafts have been applied. In PNS, both laminin-1 and the γ1-chain decapeptide allowed neurorraphy and reduced autotomy pain caused by sciatic nerve injuries. However, the laminin-grafts were thick and caused severe compression, if used to repair thin nerves. Therefore, they could only be used on large nerves. We initiated trials to develop more suitable thin materials that could be used to form the back bone of the grafts for attachment of laminin-1 and its biologically active peptides. These grafts could then be tested in attempts to regenerate both peripheral nerve and spinal cord injuries.

Laminin-1 has been shown to induce neuronal differentiation of both early chick neural tube cells and retinal neuroepithelial cells. Neuronal differentiation is finalized by cessation of cell division by the fully differentiated neurons. Molecules and mechanism involved in neuronal differentiation are poorly understood. In recent years, the ability to isolate and maintain neuronal precursor cells in culture has allowed molecular and biochemical analysis of neuronal differentiation. Teratocarcinoma cells have been used as alternative model systems in studies on early neuronal development. For example, the mouse F9 teratocarcinoma cells have been shown to differentiate into neuron when cultured in the presence of both retinoic acid (RA) and dibutyryl cyclic adenosine monophosphate (dbcAMP) in low serum concentrations. However, neuronal differentiation of the F9 cells occurs in a subclass of the cells while a large proportion of the cells still remain undifferentiated. To enable the use of F9 cells in molecular studies on neuronal differentiation, we subcloned the F9 cells into a homogeneous population of cells that expressed neuronal characteristics on regular tissue culture plastics without any inductive agents. This novel cell line will be used in further studies to investigate the mechanisms of neuronal differentiation.

REVIEW OF LITERATURE

1. Laminin

1.1. General features of laminins

Laminins form a growing family of large secretory glycoproteins with diverse functions and cellular distributions. The first, and best characterized laminin molecule, laminin-1, was purified from the Engelbreth-Holm-Swarm tumor (Timpl *et al* 1979). This prototype of laminin consists of three different, but related polypeptide chains, A (400 kDa), B1 (210 kDa) and B2 (200 kDa), all coded by different genes (Beck *et al* 1990; Engel *et al* 1993) (Figure 1). Laminin-1 is a constituent of basement membranes, and antibodies raised against laminin-1 cross-react with laminins from various tissues and species. At present, 14 different laminin chains have been cloned (Table 1) and shown to assemble into 12 different heterotrimeric molecules (Table 2). Currently, there are five different α-chains, three different β-chains and six different γ-chains, including netrins 1-3. The domain assembly of the different laminins is shown in Figure 2.

Table 1. Different chains of laminin and their respective genes in humans

Chain	Gene	Reference
α1	LAMA1	Nissinen *et al*., 1991
α2	LAMA2	Vuolteenaho *et al*., 1994
α3A	LAMA3A	Ryan *et al*., 1994
α3B	LAMA3B	Ryan *et al*., 1994
α4	LAMA4	Iivanainen *et al*., 1995
α5	LAMA5	Durkin *et al*., 1997 (partial)
β1	LAMB1	Pikkarainen *et al*., 1987
β2	LAMB2	Wewer *et al*., 1994
β3	LAMB3	Gerecke *et al*., 1994
γ1	LAMC1	Kallunki *et al*., 1991
γ2	LAMC2	Kallunki *et al*.,1992
γ3	LAMC3	Koch *et al*., 1999
γ4 (netrin-1)	LAMC4	Meyerhardt *et al*., 1999
γ5 (netrin-2)	LAMC5	van Raay *et al*., 1997

Table 2. Chain assembly of laminins.

Old name	New name	Chain assembly	Reference
EHS-laminin	laminin-1	α1β1γl	Timpl *et al*., 1979
merosin	laminin-2	α2β1γl	Ehrig *et al*., 1990
S-laminin	laminin-3	α1β2γl	Engvall *et al*., 1990
S-merosin	laminin-4	α2β2γl	Engvall *et al*., 1990
kalinin/ nicein/ epiligrin	laminin-5	α3β3γ2	Rousselle *et al*., 1991
K-laminin	laminin-6	α3β1γl	Marinkovich *et al*., 1992
KS-laminin	laminin-7	α3β2γl	Champliaud *et al*.,1996
	laminin-8	α4β1γl	Miner *et al*., 1997
	laminin-9	α4β2γl	Miner *et al*., 1997
	laminin-10	α5β1γl	Miner *et al*., 1997
	laminin-11	α5β2γl	Miner *et al*., 1997
	laminin-12	α2β1γ3	Koch *et al*., 1999
netrin-1	laminin-13	γ4	Serafini *et al*., 1994
netrin-2	laminin-14	γ5	Serafini *et al*., 1994
netrin-3	laminin-15	γ6	Wang *et al*., 1999

1.2. Nomenclature of laminins

The new nomenclature of laminins (Burgeson *et al.*, 1994) was established to allow the identification of a growing number of proteins composed of different α, β and γ subunits. The first cloned chains of laminin, A, B1 and B2 chains, were re-named as α1, β1 and γ1 chains, respectively. The newly identified laminin chains, bearing homology to the first α, β, and γ chains, are then named as α, β and γ 2,3,4 etc. in the chronological order of

publication. The EHS-tumor laminin is re-named as laminin-1 ($\alpha1\beta1\gamma1$). The laminin containing a variant A-chain, merosin, is called laminin-2 ($\alpha2\beta1\gamma1$), and that containing a variant B1 chain, s-laminin, is called laminin-3 ($\alpha1\beta2\gamma1$). S-merosin, a variant of both A- and B-chains, is laminin-4 ($\alpha2\beta2\gamma1$). Kalinin/nicein, a variant of all three chains is laminin-5 ($\alpha3\beta3\gamma2$), k-laminin, another variant of the A chain is laminin-6 ($\alpha3\beta1\gamma1$), and ks-laminin that has both variant A- and B1-chains is laminin-7 ($\alpha3\beta2\gamma1$; Burgeson *et al* 1994).

1.3. Structure of laminin-1

Rotatory shadowing electron microscopy revealed laminin-1 as a cross-shaped molecule with one long and three short arms (Engel *et al.*, 1981). The primary structures of both mouse and human laminin-1 (Pikkarainen *et al.*, 1987; Pikkarainen *et al.*, 1988; Nissinen *et al.*, 1991; Sasaki *et al.*, 1987; Sasaki and Yamada, 1987; Sasaki *et al.*, 1988) are consistent with the ultrastructure of the purified protein (Sasaki *et al.*, 1988). The polypeptide chains of laminin-1 consist of several independent domains. The $\alpha1$-chain has nine distinct domains (Sasaki *et al.*, 1988), the $\beta1$-chain is composed of seven different domains (Sasaki *et al.*, 1987), and the $\gamma1$-chain has six unique domains (Sasaki and Yamada, 1987; Figure 1). The primary structural data suggest that laminin-1 consists of three closely related short arms, each composed of the N-terminal regions of the $\alpha1$, $\beta1$ or $\gamma1$ chains (domains III-VI), one long arm (domains I and II) in which all the three chains associate into one rod like structure, and a globular G-domain present in the $\alpha1$-chain only (Figure 1). The three polypeptide chains ($\alpha1$, $\beta1$, and $\gamma1$) of laminin-1 are linked to each other by disulfide bonds in domain II near the center of the laminin cross and in domain I near the globular C-terminal part of the $\alpha1$-chain (Engel 1993).

Laminin-1 contains a large number of cysteine-rich motifs in its short arms. These motifs bear a structural homology to epidermal growth factor (EGF; Engel, 1989). The $\alpha1$-chain has three cysteine-rich motifs in domains IIIa, IIIb and V (Sasaki *et al.*, 1988). In the $\beta1$- and γ-1 chains two cysteine-rich repeats are located in domains III and V (Sasaki *et al.*, 1987; Sasaki and Yamada, 1987).
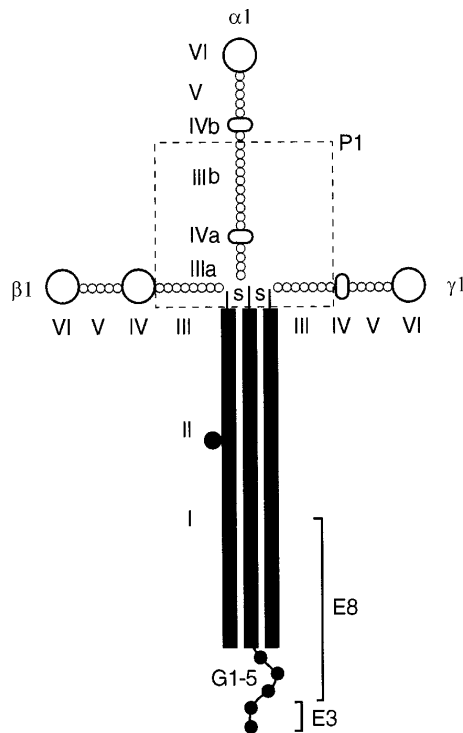
Figure 1. The domain structure and proteolytic fragments of laminin-1. The short arms of all the laminin chains have the same domain organization. Each short arm has two kinds of globular domains, VI and IV, which are separated by rod-like domains V and III. According to sequence analysis, domain VI has a mixture of α-helix, β-sheet and random coil structures. Domain VI has several cysteine residues. Domain IV has two unrelated structures, IV typical for most α and γ chains and IV′ specific for the β-chains. The IV domains, like the VI domains, have a mixture of α-helix, β-sheet and random coil structures, but no cysteines to allow the formation of disulfide bonds. The α-chain has two IV- domains, IVa and IVb. The rod-like domains V and III have cysteine-rich repeats, that show structural similarity to the EGF-like repeats, with the exception that the laminin repeats have eight cysteines, whereas the EGF-like repeats have only six cysteines. The cysteine repeats of laminin are named "laminin-type cysteine-rich repeats". The long arm is a heterotrimeric rod-like structure composed of all three chains forming domains I and II and a globular G domain composed of α-chain only. In the β-chain there is also an α-domain between domains I and II. The proteolytic enzymes used for limited proteolysis of laminin to produce various proteolytic fragments are pepsin (P), pancreatic elastase (E) trypsin and cathepsin G. The proteolytic fragments are named according to the enzyme used for proteolysis.

Laminin-1 is a highly glycosylated protein with a carbohydrate content of approximately 13-15% (Chung *et al.*, 1979; Arumugham *et al.*, 1986) that accounts for up to 25-30% of its molecular weight (Knibbs *et al.*, 1989). There are 74 potential N-glycosylation sites in the laminin-1 molecule (Beck *et al.*, 1990). A functional role for glycosylation of laminin has been reported in tumor cell adhesion, cell spreading, neurite outgrowth and integrin-laminin interactions (Dennis *et al.*, 1984; Bouzon *et al.*, 1990; Dean *et al.*, 1990; Chandrasekaran *et al.*, 1991; Chammas *et al.*, 1991). Some of these functions have also been reported for synthetic peptides derived from the laminin-1 sequence devoid of the sugar chains (see Table 4 for references). Thus, it is possible that glycosylation may have a regulatory role in multiple functions of laminin-1.

A short sequence (up to 100 to 200 amino acid residues) at the carboxy terminus of each chain is required for the assembly of laminin-1 polypeptide chains into double- and triple-stranded coiled-coil structures (Utani, *et al.*, 1994, 1995b). The β and γ chains first form a dimeric structure followed by a more stable trimer formation with the α chain (Peters *et al.*, 1985; Utani, *et al.*, 1994, 1995b; Nomizu *et al.*, 1996).

Thusfar, primary structures of human α1-α5 (partial), β1-β3 and γ1-γ5 chains have been identified (for references see Table1). The primary structures of the α1-α5, β1-β3, γ1-γ4 and γ6 chains of the rodent laminins are sequenced (Sasaki *et al.*, 1988; Bernier *et al.*, 1994, Galliano *et al.*, 1995; Miner *et al.*, 1997; Liu and Mayne, 1996; Miner *et al.*, 1995; Sasaki *et al.*, 1987; Hunter *et al.*, 1989a; Utani *et al.*, 1995a; Sasaki and Yamada 1987; Sugiyama *et al.*, 1995; Iivanainen *et al.*, 1999; Serafini *et al.*, 1996; Wang *et al.*, 1999). The only laminin gene found to encode for more than a single polypeptide chain is the laminin α3 gene (Ryan *et al.*, 1994). The α3B-chain lacks domains V and VI (Ryan *et al.*, 1994). In addition to domains V and VI, the α3A- and α4-chains miss also domains IVb, IIIb and IVa (Ryan *et al.*, 1994; Iivanainen *et al*., 1995). The β3-chain does not have a domain IV´ found in the β1- and β2 chains (Gerecke *et al.*, 1994). The γ2-and γ3-chains are devoid of the globular domain VI (Kallunki, *et al.*, 1992; Koch *et al.*, 1999). The γ4-6 chains (netrins 1-3) differ from the other γ-chains by having only the domains VI and V in addition to the C-terminal domain unrelated to other laminins (Meyerhardt *et al*., 1999; van Raay, *et al.*, 1997; Wang *et al.*, 1999). It is also believed, although not verified, that netrins are secreted from cells as a single polypeptide chain. The domain organization of different laminin chains is presented in Fig. 2. All variants of the α-, β- and γ-chains have

structural homology within domains I and II, although sequences coding for these domains are only 20-40% conserved (Engvall and Wewer, 1996). The sequence of the domain VI is the most highly conserved amongst the various laminin chains and amongst the same subunits from different species (Engvall and Wewer, 1996). The sequence of the globular IV domain is also highly conserved (Engvall and Wewer, 1996).
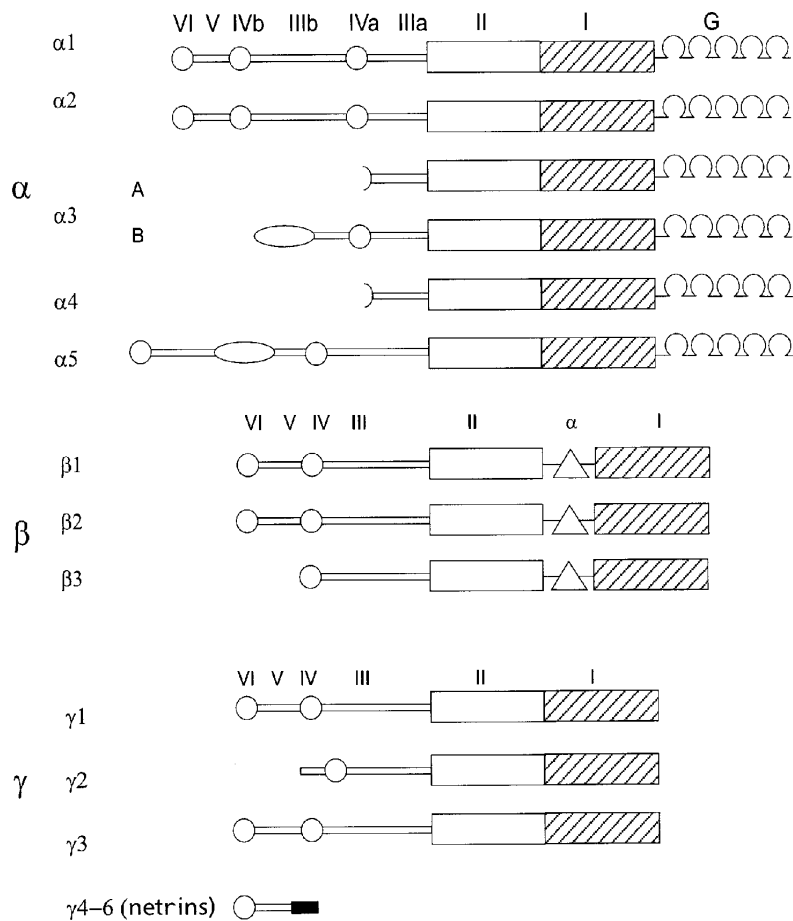

Figure 2. The domain (I-IV) organization of different laminin subunits.

1.4. The biological functions of laminin

The importance of laminins in mammalian development is emphasized by the fact that laminin-1 is the first extracellular matrix protein present in between cells of the developing mouse embryo. This was first demonstrated at the morula stage (Leivo *et al.*, 1980), and more recently by using different antibodies, at the 2-cell stage (Cooper and McQueen, 1983; Dziadek and Timpl, 1986). Expression of the specific chains of laminin is spatially and temporarily regulated, which means that each chain is present in a particular tissue at defined periods of time (Aumailley and Krieg, 1996). For example, the laminins in the adult brain are largely components of basement membranes whereas in developing brain laminins exist also in a soluble less polymerized state (Liesi, 1990; Edgar, 1991). The differential expression of the various subunits of laminin has been demonstrated for both developing and adult nervous tissue (Liesi, 1990; Edgar, 1991; Hunter *et al.*, 1992a,b: Jucker *et al.*, 1996; Luckenbill-Edds, 1997; Raabe *et al.*, 1997; Powell *et al.*, 1998; Table 5). The laminins of both *Drosophila* (Fessler *et al.* 1987) and sea urchin (McCarthy *et al.* 1987) resemble mammalian laminin-1 both structurally and functionally. Goldfish and frog central nervous system (CNS) laminins are antigenically close enough to the mammalian laminin-1 to be recognized by antibodies against mouse laminin-1 (Liesi 1985b). Leech laminin β1-chain shows homology to human, *Drosophila,* and mouse laminin β1-chains (Luebke *et al.*, 1995). Furthermore, both the mammalian netrin genes and the *unc-6* genes of *C. elegans* code for proteins that are homologues of the γ1-chain of laminin-1 (Serafini *et al.*, 1994; Wang *et al.*, 1999; Hedgecock *et al.*, 1990; Ishii *et al.*, 1992). These results indicate that laminins are highly concerved molecules throughout evolution. The high phylogenetic preservation of laminins implies that these proteins serve in fundamental roles throughout the species.

Laminins are essential for the architecture of basement membranes by participating in their assembly, and by maintaining the differentiated states of epithelial and endothelial cell layers, intimately associated with the basement membranes (Yurchenco *et al.*, 1992; Yang *et al.*, 1998). Formation of a laminin-1 and type IV collagen network is considered a major event in the supramolecular organization of basement membranes (Timpl and Brown, 1994; Timpl, 1996). Laminin-collagen networks are thought to be stabilized by other molecules, such as nidogen (Dziadek, 1995), heparan sulfate proteoglycans (Timpl, 1994), agrin (Denzer *et al.*, 1995), perlecan (Iozzo *et al.*, 1994), fibulin-1 (Sasaki *et al.*, 1995b), fibulin-2 (Sasaki *et al.*, 1995a), and BM-40 (Hohenester *et al.*, 1996), an anti-adhesive glycoprotein involved in tissue remodeling.

The identified functions of laminins include promotion of attachment and spreading of various cell types, such as hepatocytes (Johansson *et al.*, 1981), fibroblasts (Couchman, *et al.*, 1983), tumor cells (Vlodavsky and Gospodarowicz, 1981; Terranova *et al.*, 1982, 1983; Malinoff *et al.*, 1983), and neuronal cells (Baron van Evercooren, 1982; Liesi *et al.*, 1984a). Laminin-1 also stimulates the growth of Schwann cells (McGarvey *et al.*, 1984), primary renal cortical tubular epithelial cells (Oberley and Steinert, 1983), F9 cells (Rizzino *et al.*, 1980), and bone-marrow-derived macrophages (Ohki and Kohashi, 1994). Laminin-1 can stimulate DNA synthesis and growth of cells possessing receptors that are functionally or topologically associated with the EGF receptors (Panayotou *et al.*, 1989). Laminin-1 also functions in promoting epithelial cell polarity (Martin and Timpl, 1987; Klein *et al.*, 1988), neurite outgrowth (Baron van Evercooren *et al.*, 1982; Liesi *et al.*, 1984a; 1989), axon guidance (Liesi and Silver, 1988; Letourneau *et al.*, 1988), neuronal migration (Liesi 1985a, Liesi *et al.*, 1995), and regeneration of both central and peripheral nervous systems (Hopkins *et al.*, 1985; Liesi 1985b; Madison *et al.*, 1985, 1987; Kauppila, 1993). Laminin-1 induces the formation of capillary-like structures by endothelial cells *in vitro* (Kramer *et al.*, 1989), and influences macrophage development and function by inducing secretion of cytokines, such as IL-6 and TNF-alpha (Armstrong and Chapes, 1994).

Laminins are involved in wound healing, and regeneration of several non-neuronal tissues. E.g. laminin-5 expression is upregulated in skin wounds (Ryan *et al*., 1994). The α3-chain (Ryan *et al*., 1994; Goldfinger *et al*., 1999) and γ2-chain (Kainulainen *et al*., 1998) of laminin-5 are shown to be essential for epithelial migration and wound closure. The interaction of hepatocytes and laminin-1 has also been shown to be important in liver regeneration, possibly in growth stimulation of hepatocytes and/or maintenance of hepatocyte-specific functions (Kato *et al.*, 1992; Wewer *et al.*, 1992). During liver regeneration, the level of the α1-chain expression increases progressively from 12 hours after partial hepatectomy to the end of the regenerative period (Giménez *et al.*, 1995). Expression of the β1- and γ1-chains increases rapidly during the first day after resection of the liver, and decreases gradually reaching normal levels at the end of the regenerative period (Giménez *et al.*, 1995). Furthermore, during the regenerative period of intestinal epithelia, laminin β1- and γ1-chains are expressed differentially indicating their role in the healing process (Goke *et al.*, 1996).

In addition to the function of laminins in normal tissues, laminins and their cellular receptors may have a significant role in tumor cell growth and metastasis (Yamamura *et al.*, 1993; Menard *et al.*, 1998). Laminin-1 has been shown to promote metastatic activity of tumor cells by stimulating their attachment and migration as well as by promoting their degradation of extracellular matrix (Yamamura *et al.*, 1993; Menard *et al.*, 1998). Several synthetic peptides derived from laminin-1 play a role in tumor cell metastasis (see Table 4). E.g. the YIGSR peptide, derived from the short arm of β1 chain of laminin-1, has been shown to inhibit tumor cell growth and metastasis (Nomizu *et al.*, 1993; Iwamoto *et al.*, 1996). Another β1-chain peptide, PDSRG, has also been shown to inhibit tumor cell metastasis (Kleinman, *et al.*, 1989). The RGD peptide, derived from the cell recognition site of fibronectin inhibits tumor invasion and metastasis (Saiki *et al.*, 1990; Fujii *et al.*, 1996). This RGD sequence is also identified in the α1-chain of laminin-1 (Tashiro *et al.*, 1991).

A number of point mutations in the various chains of laminins have been linked to human diseases. For example, mutations in α3, β3 and γ2 -chains of laminin-5 have been reported in patients with junctional epidermolysis bullosa (McGrath *et al.*, 1995; Aumailley and Grieg, 1996; Aumailley and Smyth, 1998). The dy^{2j} mice with a truncated domain VI of the α2-chain of laminin-2 lack a structurally identifiable basement membrane in their peripheral nerves (Xu *et al.*, 1994). In humans, mutations in domains VI and I of the laminin-2 α2-chain (laminin-2) gene have been found in patients with congenital muscular dystrophy (CMD; Helbling-Lecler *et al.*, 1995; Hayashi *et al.*, 1997; Gullberg *et al.*, 1999). Complete absence of the α2-chain is a characteristic of some congenital muscular dystrophies (Tomé *et al.*, 1994; Gullberg *et al.*, 1999). The α2-chain deficiency of CMD is also reported to lead to extensive brain abnormalities (Sunada *et al.*, 1995). This result suggests that the α2-chain of laminin is important in the development of CNS. In *Drosophila*, mutations of the α1-chain, the only α-chain identified in this species, is embryonically lethal (Henchcliffe *et al.*, 1993), leading to defects in numerous tissues, such as brain, heart, gut and somatic muscles (Yarnitzky and Volk, 1995; Garcia-Alonso *et al.*, 1996).

Attempts to produce knockouts for most chains of laminins have failed, which further emphasizes their fundamental importance for embryonic development. The α2-chain null mutant mice are characterized by growth retardation and symptoms of severe muscular dystrophy. These animals die by the age of 5 weeks (Miyagoe *et al.*, 1997). In the α3-chain deficient mice the formation of hemidesmosomes is perturbed and functional interaction between laminin-5 (α3β3γ2) and integrin α6β4 is disrupted (Ryan *et al.*, 1999). The α3-chain deficient mice die 2-3 days after birth (Ryan *et al.*, 1999). Mice lacking the α5-chain of laminin have multiple developmental defects, including a failure of closure of the anterior neural tube (Miner *et al.*, 1998). Embryos lacking laminin α5-chain die late in embryogenesis (Miner *et al.*, 1998). The function of the β2-chain of the laminin-3 has been tested in β2-chain knockout mice (Noakes *et al.*, 1995a, b). Mice lacking the β2-chain show aberrant synaptic differentiation of their neuromuscular junctions and die within 4 weeks after birth (Noakes *et al.*, 1995a). These results suggest that laminin-3 regulates the formation of motor nerve terminals (Noakes *et al.*, 1995a). The β2-chain deficient knockout mice also show impaired glomerular ultrafiltration and kidney function (Noakes *et al.*, 1995b) as well as abnormal retinal development (Libby *et al.*, 1999). Targeted deletions of the γ1-chain in mouse embryonic stem cells cause early embryonic lethality (Smyth *et al*., 1999). The γ1-chain-deficient embryos lack basement membranes and die by the embryonic day 5.5 (Smyth *et al*., 1999). Netrin-1 knockout mice exhibit defects in spinal commissural axonal projections and several forebrain commissures and die within a few days (Serafini *et al.*, 1996). Mice lacking a functional netrin-1 receptor (DCC, deleted in colorectal cancer) have similar defects in their axonal projections as shown in netrin-1 knockout mice (Fazeli *et al.*, 1997; Deiner and Sretavan, 1999).

1.5. Analysis of the functions of laminin-1 using proteolytic fragments and synthetic peptides

Several studies have identified specific functional domains of laminin-1 polypeptide chains. Initially such functional domains were identified by isolating proteolytically cleaved and biochemically purified fragments of laminin-1 (Timpl and Martin, 1987; Paulsson, 1992). The location and biological functions of these proteolytically cleaved fragments are summarized in Table 3.

Table 3. Functions of proteolytic fragments of laminin. Abbreviations C, E, P and T indicate the proteolytic enzyme used; C, chymotrypsin; E, elastase; P, pepsin and T, trypsin. See Fig 1. for positions of proteolytic fragments in the laminin-1 molecule.

Proteolytic fragment	Functions of the fragment	Reference
P1	Cell attachment, Type IV collagen binding Nidogen binding Mitogenic	Aumailley *et al*., 1987 Paulsson *et al*., 1987 Panayotou *et al*., 1989
E1	Mitogenic	Panayotou *et al*., 1989
E4	Inhibition of Ca^{2+} induced aggregation of laminin	Schittny and Yurchenco, 1990
C1-4	Ca^{2+} dependent polymerization	Bruch *et al*., 1989
C8-9	Not detected	Bruch *et al*., 1989
E8	Cell attachment Promotion of neurite outgrowth Heparin binding	Aumailley *et al*., 1987 Edgar *et al*., 1984 Edgar *et al*., 1984
T8	Cell attachment	von der Mark, *et al*., 1991
E3, C3	Antibodies against 25K inhibit neurite outgrowth Binding of heparin and heparansulfate	Edgar *et al*., 1988 Ott *et al*., 1982

More accurate information on the specific functional domains of laminin-1 has been obtained using synthetic peptides. The peptides, their functions, and their positions within the laminin-1 molecule are listed in Table 4. Some of the identified peptides exhibit multifunctional properties.

Neurite outgrowth function of laminin-1 has been mapped to five different peptide domains. One is in the domain I of the γ1-chain (Liesi *et al.*, 1989b), another in the α1-chain just before the G-domain (Tashiro *et al.*, 1989), and the remaining three in the domain G of the α1-chain (Skubitz *et al.*, 1991). Other neuronally active peptides have been localized in the β2-chain of laminin-3 (LRE; Hunter *et al.*, 1989b, 1991).

Table 4. Some of the biologically active synthetic peptides of laminins and their functions. Abbreviations for amino acids: A alanine, R arginine, N asparagine, 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.

	Peptide	Function	Reference
α1 I	CSRARKQAAS IKVAVSADR	Cell adhesion, neurite outgrowth	Tashiro *et al*., 1989
		Stimulation of metastasis, collagenase production	Kanemoto *et al*., 1990
		Bone cell differentiation	Vukicevic *et al*., 1990
		Binding of 110 kDa cell surface protein	Kleinman *et al*., 1991
		Stimulation of plasminogen activation	Stack *et al*., 1991
		Signal transduction, cell growth	Kubota *et al*., 1992
		Promotion of angiogenesis and tumor growth	Kibbey *et al*., 1992
		Tumor growth, colony formation	Yamamura *et al*., 1993
		Binding of APP	Kibbey *et al*., 1993
		T-lymphocyte adhesion	Weeks *et al*., 1994
		Disruption of gastulation in sea urchin	Hawkins *et al*., 1995
α1 G α1 III	RGD	Endothelial differentiation	Grant *et al*., 1989
		Cell adhesion	Aumailley *et al*., 1990
		Cell adhesion, spreading,	Tashiro *et al*., 1991
α1 IVa	LSNIDYILIKAS	Metastasis promotion	Kuratomi *et al*., 1999
α1 IVb	RDQLMTVLAN VT	Metastasis promotion	Kuratomi *et al*., 1999
α1 IVb	SINNTAVMQRL T	Metastasis promotion	Kuratomi *et al*., 1999
α1 VI	RQVFQVAYIIIK A	Metastasis promotion	Kuratomi *et al*., 1999

α1 G	KQNCLSSRASF RGCVRNLRLSR	α3β1 integrin binding	Gehlsen et al., 1992
α1 G	SINNNR	Alveolar formation, cell adhesion Cell adhesion	Matter and Laurie, 1994 Chen et al., 1997b
α1 G	KATPMLKMRT SFHGCIK,	Cell adhesion, heparin binding, neurite outgrowth	Skubitz et al., 1991
α1 G	KEGYKVRDLNI TLEFRTTSK	Cell adhesion, heparin binding, neurite outgrowth Binding of α3β1 integrin	Skubitz et al., 1991 Pattaramalai et al., 1996
α1 G	KNLEISRSTFDL LRNSYGRK	Cell adhesion, neurite outgrowth	Skubitz et al., 1991
α1 G	DGKWHTVKTE YIKRKAF	Cell adhesion, neurite outgrowth	Skubitz et al., 1991
α1 G	RKRLQVQLSI RT	Neurite outgrowth Metastasis promotion Inhibition of branching epithelial morphogenesis Stimulation of matrix metalloproteinase secretion	Richard et al., 1996 Kim et al.,1998 Kadoya et al., 1998 Weeks et al., 1998
α2 G	KNRLTIELEVR T	Neurite outgrowth	Richard et al., 1996
β1 III	YIGSR	Cell adhesion, chemotaxis, binding to 67 kDa receptor, neuronal attachment Inhibition of metastasis Bone cell differentiation Endothelial differentiation Seroli cell morphogenesis Inhibition of angiogenesis and tumor growth Disruption of gastrulation in sea urchin	Graf et al., 1987 Iwamoto et al., 1987 Nakai et al., 1992 Yamamoto et al., 1994 Vukicevic et al., 1990 Grant et al., 1989 Hadley et al., 1990 Sakamoto et al., 1991 Hawkins et al., 1995
β1 III	PDSRG	Cell adhesion, inhibition of metastasis	Kleinman et al., 1989
β1 III	YGYYGDALR	α2β1 integrin binding	Underwood et al., 1995

β1 IV	RYVVLPRPVCF EKGMNYTVR	Heparin binding, cell adhesion	Charonis *et al.*, 1988
β1 VI	RIQNLLKITNLR IKFVK	Heparin binding	Kouzi-Koliakos *et al.*, 1989
β1 V	LGTIPG	Binding to 67 kDa elastin receptor	Mecham *et al.*, 1989
β2 I	LRE	Motoneuron stop signal, inhibition of neurite outgrowth	Hunter *et al*, 1989b,1991
		Promotion of motor axon growth	Brandenberger *et al*, 1996
γl I	RNIAEIIKDI	Neurite outgrowth, neurotrophic effect, neurotoxic effect	Liesi *et al.*, 1989
		Neuronal migration	Liesi *et al.*, 1995, 1996
		Nuclear translocation	Liesi *et al.*, 1995
		Required for di/trimerization of laminin	Utani *et al.*, 1994
		Axonal differentiation	Matsuzawa *et al.*, 1996
		Axon guidance	Matsuzawa *et al.*, 1998
		Modulation of electrical activity of neurons	Hager, *et al.*, 1998

1.6. Molecular interactions of laminin-1

Laminin-1 has the ability to self-associate into polymers by aggregation of the amino-terminal globular domains VI (Yurchenco *et al.*, 1992; Yurchenco and Cheng, 1993). This self-association requires calcium to induce the required conformational change (Yurchenco and Cheng, 1993).

Various molecules interact with laminin-1 either directly or indirectly and can therefore modulate its biological effects. Nidogen-1 (entactin) binds to the fourth EGF-like repeat of the domain III in the γ1-chain (Mayer *et al.*, 1993) increasing hepatocyte adhesion and spreading (Levavasseur *et al.*, 1994). Nidogen-2 binds with lower affinity to the same binding site as nidogen-1 (Kohfeldt *et al.*, 1998). A basement membrane-associated glycoprotein, fibulin, binds close or directly to the G-domain of laminin-1 (Pan *et al.*, 1993). Thrombospondin binds laminin-1 directly (Frazier, 1987), as does heparansulfate proteoglycan that binds to the long arm of laminin-1 (Laurie *et al.*, 1986). Agrin, a large

multidomain heparansulfate proteoglycan, attaches to the central region of the three-stranded, coiled-coil oligomerization domain in the long arm of laminin-1 (Denzer *et al.*, 1998). Heparin has several binding sites in the laminin-1 molecule, with the most important one being in the G-domain of laminin-1 (Ott *et al.*, 1982; Laurie *et al.*, 1986). In addition, the α-chain of laminin-1 has a heparin-binding site in domain VI (Colognato-Pyke *et al.*, 1995). Electron microscopy has revealed two additional heparin-binding sites in the globular domains IV and VI of β1-chain (Charonis *et al.*, 1988 for domain IV; Kouzi-Koliakos *et al.*, 1989 for domain VI). Also in the long arm of laminin-1 an additional heparin binding site is present in domain II close to the region where the α1, β1, and γl chains intersect (Skubitz *et al.*, 1988). Type IV collagen has been found to bind laminin-1 either directly to the long arm (Charonis *et al.*, 1986) or indirectly via nidogen-1 (Fox *et al.*, 1991) to domain III of the γl-chain (Mayer *et al.*, 1993). Type VII collagen binds directly to the β3 chain of laminin-5 using its non-collagenous, globular domain (Chen *et al.*, 1999). Although fibronectin does not bind directly to laminin-1 it can interact with laminin-1, because both laminin-1 and fibronectin bind heparin (Hayashi *et al.*, 1980; Ott *et al.*, 1982; Timpl, 1989). An extracellular matrix molecule tenascin-C does not bind directly to laminin-1 (Lightner and Erickson, 1990). However, indirect binding of tenascin-C to laminin-1 or laminin-2 may occur via agrin. Agrin binds directly tenascin-C, laminin-1, and laminin-2 (Cotman *et al.*, 1999).

Apolipoprotein E binds laminin-1 directly (Huang *et al.* 1995). It increases neuronal adhesion to laminin-1 and alters neurite morphology (Huang *et al.* 1995). The amyloid precursor protein (APP) has also been shown to bind laminin (Narindrasorasak *et al.*, 1992). APP has been suggested to be a laminin receptor, and it has been found to bind to the IKVAV-sequence in the carboxyl terminus of the α1-chain of laminin-1 (Kibbey *et al.*, 1993). Serum amyloid P (SAP), a component of all amyloid plaques (Pepys *et al.*, 1994) and a normal component of a number of basement membranes (Dyck *et al.*, 1980; Al Mutlag *et al.*, 1993), binds to laminin-1 (Zahedi, 1997). Chicken neuronal-glial cell adhesion molecule (Ng-CAM) also binds to the short arms of laminin-1 (Grumet *et al.*, 1993). The mammalian homologue of Ng-CAM, the L1-antigen, also binds to the G2 domain of laminin-1 (Hall *et al.*, 1997a). However, laminin binding of the L1-molecule take place via sulfated HNK-1 carbohydrate epitopes (Hall *et al.*, 1997a). Some sulfated glycolipids also bind laminin-1 (Roberts *et al.*, 1985; Kobayashi *et al.*, 1994; Hillery *et al.*, 1996; Hall *et al.*, 1997b). Lectins, plant proteins that have high affinity for specific sugar residues of laminin-1 (Woo *et al.*, 1990; Ozeki, *et al.*, 1995; Saarela *et al.*, 1996), and calreticulin, a molecular chaperone with lectin like properties (McDonnell *et al.*, 1996), are also known to bind to laminin-1 carbohydrates.

Several receptors that mediate interactions of various domains of laminins with cells have been identified. Generally the laminin receptors are divided into two categories, the non-integrin-type and the integrin-type cell surface receptors (Hemler, 1990; Mecham, 1991; Liesi, 1991; Timpl and Brown, 1994). The first non-integrin type laminin receptor identified is the 67 kDa laminin receptor isolated from melanoma cells (Rao *et al.*, 1983). Later this receptor was identified in several other cell types, including primary neuronal cells (Douville *et al.*, 1988). Another non-integrin type receptor, the 110 kDa receptor (Kleinman *et al.*, 1991), is localized in brains of embryonic and postnatal mice (Luckenbill-Edds *et al.*, 1995). This receptor has been proposed to be the amyloid precursor protein (APP) also binding to the IKVAV sequence of the $\alpha 1$-chain of laminin-1 (Kibbey *et al.*, 1993). Dystroglycan-α is a non-integrin laminin receptor that binds laminin-1, laminin-2, and laminin-4 (Hemler, 1999). Binding of the laminin-dystroglycan-α complex to the cell surface is mediated by attachment of dystroglycan-α to dystroglycan-β that is a transmembrane protein (Hemler, 1999). In laminin-1, the binding site of dystroglycan-α is in the major heparin binding domain in the E3 fragment (Gee *et al.*, 1993). Cranin, a brain isoform of dystroglycan-α, (Smalheiser and Kim, 1995) binds to the laminin E3 fragment in the long arm of the $\alpha 1$-chain (Smalheiser, 1993) and is involved in neurite outgrowth (Smalheiser and Schwartz, 1987). At least seven integrins ($\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_6\beta_4$, $\alpha_7\beta_1$, and $\alpha_9\beta_1$) are known to bind laminins, and the integrins $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_6\beta_1$, $\alpha_6\beta_4$, $\alpha_7\beta_1$, and $\alpha_9\beta_1$ bind specifically laminin-1 (Hemler, 1991; Timpl and Brown, 1994; Forsberg *et al.*, 1994; McKerracher *et al.*, 1996).

1.7. Laminins in the nervous system

One of the best characterized functions of laminin-1 is its involvement in neuronal development (Sanes, 1989; Liesi 1990; Luckenbill-Edds, 1997). Laminin-1 promotes neurite outgrowth, neuronal migration, and nerve regeneration (Sanes, 1987; Liesi 1990; Luckenbill-Edds, 1997). Laminin-1 also plays a role in neuronal differentiation (Heaton and Swanson, 1988; Frade *et al.*, 1996). The roles of laminins in the nervous system are discussed in sections 2.1.1., 2.2.2.a, 2.3.1., 2.4.1.3. and 2.5.4.1. Knockout studies further demonstrate the significance of laminins in the nervous system development (see section 1.4.).

In adult mammalian brain, antibodies against native laminin-1 demonstrate this protein mostly in basement membranes, whereas in those CNS areas and systems that support axon growth and neuronal regeneration laminin-1 is detected in astrocytes (Liesi, 1985a). In the developing brain, laminin-1 is expressed along the routes of the migratory neurons and pioneer axons (Liesi, 1985a,b; Cohen *et al.*, 1987; Liesi and Silver, 1988; Letourneau *et al.*, 1988). Transient expression of laminin-1 is induced in reactive astrocytes of the adult injured rat brain (Liesi *et al.*, 1984). The hypothesis that induction of laminin-1 in the injured aduld CNS supports regeneration comes from the fact that astrocytes of the adult rat olfactory bulb, the only regenerative area in adult mammalian brain, express laminin continuously (Liesi, 1985b). Furthermore Schwann cells of the adult peripheral nervous system (PNS) (Cornbrooks *et al.*, 1983) and astrocytes of the adult CNS of the lower vertebrates that can regenerate their injured nerves (Liesi, 1985b; Hopkins *et al.*, 1985) produce laminin-1.

Laminin-2 has been localized in retinal ganglion cells and along the optic pathway in chicks (Morissette and Carbonetto, 1995). Laminin-2 is also expressed in other parts of the CNS, e.g. in the dendritic spines of the adult mammalian hippocampus (Tian *et al.*, 1997), in neuronal fibers and structures of the limbic brain region (Hagg *et al.*, 1997) as well as in the developing mammalian olfactory system (Raabe *et al.*, 1997). In the developing mouse cerebellum laminin-2 immunoreactivity is restricted to the Purkinje cells (Powell *et al.*, 1998). In the PNS laminin-2 the predominant laminin isoform present in the endoneurial basement membranes of the peripheral nerves (Sanes *et al.*, 1990), is produced by Schwann cells (Leivo and Engvall, 1988).

Laminin-3 is concentrated in the basement membranes of the neuromuscular junctions (Hunter *et al.*, 1989a,b) but is also transiently expressed during development of the rat neocortex (Hunter *et al.*, 1992a). During CNS development laminin-3 is present in the pial surface that overlies the floor plate of the spinal cord (Hunter *et al.*, 1992a). Prenatally, laminin-3 appears transiently in the capillary basement membranes throughout the CNS (Hunter *et al.*, 1992a).

In the developing human nervous system, mRNA for all five laminin α-chains has been detected. The α-1 chain mRNA is present in the 17 wk human embryo brain in the neural retina, olfactory bulb, cerebellum, and in the meninges whereas the α-2-mRNA is detected

in the choroid plexus and the meninges (Vuolteenaho *et al.*, 1994). The mRNAs for the α3-, α4-, and α5-chains are present in the 6-12 wk human embryo brain (Liesi, unpublished result). In 20 wk embryo brain, the α4-chain mRNA is not detected (Iivanainen *et al.*, 1995). The β1-chain mRNA has been detected in 6-12 wk human embryo brain (Liesi, unpublished result) and the β2-chain in the 17 wk human embryo brain (Iivanainen *et al.*, 1994). In 18-19 wk embryos the mRNA for the γl-chain and in 17 wk embryos the mRNA for the γ2 have been demonstrated (Kallunki *et al.*, 1992). The γ4-chain mRNA was found already in the 6-12 wk embryo brain (Liesi, unpublished result). In the adult human CNS, the expression of laminin chains is more restricted. Expression of the mRNA for γ1-, γ3- and γ5-chains of laminins (I; Koch *et al.*, 1999; Van *et al.*, 1997) has been detected. In rodents the qualitative expression of laminin mRNAs is largely similar to that found in the human CNS. However, unlike the adult human CNS the mRNAs for α4, α5, and β2-chains are expressed in the adult mouse (for reference see Table 5b). Listing of expression of mRNAs for laminins in the human and rodent CNS is shown in Table 5.

Table 5a. Expression of mRNAs for different chains of laminins in the human CNS.

Laminin chains	mRNA expression		
α1	+	embryo 17 wk	Vuolteenaho *et al.*, 1994
	-	adult	I
α2	+	embryo 17 wk	Vuolteenaho *et al.*, 1994
α3	+	embryo 6-12 wk	Liesi, unpublished
α4	+	embryo 6-12 wk	Liesi, unpublished
	-	embryo 20 wk	Iivanainen *et al.*, 1995;
	-	adult	Iivanainen *et al.*, 1995
α5	+	embryo 6-12 wk	Liesi, unpublished
	-	adult	Durkin *et al.*, 1997;
β1	+	embryo 6-12 wk	Liesi, unpublished
	+	adult	I
β2	+	embryo 17 wk	Iivanainen *et al.*, 1994
β3	-	embryo 6-12 wk	Liesi, unpublished
γ1	+	embryo 18-19 wk	Kallunki *et al.*, 1992
	+	adult	I
γ2	+	embryo 17 wk	Kallunki *et al.*, 1992
γ3	-	embryo	Koch *et al.*, 1999
γ3	+/-	adult	Koch *et al.*, 1999
γ4 (netrin-1)	+	embryo 6-12 wk	Liesi, unpublished
γ5 (netrin-2)	+	adult	Van Raay *et al.*, 1997

Table 5b. Expression of mRNAs for different chains of laminins in the rodent CNS.

Laminin chains	mRNA expression		
α1	+	embryo 15.5 d	Miner *et al.*, 1997
	-	adult	Miner *et al.*, 1997
α2	-	nb*	Bernier *et al.*, 1994
	-	adult	Miner *et al.*, 1997
α3	+	embryo 13-17 d	Galliano *et al.*, 1995
	-	adult	Miner *et al.*, 1997
α4	+	embryo 17.5d, adult	Miner *et al.*, 1997
α5	+	embryo 17,5d adult	Miner *et al.*, 1995
β1	+	embryo, nb, adult	Sarthy and Fy, 1996
β2	+	nb*, adult	Libby *et al.*, 1997
β3	-	embryo 14 d, nb*	Utani *et al.*, 1995a
γ1	+	embryo 14 d	Sugiyama *et al.*, 1995
γ2	+	embryo 14d	Sugiyama *et al.*, 1995
γ3	+	adult	Iivanainen *et al.*, 1999
γ4 (netrin-1)	+	embryo 10,5-11,5	Serafini *et al.*, 1996
γ6 (netrin-3)	+	embryo 11,5-14,5d	Wang *et al.*, 1999

*nb, newborn

Schwann cells of the PNS (Davis *et al.*, 1985) and astrocytes of the CNS (Liesi and Risteli, 1989) produce a variant form of laminin with the conventional α1-chain missing. Recently, human glial laminin has been shown to contain a novel β-chain together with a regular γ1-chain and a shorter α-chain, possibly related to α3B-, α4-, and α5-chains (LeMosy *et al.*, 1996). Glial expression of laminin-1 is shown to correlate with neuronal migration in all areas of the central nervous system investigated (Liesi 1985a). In the developing optic nerve axons are in contact with punctate extracellular deposits of laminin (Cohen *et al.*, 1987; Liesi and Silver, 1988; Letourneau *et al.*, 1988; Liesi and Risteli, 1989). The fine punctate deposits of laminin-1 localized in association with growing axons are composed of β1- and γ1-chains of laminin, whereas the α1 immunoreactivity is present in the basement membranes (Liesi and Risteli, 1989).

2. Nervous system development, degeneration and regeneration

In recent years a large number of molecules, involved in nervous system development, degeneration, and regeneration have been identified. The functions of these molecules, including laminins, are only partially understood and many questions remain to be answered concerning the mechanisms that allow these molecules to orchestrate the behavior of neuronal cells.

2.1. Neuronal differentiation

The differentiated cell types of the vertebrate nervous system arise from the neurogenic epithelium (neuroectoderm) of the neural plate. In recent years major advances have been made in understanding the inductive signals of neurogenesis during early embryonic development. Studies on dissociated ectodermal cells during the gastrula stage showed that these cells reaggregate after long dissociation and express neuronal markers (Godsave and Slack, 1989; Sato and Sargent, 1989). This suggested that neuronal induction may occur without any inductive signals from the dorsal mesoderm. The results indeed imply that embryonic cells would become nerve cells if they do not get any other inductive signals. Thus, the early neuronal differentiation is under a negative control. One of the molecules that inhibit neurogenesis is the bone morphogenetic protein (BMP) 4 (Wilson and Hemmati-Brivanlou, 1995). Several results indicate that the ectoderm responds to BMP-activation in a dose-dependent manner: The neural fate associates with the lowest BMP activity, the epidermal fate to the highest, and the neural crest to the intermediate level (Chitnis, 1999). The inhibitory effect of BMP is inactivated by neural inducers such as noggin, follistatin, and chordin (Sasai *et al.*, 1995). All these molecules are secreted by the Spemann organizer (Smith and Harland, 1992; Hemmati-Brivanlou *et al.*, 1995; Sasai *et al.*, 1994). These molecules may bind to BMP and block the activation of BMP receptors (Piccolo *et al.*, 1996; Zimmerman *et al.*, 1996). This suppresses BMP signaling and leads to activation of a number of genes that promote neuronal fate in the dorsal ectoderm (Sasai, 1998).

The specificity of neuroectodermal cells depends on the position they initially occupy in the neural plate. The dorsoventral development of the neural tube is mediated by two different signaling systems (Tanabe and Jessell, 1996). The dorsal specification is

mediated by BMPs that are initially expressed by epidermal ectoderm and later by the roof plate (Tanabe and Jessell, 1996). BMP signaling induces the faiths of several sensory interneurons in the dorsal spinal cord. Recently genetic ablation studies have shown that the roof plate is essential for specification of certain dorsal interneurons (Lee *et al*., 2000). The ventral specification of the neural tube is mediated by Sonic hedgehog (Shh; Tanabe and Jessell, 1996). Initially, Shh is expressed by the notochord, inducing the formation of the floor plate, where it also will be expressed. The expression pattern of Shh is closely linked to the development and differentiation of the entire ventral neural tube (Martí *et al*., 1995). Shh expressed by the notochord defines the differentiation of motor neurons and ventral interneurons (Ericson *et al*., 1996). Shh-deficient mice show perturbed ventral patterning of the CNS (Chiang *et al*., 1996). Shh prevents the dorsalization of the ventral neuronal tube by antagonizing the effect of BMPs (Liem *et al*., 1995).

During closure of the neural tube the cells in the anterior part of the neural tube begin to form the primitive forebrain, midbrain, and hindbrain. Posteriorly, the neural tube forms spinal cord. Shortly after closure of the neural tube the neuroepithelial cells proliferate rapidly**.** During this proliferative phase the cells can either remain as undifferentiated neuronal stem cells or they may differentiate into neurons and glial cells. The mechanisms controlling the undifferentiated and differentiated state of neurons is not fully understood. The cell surface protein Notch and its ligand Delta-1 have been shown to participate in the regulation of the choice between the undifferentiated and differentiated states of neurons (Henrique *et al.*, 1997). In different parts of the CNS different sets of homeotic genes have been found to determine the direction of cellular development. E.g. the development of the midbrain and cerebellum depends on an organizing center at the midbrain-hindbrain junction known as isthmus (Marin and Puelles, 1994). In the isthmus region several important secreted and regulatory genes are expressed, such as *Wnt-1* (McMahon and Bradley, 1990) and *En1* (Wurst *et al.*, 1994; Danielian and McMahon, 1996). Knockout experiments on these genes eliminate cerebellum either totally or to a large extent (McMahon and Bradley, 1990; Wurst *et al.*, 1994; Danielian and McMahon, 1996). *En1* may further be regulated by a secreted glycoprotein WNT (McMahon *et al.*, 1992). Expression of WNT protein is regulated by the *Pax*-gene family (Song *et al.*, 1996). Inactivation of *Pax2* leads into a massive loss of cerebellum and posterior midbrain (Favor *et al.*, 1996). Recent studies indicate that *Pax6* is expressed in the rhombic lip and in the presumptive early granule cell moving away from the rhombic lip (Engelkamp *et al.*,

1999). *Math1*, the mouse homologue of the Drosophila gene *atonal* encoding for a basic helix-loop-helix transcription factor, has been shown to be essential for the genesis of cerebellar granule neurons *in vivo* (Ben-Arie *et al.*, 1997). Segmentation plays a prominent role in the hindbrain (Lumsden and Krumlauf, 1996). The hindbrain neural tube is divided in segmental units called rhombomeres. Expression of Hox genes patterns an ordered set of domains along the neuraxis (Lumsden and Krumlauf, 1996). Thus, it is possible that the identity of individual rhombomeres could be defined by a cooperative action of the Hox proteins. Retinoic acid (RA) regulates the expression of Hox genes and overexpression of RA has been shown to result in an anterior shift of the Hox gene expression (Lumsden and Krumlauf, 1996).

2.1.1. Laminin in neuronal differentiation

The involvement of laminin-1 in neuronal differentiation became evident in studies on frog retinal pigment epithelial (RPE) cells (Reh 1987). RPE cells, grown on laminin-1, transdifferentiate into neurons instead of the lens cells (Reh 1987). Laminin-1 is also known to influence neuronal differentiation of the early chicken neural tube (Heston and Swanson 1988). The E8 fragment of laminin-1 stimulates proliferation and differentiation of neuroepithelial cells into neurons *in vitro* (Drago *et al.*, 1991; Frade *et al.*, 1996). Integrin α1β6 receptors have been found to be down-regulated when retinal ganglion cells progressively differentiate (de Curtis *et al.*, 1991; Cohen and Johnson, 1991). Transformation of chromaffin cells into sympathetic neurons in response to basic fibroblast growth factor occurs if laminin-1 is used as culture substratum (Chu and Tolkovsky, 1994). The IKVAV domain of the α1-chain of laminin-1 interacts with the plasmalemmal protein LBP110 (Chalazonitis *et al.*, 1997). This protein is acquired by the neural crest-derived precursors of the enteric neurons after they colonize the gut and, therefore, promote the development of the enteric neurons (Chalazonitis *et al.*, 1997). Laminin β2-chain may also be involved in the differentiation of rod photoreceptors (Hunter *et al.*, 1992b) and may control their developmental choices between the rod photoreceptor and bipolar cell fates (Hunter and Brunken, 1997).

2.1.2. Neuronal cell lines

Different kinds of neuronal cell lines have been developed to study the molecular mechanisms of neuronal differentiation. These cell lines may be divided in different groups depending of the origin. Teratocarcinoma cells, derived from tumors of the fetal germ cells, e.g. teratomas, provide cell lines in which the entire differentiation process from pluripotent cells to terminally differentiated cells can be followed (Martin, 1980). See also section 2.1.3

Human neuroblastoma cell lines are derived from childhood solid tumors containing of primitive cells of precursors of the autonomic nervous system. These neuroblastoma cell lines have been shown to undergo spontaneous and chemically induced differentiation into cells resembling those of the mature nervous system. Neuroblastoma cell lines have been used to study the mechanism of neuronal differentiation (Abemayor and Sidell, 1989; Påhlman *et al.*, 1995; Maggi *et al.*, 1998) and degeneration (Omar and Pappola 1993; Neill *et al.*, 1994). In addition to human neuroblastomas mouse neuroblastoma cell lines have been chemically induced from both central and peripheral neurons (Liesi, 1984).

Immortalized neural cell lines can be produced using somatic cell fusion to neuroblastoma cell lines, isoforms of transforming agents such as *myc* and *neu* or by using SV40 large tumor antigen (Martinez-Serrano and Björklund, 1997). These gene transfer techniques have allowed introduction of growth factors, neuropeptides, neurotransmitters as well as biosynthetic and metabolic enzymes into the neural progenitor cells (Gökhan *et al.*, 1998). Using this technique several conditionally immortalized neural stem/progenitor cell lines have been established from embryonic hippocampus, cerebellum (Gökhan *et al.*, 1998), and spinal cord (Li *et al.*, 2000). The conditionally immortalized neural cell lines can be transplanted back into the mammalian brain and may represent an experimental resource for characterization of molecular mechanism involved in CNS development, plasticity, and regeneration (Gökhan *et al.*, 1998). Undifferentiated stem cells of the adult brain have been shown to differentiate into neurons, astrocytes, and oligodendrocytes *in vitro* (Reynolds and Weiss, 1992, 1996; Palmer *et al.*, 1997; Johansson *et al.*, 1999; Chiasson *et al.*, 1999). The neural stem cells are most abundant in the walls of the lateral ventricles (Weiss *et al.*, 1996) and in the hippocampus (Palmer *et al.*, 1997). Stem cells that associate with the ventricular systems have been shown to originate either from the ependyma (Johansson *et al.*, 1999) or the subependyma (Chiasson *et al.*, 1999).

2.1.3. Teratocarcinoma cells as a model system for neuronal differentiation

Teratocarcinoma cells have been used as a model system for early embryonic development (Strickland and Mahdavi 1978; Martin 1980,). These pluripotent cells are capable of differentiating into various organs and cell types. The inductive signals of this differentiation have been studied as putative morphogens, e.g. factors that may control cell determination during early embryonic development (Martin and Evans 1975; Sell and Pierce, 1994; Andrews, 1998). Depending on cell density laminin-1 induces differentiation of teratocarcinoma cells either into neurons or multinucleated striated muscle cells (Darmon, 1982; Sweeney *et al.*, 1990; Kim *et al.*, 1995).

Retinoic acid (RA) alone or in combination with dibutyryl cyclic AMP (dbcAMP) has been used to differentiate teratocarcinoma cells into visceral or partietal endoderm (Strickland and Mahdavi 1978, Sporn and Roberts 1983). DbcAMP alone does not provoke differentiation of the F9 cells but the cells must first be exposed to RA in order to be permissive to the effects of dbcAMP (Darrow *et al.*, 1990). Serum deprivation together with RA and dbcAMP has been shown to induce neuronal differentiation of various teratocarcinoma cell lines (Pleiffer *et al.*, 1981, McBurney *et al.*, 1982, Levine and Flynn 1986, Kubo 1989), including the F9-cells (Kuff and Fewell, 1980, Liesi *et al.*, 1983).

2.2. Neuronal migration

2.2.1. Current models of neuronal migration

The classical viewpoint on neuronal migration is that migration of embryonic and early postnatal neurons occurs via physical guidance of the radially oriented glial cell processes (Jacobson, 1991). According to this point of view all radial migration of neurons proceeds along the radial glial cells while all horizontal neuronal migration proceeds along other neuronal fibers (Rakic, 1990). Thus, the glial cells are considered to have all the molecular and physical guidance cues that the migratory neurons need (Sidman and Rakic 1973; Rakic, 1990) and to provide all the directionality required for neuronal movement.

In the past years an alternative opinion, the nuclear translocation model of neuronal migration, has emerged. It implies that neurons move without the glial guidance by nuclear translocation inside their preformed processes (Morest, 1970; Domesick and Morest, 1977; Nakatsuji and Nagata, 1989; Book and Morest, 1990; Liesi, 1992). Nuclear translocation of neuroblasts has been described as early as in 1935 in the young cerebral cortex (Sauer, 1935), but it was then thought to relate to the division of neuroblasts rather than to their migration. The fact that CNS neurons migrate via nuclear translocation inside a preformed processes was first predicted by Morest based on his careful studies on the Golgi-stained sections of different brain regions (Morest, 1970). However, the lack of direct visual evidence prevented the acceptance of the nuclear translocation model, although experiments using intraperitoneal injections of specific gliotoxins indicated that cerebellar granule neurons can migrate in the absence of the glial cells (Sotelo and Rio, 1980).

In recent time lapse video microscopy studies, the cerebellar granular neurons were seen to migrate without glial cells in association with other neurites (Nakatsuji and Nagata, 1989). When cerebellar granule neurons were cultured on a laminin substratum, the granule neurons migrated via neurite extension and contact formation followed by nuclear translocation inside preformed neuronal processes without the presence of the glia (Liesi, 1992). Novel developments of infrared video microscopy allowed the visualization of neuronal migration in slices of living postnatal cerebellum (Hager *et al.*, 1995). Using this modern methodology, the nuclear-translocation-type neuronal migration was found to be the primary form of neuronal movement in postnatal cerebellum (Hager *et al.*, 1995; Liesi *et al.*, 1995). The nuclear translocation mode of neuronal migration was found to involve two distinct phases. During the first, the neurite extension phase, granule neurons extend neurites towards the presumptive internal granule cell layer. Interestingly, the stop signal for granule neuronal migration is suggested to be their contact with mossy fibers (Baird *et al.*, 1992), their final synaptic counterparts. Thus, it is possible that the neurite-extension phase continues until the granule neurons make contact with their appropriate targets. After the contact is formed the second phase, the nuclear translocation phase, follows. The nuclei then translocate inside their preformed neurites into the internal granule cell layer. In the cerebral cortex a glia-independent form of neuronal migration has also been detected using DiI-labeling of living migratory neurons (O'Rourke *et al.*, 1992, 1995).

Confocal studies indicate that migratory cortical neurons first proceeded vertically (as if they followed the radial glial fibers), but then turned 90 degrees and moved horizontally (O'Rourke *et al.*, 1992, 1995). The horizontal mode of migration cannot be guided by the radial glia.

2.2.2. Molecules involved in neuronal migration

Molecules involved in neuronal migration belong to two primary categories: The ECM proteins and the cell adhesion molecules (CAMs). Many molecules affecting axon guidance also seem to have an effect on neuronal migration. According to the neuronal translocation model, neuronal migration and axon guidance are closely related events. Therefore it is feasible that the same molecules may be involved in both neuronal migration and axon guidance.

2.2.2.a ECM molecules

A number of ECM proteins have been shown to play a role in neuronal migration (Liesi, 1990; Sanes, 1990). In developing brain, laminin-1 is localized along the routes of migratory neurons (Liesi, 1985a). In chick CNS β1-integrin antisense RNA inhibited neuronal migration *in vivo* (Galileo *et al.*, 1992). These results provide functional evidence for the role of laminin in neuronal migration, because β1-integrin-type laminin receptors have been shown to mediate the effects of laminin on both central and peripheral neurons in culture (Tomaselli *et al.*, 1986; Hall *et al.*, 1987; Cohen *et al.*, 1986, 1987). Antibodies against the native EHS-tumor laminin-1 fail to inhibit neuronal migration on a laminin-1 substratum (Linder *et al.*, 1986), although recent experiments indicate that antibodies against the neurite outgrowth promoting domain of the γl chain of laminin-1 inhibit neuronal migration both *in vitro* (Liesi *et al.*, 1992) and *in vivo* (Liesi *et al.*, 1995). The non-functional antibodies against native laminin-1 (used by Linder *et al.*, 1986) recognize the P1- fragment in the N-terminus of laminin-1 (Ott *et al.*, 1982), while the neurite outgrowth domains of laminin-1 localize in the C-terminal parts of this molecule (Martin and Timpl, 1987; Liesi *et al*., 1989; Paulsson, 1992). Thus, antibodies (I) against the C-terminal neurite outgrowth promoting domain inhibit neuronal migration in cerebellar microcultures (Liesi *et al*., 1992) and in living slices of postnatal rat cerebellum microcultures (Liesi *et al*., 1995).

Laminin-2 promotes olfactory neuronal migration (Calof and Lander, 1991), whereas laminin-3 inhibits neuronal migration by a gated mechanism, e.g. neurons can migrate on laminin-3 but do not migrate from laminin-3-free substratum towards laminin-3 substratum (Porter and Sanes, 1995). Laminin-3 is transiently expressed along the routes of the migratory cortical neurons (Hunter *et al.*, 1992a).

Netrins, the vertebrate homologues of the UNC-6 gene and homologues of the γ1-chain of laminin-1, are also involved in neuronal migration (Hedgecock *et al.*, 1990; Kennedy *et al.*, 1994). In the reeler mutant mice the orderly inside-out deposition of neocortical cells is disturbed (Frotscher, 1997). The affected gene in the reeler mutation has been found to code for reelin, a large extracellular matrix protein that is secreted by the Cajal-Retzius cells (D'Arcangelo *et al.*, 1995; Frotscher, 1997). Antibodies against thrombospondin-1 inhibit granule neuronal migration by a dose-dependent manner (O'Shea, 1990). Tenascin-C either promotes or inhibits neuronal migration (Faissner and Kruse, 1990; Husmann *et al.*, 1992, 1995; Faissner, 1997). Antibodies against tenascin-C inhibit cerebellar neuronal migration (Chuong, 1990). A chondroitin sulfate proteoglycan (CSPG), astrochondrin, has been shown to be involved in cerebellar neuronal migration (Streit *et al.*, 1993). Apart from stimulation of neuronal migration (Streit *et al.*, 1993; Garwood *et al.*, 1999) CSPGs have also been shown to act as molecular barriers of neuronal migration (Grumet *et al.*, 1996; Landolt *et al.*, 1995) and their inhibitory effect can be modulated by cell adhesion molecules (Grumet *et al.*, 1996; Dou and Levine, 1995). The involvement of fibronectin in CNS neuronal migration has been proposed by studies in which fibronectin has been localized along the migratory cell pathway of embryonal cerebellar neurons (Schachner *et al.*, 1978; Hatten *et al.*, 1982). These studies have been confronted by opposite results indicating that fibronectin is not involved in cerebellum neuronal migration (Hynes *et al.*, 1986). Neuronal migration of neural crest cells has been blocked by antibodies against fibronectin (Rovasio *et al.*, 1983). The CSAT antibodies which recognize a cell surface receptor for both fibronectin and laminin (Bronner-Fraser, 1986) or peptides containing the cell-binding site of fibronectin (Boucaut *et al.*, 1984) also interfere with neural crest cells migration.

2.2.2.b Cell Adhesion molecules

Several cell adhesion molecules are involved in neuronal migration. Neural cell adhesion molecule (N-CAM), Ng-CAM, and L1 antigen (Chuong, 1990; Matsushita *et al.*, 1997; Rønn *et al.*, 1998) as well as astrotactin (Hatten and Mason, 1990; Zheng *et al.*, 1996) are all present in the cerebellum during the time of neural migration. Antibodies against all these adhesion molecules inhibit neuronal migration (Chuong, 1990; Hatten and Mason, 1990). Inactivation of N-CAM by genetic deletion have been shown to disturb the migration of several types of neurons (Cremer *et al.*, 1994; Ono *et al*., 1994). The adhesion molecule on glia (AMOG), the β subunit of the Na/K-APTase, is expressed in the cerebellum during the time of neuronal migration and neuronal migration is inhibited by antibodies against AMOG (Antonicek *et al.*, 1987; Gloor *et al.*, 1990).

2.2.2.c Other mediators of neuronal migration

Ion channels also play a role in neuronal migration. Blockers of both voltage-gated N-type calcium channels (Komuro and Rakic, 1992) and N-methyl-D-aspartate (NMDA) receptors (Komuro and Rakic, 1993) inhibit neuronal migration of the cerebellar granule neurons. The rate of granule neuronal migration depends on both the extracellular concentrations of Ca^{2+} and the Ca^{2+} influx through the calcium channels (Komuro and Rakic, 1992, 1993, 1996). In these experiments inhibitors of potassium and sodium channels or L- and T-type calcium channels had no effect on neuronal migration (Komuro and Rakic, 1992).

Proteolytic enzymes play a role in neuronal migration (Kalderon, 1982; Moonen *et al.*, 1982). Cultured cerebellar granule neurons secrete tissue plasminogen activator (tPA), a serine protease (Krystosek and Seeds, 1981), and bind tPA with high affinity onto their surfaces (Verrall and Seed, 1988). Both tPA activity and tPa mRNA levels of the postnatal cerebellum are maximal at P7 (Friedman and Seeds, 1995), which coincides with the most active period of granule cell migration. Inhibitors of serine proteases inhibit granule cell migration both *in vitro* and *in vivo* (Seeds *et al.*, 1990; Seeds *et al*, 1997).

In addition to the above mentioned molecules additional gene defects and mutations may disturb the neuronal migration process (Goldowitz and Hamre, 1998). Staggerer, weaver, and reelin mice are examples of naturally occuring mutations that affect neuronal migration in the cerebellum (Sidman and Rakic, 1973; Goldowitz and Hamre, 1998). In

humans lissencephaly is a neuronal migration disorder resulting in brain malformation, epilepsy, and mental retardation (Pilz *et al.*, 1998). Two genes associated with lissencephaly have been identified. The gene for X-linked lissencephaly is called doublecortin and its function is to stabilize microtubules during neuronal migration (Allan and Walsh, 1999; Francis *et al.*, 1999; Gleeson, 1999). Another lissencephaly associated gene, LIS1, the gene for the β subunit of platelet activating factor acetylhydrolase, also influences microtubule dynamics (Sapir *et al.*, 1997). LIS1 knockout mice exhibit abnormal neuronal migration (Hirotsune *et al.*, 1998). Neuronal migration is also influenced by a number of external physical (e.g. ionizing radiation, heat), chemical (e.g. toxins, various drugs, alcohol) or biological factors (e.g. some viruses) (Jacobson, 1991).

2.2.3. Neuronal migration during cerebellar development

In the adult cerebellum five types of neurons are present: Purkinje cells, granule cells, basket cells, Golgi II cells, and stellate cells. The Purkinje cells are the only efferents of the cerebellum whereas all other neurons are interneurons. The adult cerebellum is composed of three cortical layers in which the basket and stellate cells are in the outmost layer called the molecular layer. Purkinje cells occupy the central layer, the Purkinje cell layer, and the inner cell layer, the granule cell laye,r is largely formed by the granule neurons (Fig. 3). Impulses are conducted into the cerebellar cortex by climbing fibers and mossy fibers. Climbing fibers synapse with the dendrites of the Purkinje cells. Mossy fibers synapse with both dendrites of the granule cells and axons of the Golgi II cells. The axons of the granule cells, the parallel fibers, make synaptic connections with dendrites of the Purkinje, basket, Golgi II, and stellate cells. Stellate cells synapse with dendrites of the Purkinje cells, and basket cells with the somas of the Purkinje cells. The Golgi II cells synapse with the granule cell dendrites.

Cerebellar neurons originate from two separate germinal zones. The Purkinje and Golgi II cells originate from the neuroepithelial ventricular zone of the fourth ventricle. Recently the basket and stellate cells have also been shown to originate from the same germinal zone as the Purkinje cells (Zhang and Goldman, 1996). The granule cells originate from the germinal cells of the rhombic lip, the lateral most area of the presumptive floor of the 4th ventricle. These germinal cells migrate to form the external granule cell layer (EGL) on the surface of the cerebellar plate (Jacobson, 1991; Goldowitz and Hamre, 1998).
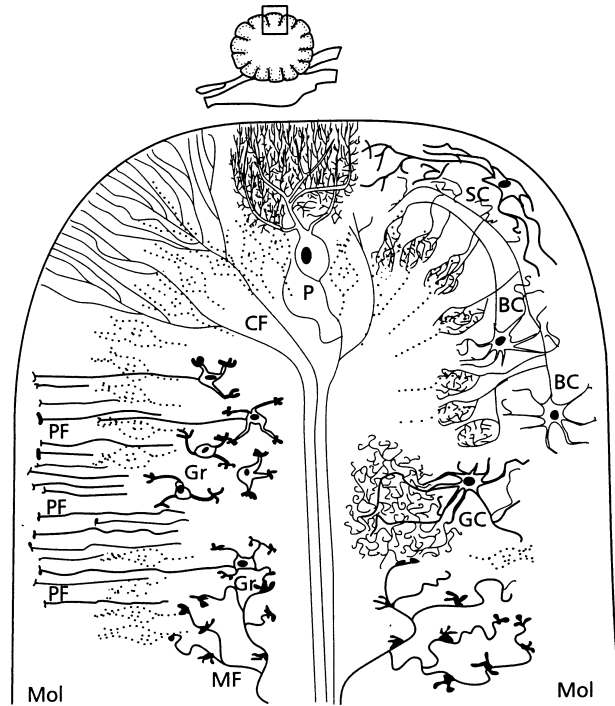
Figure 3. Organization of adult mammalian cerebellar neurons into the cortical layers; Mol = molecular layer, Pur = Purkinje cell layer, Gran = granule cell layer, BC = basket cells, CF= climbing fiber, GC = Golgi II cells, gr = granule cells, Pur= Purkinje cells, SC = stellate cells, PF=parallel fibers.

During neuronal migration neuroblasts move from their origin of birth to their final destinations. Migration of cerebellar neurons occurs in two phases. During the first phase neuroblasts migrate from the ventricular germinal zone of the fourth ventricle along the basal lamina outward to create the mantle layer of the cerebellar plate. This first migratory phase that occurs in the mouse around E11-E13 (Miale and Sidmam, 1961) gives rise to the Purkinje cells. The Golgi II cells originate from the same ventricular zone around E12-E15 and migrate directly into the presumptive molecular layer (Miale and Sidmam, 1961; Zhan and Goldman, 1996; Goldowitz and Hamre, 1998). The neuroblasts that generate granule neurons divide in the rhombic lip at E13 to E15 and migrate over the surface of the cerebellar plate to form the external granular cell layer (EGL). After having formed the

external granule cell layer these precursors of granule neurons enter a proliferative phase (Miale and Sidmam, 1961) before they enter their second phase of neuronal migration. During this second postnatal phase the granule neurons migrate from the EGL passing the Purkinje cells to form the internal granule cell layer of the mature cerebellum (Miale and Sidmam, 1961; Zhan and Goldman, 1996; Goldowitz and Hamre, 1998). The migration of granule neurons in the developing cerebellum is schematically presented in Figure 4. Progenitors of the basket and stellate cells migrate first from the primary germinal zone to the cerebellar white matter and divide there postnatally (Zhang and Goldman, 1996). Finally they enter the cerebellar cortex (Zhang and Goldman, 1996).
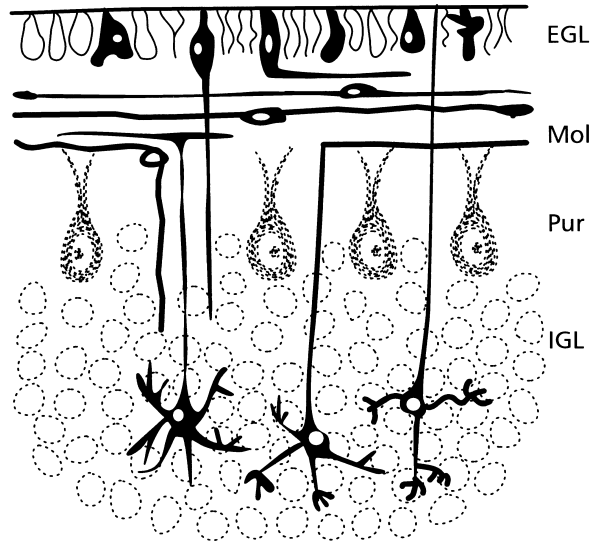

Figure 4. Migration of granule neurons from the external granule cell layer to the internal granule layer. Abbreviations; EGL, external granule cell layer; Mol, molecular layer; Pur, Purkinje cell layer; IGL, internal granule cell layer.

2.2.4. The weaver mutant mouse as a model of neuronal migration and death

The molecular and cellular mechanisms coverning neuronal migration can be studied using neurological mutants having defects in neuronal migration. E.g, 20 mutations that alter the development of cerebellum have been identified (Sidman *et al.*, 1965). The weaver mouse is a naturally occurring, autosomally recessive mutation that has been genetically mapped to chromosome 16 in the mouse (Reeves *et al.*, 1989). The weaver mice have a neuronal defect that disturbs both cerebellar and nigro-neostriatal development (Sidman 1965, Roffler-Tarlov and Graybiel 1986). The early postmitotic granule cells are unable to migrate or form bipolar neurites (Sotelo, 1975, Rakic and Sidman 1973a,b,c). A schematic presentation of cell layers in both the weaver mutant and normal mouse cerebella is shown in Fig. 5. In the weaver mouse cerebellum a large majority of the vermal granule neurons die within the first two postnatal weeks (Rakic and Sidman, 1973c) leaving the adult weaver cerebellum low in granule neurons (Sotelo, 1975; Rakic and Sidman 1973c). Purkinje cells appear normal at the start of their postnatal life, but are later misplaced and a proportion of them die during the third postnatal week of life (Smeyne and Goldowitz, 1990; Maricich *et al.*, 1997). Neuronal degeneration in the substantia nigra (SN) is most abundant during the postnatal days 24-25 (Schmidt, *et al.*, 1982; Oo *et al.*, 1996). In addition to the disturbed cerebellar and SN development the weaver mice have a number of other abnormalities. Most of the homozygous weaver males are sterile due to of a failure in spermatogenesis (Harrison and Roffler-Tarlov, 1994). Germ cells are shown to die during the development of the testes (Harrison and Roffler-Tarlov, 1998). Furthermore, both heterozygous and homozygous weaver mice undergo tonic-clonic seizures (Eisenberg and Messer, 1989), which are thought to be the most common cause of their death. Cell death in the weaver cerebellum has been suggested to occur via apoptosis (Migheli *et al.*, 1995; Wullner *et al.*, 1995), whereas apoptosis is not the mechanisms of neuronal death in the weaver SN (Oo *et al.*, 1996). The weaver mutant mouse is used as an animal model for studies on neuronal migration, neuronal death, and neurodegenerative disorders, such as Parkinson's disease.

a)   b)
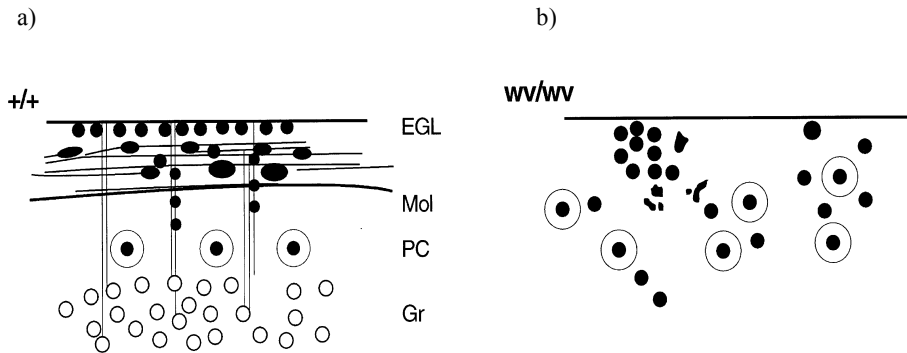
Figure 5. Schematic presentation of cell layers in early postnatal cerebellum of the a) normal and b) weaver mutant mouse.

The weaver gene defect impairs neuronal migration both *in vivo* and *in vitro* (Rakic and Sidman, 1973a-c; Trenkner *et al.* 1978; Willinger and Margolis, 1985). Several studies have shown that the weaver granule neurons fail to migrate and die under normal cell culture conditions (Trenkner *et al.* 1978; Willinger and Margolis, 1985). Studies using chimeric mice indicate that the weaver defect is intrinsic to the granule neurons (Goldowitz, 1989). This is further verified by *in vitro* and *in vivo* transplantation studies in which the weaver granule cells can be rescued by the wild-type granule neurons (Gao and Hatten, 1993) or their membrane extracts (Gao *et al.*, 1992). Although the weaver gene defect is intrinsic to the granule neurons the weaver Bergmann glial cells are also abnormal. They project normally, but appear immature compared to the normal Bergmann glial cells (Bignami and Dahl, 1974a,b; Sotelo and Changeux, 1974) and resemble "reactive" astrocytes by their increased expression of both GFAP and laminin (II).

Recent reports suggest that a point mutation in a G-protein coupled inwardly rectifying K^+ channel gene, GIRK2 (Lesage *et al.*, 1994), could be the weaver gene (Patil *et al.*1995; Slesinger *et al.*, 1996). This mutation maps close to the weaver locus in chromosome 16 and is a single amino acid substitution (glysine $\rightarrow$serine) in the pore-forming H5 region of the GIRK2 channel protein (Patil *et al.*1995; Fig. 6.).
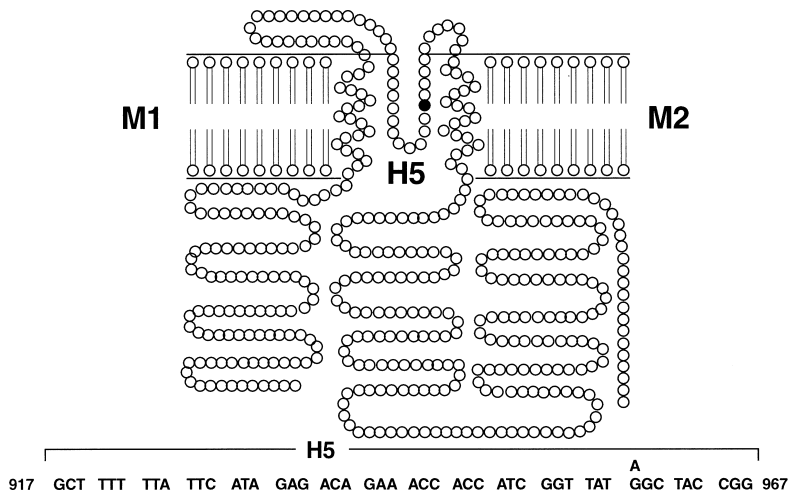
Figure 6. Proposed weaver-mutation in the GIRK2 channel gene. The position of the mutated amino acid (nucleotide change G→A) in H5 domain is marked with a black dot.

A point mutation in the GIRK2 channel pore domain creates a G protein-insensitive, non-selective cation channel that gates not only K^+ but also Na^+ and Ca^{2+} (Navarro *et al.*, 1996). Both GIRK2 and the related protein GIRK 1 have been localized in the granule neurons of the external granule cell layer of the normal postnatal cerebellum during the period of neuronal differentiation and neuronal migration (Slesinger *et al.*, 1996). Interestingly, the GIRK2 channel protein was not studied in the postnatal weaver cerebellum (Slesinger *et al.*, 1996).

In spite of these results it is becoming increasingly clear that the GIRK2 point mutation can not explain the weaver phenotype. The resting membrane potentials of weaver neurons can be rescued by aprotinin (II), a L-type Ca^{2+}-channel blocker verapamil (Liesi and Wright, 1995), antibodies against the γl-chain of laminin-1 (Liesi and Wright, 1996), and ethanol (Liesi, *et al.*, 1997). Several recent experiments have shown that the GIRK2 channel is inactive in weaver neurons during the developmental stages when the neurons are dying (Mjaatved *et al.*, 1995; Surmeier *et al.*, 1996). Furthermore, mice lacking the

GIRK2 channel gene have normal cerebella (Signorini *et al.*, 1997). As weaver granule neurons can be rescued by inhibiting the calcium entry into the neurons or chelating calcium by BABTA-AM (Liesi and Wright, 1996; Liesi *et al.*, 1997), it is possible that any measure that reduces intracytoplasmic levels of Ca^{2+} will be sufficient to prevent death of the weaver neurons. In line with this view point the weaver granule cells fail to express functional NMDA receptors (Liesi *et al.*, 1999), which helps to reduce influx of Ca^{2+} that would occur via activated NMDA receptor channels.

2.3. Neurite outgrowth and axonal targeting

During the development of the nervous system pioneer axons have to find their right targets to establish specific neuronal connections. Growth cones of growing axons responds to different guidance cues, such as soluble chemoattractants and/or chemorepellents as well as contact repulsion and/or attraction. Some of these guidance cues have been identified and found to be extracellular matrix molecules and cell adhesion molecules (Tessier-Lavigne and Goodman, 1996).

2.3.1. Laminin and other extracellular matrix molecules in neurite outgrowth

Laminin-1 is shown to promote neurite outgrowth in several *in vitro* (Baron-Van Evercooren *et al.*, 1982; Manthorpe *et al.*, 1983; Liesi *et al.*, 1984a, 1989; Edgar *et al.*, 1984; Gundersen, 1987; Lein *et al.*, 1992; Matsuzawa *et al.*, 1996; 1998) and *in vivo* studies (Liesi *et al*, 1985b; Hopkins *et al.*, 1985; Cohen *et al.*, 1987; Letourneau, 1988; Liesi and Silver, 1988).

Five different neurite outgrowth promoting peptides, one in the γ1-chain (Liesi *et al.*, 1989b), and four in the α1-chain (Tashiro *et al.*, 1989; Skubitz *et al.*, 1991), have been identified in the laminin-1 molecule. Axonal differentiation and directional axon growth of rat hippocampal neurons have been shown to be promoted by patterned substrates of a decapeptide derived from the γ1-chain neurite outgrowth domain of laminin-1 (Matsuzawa *et al.*, 1996; 1998). The neurite outgrowth promoting peptide of the γ1-chain of laminin-1 modulates the electrical properties of neocortical neurons (Hager *et al.*, 1998).

The first genetic evidence for the involvement of laminin in neuronal migration and axon growth came from studies on neuronal development in C. elegans (Hedgecock *et al.*, 1990; Ishii *et al.*, 1992). In this species a mutation of the *Unc-6* gene affects neuronal migration and pioneer axon growth indicating that *Unc-6* takes part in these processes (Hedgecock *et al.*, 1990; Ishii *et al.*, 1992). The *Unc-6* gene product is homologous to the IV and V domains of the γ-1 chain of laminin-1 and to netrins, recently identified homologues of the γ1-chain of laminin-1 (Serafini *et al.*, 1994).
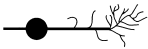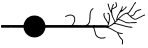
In addition to the role of laminin-1 in development of the optic pathway (Liesi and Silver, 1988) laminin-2 has also been proposed to play a role in the development of the visual system. Retinal ganglion cells (RGC) lose their ability to respond to laminin-1 during the developmental period when axons grow into the optic tectum (Cohen *et al.*, 1986), whereas laminin-2 supports neurite outgrowth of chick RGC throughout their embryonic development until the postnatal day 15 (Cohen and Johnson, 1991). Neurite outgrowth of retinal ganglion cells (RGC) on both laminin-1 and laminin-2 is blocked by anti-β1 integrin antibodies (Cohen and Johnson, 1991). Laminin-2 promotes regeneration of sympathetic nerves *in vivo* (Anton *et al.*, 1994).

Laminin-3 is a potent inhibitor of neurite outgrowth of motor neurons (Porter *et al.*, 1995; Porter and Sanes, 1995). The β2-chain of rat laminin-3 bears a site, LRE, that has been shown to inhibit neurite outgrowth promoted by other extracellular matrix molecules (Porter *et al.*, 1995). Interestingly, the human β2-chain of laminin-3 lacks the LRE-site (Wewer *et al.*, 1994), which questions the importance of LRE as a general stop-signal for growing axons. Whether the LRE domain inhibits or promotes neurite outgrowth may also depend on the assembly of this peptide domain with other chains of laminin (Brandenberger *et al.*, 1996). E.g. native chick laminin-4, which contains the β2 chain, does not inhibit but rather promotes motor axon growth (Brandenberger *et al.*, 1996).

Several other ECM molecules may either promote or suppress neurite outgrowth (Table 6). Neurite outgrowth promoting ECM molecules include fibronectin (Akers *et al.*, 1981) and trombospondin (Neugebauer *et al.*, 1991; Arber and Caroni, 1995). Collagens I, IV and VII have high neurite outgrowth promoting activities on cultured neurons from embryonic mouse brain, but they are less effective on neurons from the postnatal brain (Hirose *et al.*, 1993). Tenascin-C (Bourdon *et al.*, 1983) exhibits both axon growth-

promoting and axon growth-inhibiting activities (Grierson *et al.*, 1990; Lochter *et al.*, 1991; Faissner, 1997). Proteoglycans are also found to have a dual role in axon growth. The growth promoting effects of chondroitin sulfate (CS), heparan sulfate (HS), dermatan sulfate (DS) and keratan sulfate (KS) are dependent on the neuronal growth substratum (Campagna *et al.*, 1995; Dou and Levine, 1995; Snow *et al.*, 1996). The inhibitory effects of proteoglycans are neutralized if the relative concentration of laminin-1 is increased in relation to the inhibitory proteoglycans (McKeon *et al.*, 1995; Snow *et al.*, 1996).

Table 6. Behaviour of dissociated rat central neurons on ECM-proteins. The effect of each substrate is illustrated as a schematic drawing.

Protein	Neurons	Effect	Stimul.	Inhibition
Laminin-1	Cerebellum HC, cortex SC, retina		+++	N.D.
Laminin-2 (Merosin)	Retina Cerebellum (P1-P3)		+	N.D.
Laminin-3 (s-laminin)	SC CG		+	-
γ1 chain neurite outgrowth domain	Cerebellum HC, Retina SC		++	N.D.
Netrin-1	SC, Retina		+++	-
Thrombospondin	Cerebellum Retina		+++	N.D
Fibronectin	Cerebellum Retina		+	N.D
Tenascin-C/R	HC;Retina Cerebellum		++	-
Type IV Collagen	Cerbellum Cortex		N.D	-
HSPG	Cerebellum Retina SC		++	-
CSPG	Cerebellum SC		++	- -
KSPG	Cerebellum SC		++	- -

2.3.2. Netrins, semaphorins and ephrins in axon guidance

Netrins are soluble axon outgrowth promoting molecules, homologous to the γl chain of laminin-1 and *Unc-6* (Hedgecock *et al.*, 1990; Ishii *et al.*, 1992). Netrins are found to either attract or repel different subsets of axons towards or away from the ventral midline (Hamelin *et al.*, 1993; Serafini *et al.*, 1994, 1996; Colamarino and Tessier-Lavigne, 1995). The receptors that mediate the attractive effect of netrins are DCC and neogenin (for ref. see Chisholm and Tessier-Lavigne, 1999; Livesey, 1999). The repellent effect of netrins is mediated by receptors of the UNC-5 family (for ref. see Chisholm and Tessier-Lavigne, 1999; Livesey, 1999).

Semaphorins are a large family of secreted and membrane bound molecules (Semaphorin Nomenclature Committee, 1999) that were first identified as repellents for axonal growth (Mark *et al.*, 1997; Mueller, 1999). Receptors for secreted semaphorins, neuropilins, are necessary for repulsive guidance cues (Kitsukawa *et al*., 1997; Giger *et al.*, 2000). Recently semaphorins (Sema-1a) were also shown to have attractive cues for developing axons (Wong *et al*., 1999).

Ephrins and their receptors, the Eph receptor tyrosine kinases, mediate axonal pattering and pathway selection through contact depended repulsion (Flanagan and Vanderhaeghen, 1998). Eph receptors are named ephA or ephB depending on the class of ligands they bind (Eph Nomenclature Committee, 1997). Ephrins are divided into two classes: EphinA binds to EphA and is GPI linked to membranes, while ephrinB is a transmembrane protein that binds EphB. Further evidence for the role Eph receptors in axon guidance has been obtained from targeted mutation studies. Embryos lacking the EphB2 receptor function do not have a part of the anterior commissure (Henkemeyer *et al*., 1996). Ephrins and Eph receptors are required for establishing appropriate connections in the retinotectal systems (Cheng *et al*, 1995; Drescher *et al*, 1995; Monschau *et al*, 1997; Friesen *et al*., 1998).

2.3.3. Cell adhesion molecules

Pioneer axons may serve as guidance cues for the axons following them. When a large number of axons follow each other they become grouped together, which is called fasciculation. Cell adhesion molecules function in the fasciculation process. In *Drosophila* Fasculin II (Harrelson and Goodman, 1988), a homologue of the vertebrate NCAM, is involved in fasciculation (Grenningloh *et al*., 1991; Lin *et al*., 1994). L1, NCAM-180, and

N-cadherin promote neurite outgrowth *in vitro* (Doherty *et al.*, 1989; Lemmon *et al.*, 1989; Bixby and Zhang, 1990) and *in vivo* (Tomasiewicz *et al.*, 1993; Dahme *et al.*, 1997) but they act on different steps of neurite outgrowth (Takei *et al.*, 1999). L1 functions in neurite extension and NCAM-180 in growth cone protrusion (Takei *et al.*, 1999). NrCAM and axonin-1 mediate commisural axons to cross the midline. Reagents that disturb the interactions of growth cone associated axonin-1 with NrCAM in floor plate result in pathfinding errors *in vivo* (Stoeckli and Landmesser, 1995).

2.4. Neuronal degeneration

Neuronal degeneration is a normal phenomenon during nervous system development (Hutchins and Barger, 1998). Virtually all cell populations in the vertebrate nervous system undergo massive cell death during the early stages of their development. Approximately half of all the neurons produced during neurogenesis die (Raff *et al.*, 1993). Several reasons may cause neuronal degeneration during development. E.g. the failure of neurons to obtain support from some critical neurotrophic factors (O'Leary and Cowan, 1984; Pilar *et al.*, 1980) or their inability to make appropriate axonal contacts (Oppenheim, 1990) might lead to their death. Sex hormones also play a role in neuronal degeneration during development (Dibner and Black, 1976; Wright and Smolen, 1985). Developmentally occurring cell death is suggested to occur via apoptosis, characterized by shrinkage of the cell and its nucleus, condensation of chromatin, and production of membrane-enclosed particles containing intracellular material known as "apoptotic bodies" (Kerr *et al.*, 1972; Wyllie *et al.*, 1980; Arends and Wyllie, 1991).

In the adult brain, ischemic brain injury that leads to intracellular overdose of Ca^{2+} may lead to neuronal death (Choi, 1988a). Glutamate excitotoxicity is thought to participate in pathogenesis of the ischemic brain injury (Choi, 1988b; Choi and Rothman, 1990). Glutamate-induced neuronal death may occur either via apoptosis or necrosis depending on the mitochondrial function (Ankarcrona *et al.*, 1995). Necrosis is associated with an extreme energy failure of the mitochondria (Ankarcrona *et al.*, 1995) whereas relatively intact mitochondrial activity appears to be necessary for the apoptosis to proceed (Ankarcrona *et al.*, 1995). Thus, necrosis is a passive process characterized by cell and organelle swelling and lysis of the intracellular contents of the cell into the extracellular environment. Neurodegenerative diseases, such as Alzheimer's disease, Huntington's

disease, and amyotrophic lateral sclerosis (ALS) all cause neuronal death in the adult nervous system. In these diseases neuronal death is shown to occur via apoptosis (Smale *et al.*, 1995; Anderson *et al.*, 1996; Zhang *et al.*, 1997 for AD; Dragunow *et al.*, 1995; Herdreen *et al.*, 1994 for Huntington's disease; Alexianu *et al.*, 1994; Rothstein *et al.*, 1994 for ALS).

2.4.1. Alzheimer's disease

Alzheimer's disease (AD) is a neurodegenerative disorder that leads to early impairment of memory and other intellectual functions in humans. The most important neuropathological signs of Alzheimer's disease include the accumulation of senile plaques and neurofibrillary tangles in the brain tissue combined with extensive neuronal loss in hippocampus (HC), neocortex, and other areas of the brain. Senile plaques are extracellular deposits with an amyloid core surrounded by degenerating neurons, sprouting neurites, reactive astrocytes, and activated microglia. The major constituent of the plaques is the amyloid β protein peptide (Aβ1-42; Masters *et al.*, 1985; Selkoe *et al.*, 1986). Additional proteins found in the plaques include α-antichymotrypsin (ACT; Abraham *et al.*, 1988), apolipoprotein E and J (Namba *et al.*, 1991; Wisniewski and Frangione, 1992; Choi-Miura *et al.*, 1992), IgG (Eikelenboom and Stam, 1982), several complement proteins (Kalaria and Perry, 1993), glycosaminiglycans (Snow and Wight, 1989), fibronectin (Howard and Pilkington, 1990), and laminin-1 (I).

2.4.1.1. Pathophysiology of Alzheimer's disease

Alzheimer's disease appears in sporadic and familial forms (familial AD = FAD). Certain familial forms of AD have been linked to chromosome 21 (St George-Hyslop, *et al.*, 1987). The amyloid β protein gene maps to this chromosome (Kang *et al.*, 1987, Tanzi *et al.*, 1987). In all cases of AD there is excessive deposition of Aβ (Masters *et al.*, 1985; Selkoe *et al.*, 1986), an insoluble, 42 residue proteolytic processing product of the amyloid precursor protein (APP). In Alzheimer's disease APP, normally located on cell membranes (Kang *et al.*, 1987), is proteolytically cleaved and released into the extracellular space. All mutants of βAPP that have been linked to AD lead to altered proteolytic processing of this protein. This altered proteolytic processing favors the production of amyloidogenic and neurotoxic Aβ fragment of βAPP (Selkoe, 1995). An eleven amino-acid-long peptide (amino acids 25-35) derived from the 42-amino-acid amyloid β protein peptide has been shown to promote neurite outgrowth at low concentrations and to be neurotoxic at high

concentrations (Whitson *et al.*, 1989; Yankner *et al.*, 1989, 1990a,b; Rober *et al.*, 1991). This suggests a role for this peptide both in neuronal degeneration and sprouting events detected in the plaques. Neuronal death induced by amyloid β-peptide *in vitro* occurs by an apoptotic way (Loo *et al.*, 1993; Watt *et al.*, 1994).

In addition to its linkage to chromosome 21 AD is also genetically linked to chromosomes 1, 14, and 19. Mutations of the Presenilin-1 (PS-1) gene in chromosome 14 (Alzheimer's Disease Collaborative Group, 1995; Pereztur *et al.*, 1995; Sherrington *et al.*, 1995) and of the Presenilin-2 (PS-2) gene in chromosome 1 (Levy-Lahad *et al.*, 1995; Rogaev *et al.*, 1995) are found to cause the early onset familial form of Alzheimer's disease (FAD). These PS-1 and PS-2 genes encode for two highly homologous proteins (Levy-Lahad *et al.*, 1995; Rogaev *et al.*, 1995; Sherrington *et al.*, 1995) that have been localized either intracellularly within the endoplasmic reticulum and the Golgi complex (Kovacs *et al.*, 1996) or alternatively in the nuclear membrane and in association with interphase kinetochores and centrosomes (Li *et al.*, 1997). Recently, PS-1 has also been localized to the cell surface (Schwarzman, *et al.*, 1999). In addition to the PS-1 gene a gene for α-antichymotrypsin (ACT) also localizes in chromosome 14 (Billingsley *et al.*, 1993). ACT is a protease inhibitor found in the amyloid deposits of AD and in preplaques (Abraham *et al.*, 1988). ACT has been proposed to accelerate the fibril-formation of Aβ (Ma *et al.*, 1994). In some families with the late onset form of AD a linkage to chromosome 19 has been demonstrated (Pericak-Vance *et al* 1991). This is relevant for the role of apolipoprotein E in AD, because apolipoprotein E gene localizes in chromosome 19 (Lin-Lee *et al.*, 1985). Apolipoprotein E4 is a known risk factor for Alzheimer's disease (Corder *et al*, 1993) although its mechanism of action is presently unknown (Mahley and Huang, 1999).

In addition to linkage studies several results indicate that defects in energy metabolism may contribute to the pathogenesis of AD (Blass and Gibson, 1991). In the late-onset AD mitochondrial cytochrome c oxidase (CO) activity in brains and platelets of the affected individuals is decreased (Parker *et al.*, 1990; Mutisya *et al.*, 1994; Chagnon *et al.*, 1995). Recently, mutations of the mitochondrial CO gene that associate with the late-onset AD have been found to result in overproduction of reactive oxygen species *in vitro* (Davis *et al.*, 1997). The free radicals formed may participate in the cleavage of β-amyloid protein and result in the aggregation of Aβ peptide in the AD brain tissue.

2.4.1.2. Extracellular matrix molecules in Alzheimer's disease

Several extracellular matrix molecules have been localized in the AD brain. Fibronectin is found in plaques and in crystal-like formations in the gray matter (Howard and Pilkington, 1990). Laminin-1 (I) is localized to the plaques of AD. In cerebral microvessels collagen IV content is increased in AD (Kalaria and Pax, 1995). At least four different classes of proteoglycans (PGs) have been detected in the AD brain. These include heparan sulfate PGs (Snow *et al.*, 1988; 1990; Snow and Wight, 1989,), dermatan sulfate PGs (Snow *et al.*, 1992), chondroitin sulfate PGs (DeWitt *et al.*, 1993), and keratan sulfate PGs (Snow *et al.*, 1996).

APP binds to several ECM proteins, including HSPGs (Narindrasorasak *et al.* 1991; Snow *et al.*, 1994) and laminin-1 (Narindrasorasak *et al.*1992; Kibbey *et al.*1993). In fact, APP may be a laminin receptor that binds specifically to the IKVAV-sequence of the α1-chain of laminin-1 (Kibbey *et al.*1993). Biological functions of Aβ-peptide could be regulated by its binding to ECM molecules (Koo *et al.,* 1993). E.g, low concentrations of substrate-bound Aβ was found to promote neurite outgrowth when combined with low amounts of the ECM proteins laminin or fibronectin (Koo *et al.,* 1993).

Recently, ECM molecules, including laminin-1, have been shown to influence the biogenesis of APP and the generation of amyloidogenic fragments containing amyloid β-peptide (Bronfman *et al.*1996ab, Mönning *et al.* 1995). Perlecan, a HSPG, accelerates Aβ fibril formation and stabilizes these fibrils (Castillo *et al.*, 1997). Importantly, laminin-1 has been shown to reduce the fibril formation of Aβ-peptide *in vitro* (Bronfman *et al.*, 1996a, 1996b, 1998; Monji *et al.*, 1998a, 1998b; Drouet *et al.*, 1999). In the hippocampus and the frontal cortex of Alzheimer's disease patients the expression of laminin-1 is increased compared to normal control brains (I). In AD laminin-1 was identified as large extracellular punctate deposits in all senile plaques (I).

2.5. Nervous system regeneration

Regeneration of the adult mammalian peripheral and central nervous system is limited. Injuries to the central nervous system of adult mammals regenerate mainly in the olfactory bulb (Graziadei and Monti Graziadei, 1978). The peripheral nervous system is capable of regeneration, although regeneration of peripheral nerve injuries in adult mammals occurs slowly and does not necessarily lead into functional recovery.

2.5.1.Regeneration in the peripheral and central nervous systems

Axotomy or crushing of a peripheral nerve leads to Wallerian degeneration, where the portion of the axon distal to the injury site degenerates. During Wallerian degeneration the environment in the trauma site is created for regeneration. This includes chromatolysis of the cell body and removal of the distal end of the axon with myelin-derived debris by macrophages. If the regeneration is successful the changes in the cell body are reversible and the proximal stump of the axon grows within the connective tissue remaining in the distal stump and regenerate. Schwann cells in the distal stump secrete chemotrophic factors that attract growing axons by mechanisms related to axon growth during development. (Sunderland, 1991).

Axons of adult CNS do not regenerate, whereas axons of adult PNS regenerate. Lack of regeneration in the mammalian CNS is thought to be due to the yet-unidentified factors that prevent regeneration in the CNS but not in the PNS. This has been shown by studies in which the CNS axons were shown to regenerate if they grow into grafts of the peripheral nerves (Benfey and Aguayo 1982, Villegas-Perez *et al.*, 1988).

In recent years some of the CNS factors inhibitory for CNS regeneration have been identified. Cultured CNS oligodendrocytes and CNS myelin were found to inhibit neurite outgrowth of cultured neurons (Schwab and Caroni, 1988). Some of this inhibition was mediated by two CNS myelin proteins (absent from PNS myelin), NI-35 and NI-250 (Caroni and Schwab, 1988a). The use of antibodies IN-1 and IN-2 against the NI-35 and NI-250 molecules did neutralize the inhibitory effects of these antigens (Caroni and Schwab, 1988b). Transplantion of myeloma cells producing the IN-1 antibody into the dorsal fronto-parietal cortex of rats 7-10 days before a spinal cord lesion allowed CNS axons to regenerate (Schnell and Schwab, 1990). Recently identifications of *Nogo* gene in

humans and rats, that encodes of an inhibitory myelin protein have been reported (Chen *et al.*, 2000; Prinjha *et al.*, 2000; GrandePré *et al.*, 2000). The Nogo-A isoform may be the NI-250 protein recognized by the IN-1 antibody (Chen *et al.*, 2000; Prinjha *et al.*, 2000; GrandePré *et al.*, 2000). Myelin-associated glycoprotein (MAG; Trapp, 1990), another myelin protein that inhibits neurite outgrowth (Mukhopadhyay *et al.*, 1994; McKerracher *et al.*, 1994), is present in myelin sheaths of both CNS and PNS axons (Figlewicz *et al.*, 1981; Quarles and Trapp, 1984). The inhibitory effect of MAG in the PNS is neutralized by laminin-1 (David *et al.*, 1995). Recent results indicate that adult CNS myelin does not always inhibit regeneration but the reactive glial extracellular matrix at the lesion site may be a major factor associated with the failure of axon regrowth (Davies *et al.*, 1997). Furthermore, several recent studies indicate that the failure of the axonal regrowth in the CNS but not in the PNS may be due to the inability of damaged CNS tissue to activate an appropriate inflammatory response after damage (Lazarov-Spiegler *et al.*, 1996, 1998; Rabchevsky and Streit, 1997; Rapalino *et al.*, 1998; Zeev-Brann *et al.*, 1998).

Embryonic CNS tissue regenerates (Kromer *et al.*, 1981; Björklund and Stenevi, 1984), although the mammalian brain has a critical age of development after which regeneration of the CNS does not occur (Smith *et al.*, 1986). This critical period coincides with myelination and oligodendrocyte development (Smith *et al.*, 1986; Varga *et al.*, 1995). Although CNS neurons are generally capable of regeneration (Benfey and Aguayo, 1982) and environmental factors are the major reason for their failure to regenerate, it has also been proposed that the intrinsic properties of CNS neurons may suppress regeneration. Axonal regeneration in the postnatal rat *in vitro* has been shown to depend on the maturation of axons (Li *et al.*, 1995) and injured axons of the adult rat have failed to regenerate, even in a permissive glial pathway (Davies, *et al.*, 1996). The regenerative failure of most older axons may be controlled by genetic programming absent in the developing neurons (Chen *et al.*, 1995, 1997a; Aigner *et al.*, 1995). A candidate gene that may regulate axon growth is the proto-oncogene *bcl-2* (Holm and Isacson, 1999), one of the primary factors responsible for keeping cells from choosing the apototic pathway. At the onset of a regenerative failure *in vitro* the expression of *bcl-2* is decreased (Chen *et al.*, 1997a). Retinal ganglion cells derived from transgenic mice over-expressing the *bcl-2* gene retain their ability to grow axons throughout their lifespan (Chen *et al.*, 1997a).

2.5.2. Attempts to regenerate PNS injuries

A standard method to rejoin transected peripheral nerves is neurorraphy using microscopic instruments and thin microfilament threads (Sunderland, 1991; Terzis and Smith, 1991). This methodology requires a specialist and is time consuming. Both gluing (Narakas, 1988) and laser technology (Almqvist, 1988) have been applied, but these methods are not easier and do not improve regeneration (Vastamäki, 1990). Thus, novel methods to regenerate injuries of the PNS are needed.

Tubulation of both the proximal and distal nerve ends is believed to aid regeneration. Tubulation prevents the displacement of nerve stumps, orient axon growth and vascularization, concentrates growth and trophic factors and protects regenerating tissue from scar invasion (Madison *et al.*, 1992). Various kinds of tubular prostheses have been used to bridge nerve gaps. Silicone tubes alone were found to increase regeneration compared to the untreated controls (Lundborg *et al.*, 1982). The silicone tube facilitates elongation of the axons across 10-mm gaps in rats (Lundborg *et al.*, 1982). Extended gaps could also be successfully bridged with silicone tubes filled with collagen-glycosaminoglycan (GAG; Chamberlain *et al.*, 1998) or artificial nerve grafts composed of polyamide filaments (Lundborg *et al.*, 1997). These grafts facilitated regeneration across a 15-mm gap in the rat sciatic nerve (Lundborg *et al.*, 1997; Chamberlain *et al.*, 1998). However, the use of silicone tubes necessitates surgery to suturate the nerve ends into the silicone tubes. Another more serious disadvantage in the use of silicone tube grafts is the fact that axons regenerated through the silicone tubes degenerate after one year of implantation unless the silicone tube is removed. Axonal degeneration may be caused by the tube material itself or by the constriction of the proximal and distal nerve stumps due to long-term tubulization (Le Beau *et al.*, 1988). Thus, usage of silicone tubes necessitates a second surgery to remove the silicone tubes after regeneration has taken place. To avoid the second surgery biodegradable tubes have been developed. The biodegradable tubes tested include polylactate tubes (Da Silva *et al.*, 1985; Madison *et al.*, 1987), and poly(organo)phosphazene tubes (Langone *et al.*,1995). Collagen or collagen with a GAG substrate (Collin and Donoff, 1984; Ellis and Yannas, 1996; Chamberlain *et al.*, 1998) have also been tried in addition to collagen tubes filled with Schwann cells (Kim *et al.*, 1994). Thin cellulose sheets have shown to be as effective as microsurgical neurorraphy in reconnecting the transected nerve (III).

2.5.2.1. Laminin treatment of the PNS injuries

Laminin-1 is a potent neurite outgrowth promoting molecule that is also involved in axon guidance (Baron van Evercooren *et al.*, 1982; Liesi *et al.*, 1984, 1989; Cohen *et al.*, 1987; Letourneau *et al.*, 1988). Astrocytes in the CNS of the lower vertebrate (Liesi 1985; Hopkins *et al.*, 1985) and Schwann cells of the mammalian PNS constitutively express laminin-1 (Cornbrooks *et al.*, 1983). Astrocytes of the olfactory bulb, the only regenerating area of the adult mammalian CNS, continuously express laminin-1 (Liesi, 1985b). These observations indicate that expression of laminin-1 correlates with nerve regeneration. This has suggested many researchers to use laminin-1 grafts in the treatment of PNS injuries. Laminin has been applied in both silicon and biodegradable tubes as well as in gels with additional ECM components or in collagen tubes also containing fibronectin (Madison *et al.*, 1985; Madison *et al.*, 1987; Bailey *et al.*, 1993; Tong *et al.*, 1994; Labrador *et al.*, 1998). The functional role of laminin-1 in neuronal regeneration is further emphasized by the fact that the inhibitory effect of a myelin-associated glycoprotein (MAG) on neurite outgrowth is neutralized by laminin-1 (David *et al.*, 1995). Microglial grafts that supposedly counteract the mechanisms that inhibit CNS regeneration induce laminin expression at the injury site (Zak, 1987; Rabchevsky and Streit, 1997).

The Schwann cell laminin differs from the basement membrane form of the protein in that it lacks the conventional α1-chain (Davis 1985; Edgar *et al.*, 1988). It is possible that the α-chain of Schwann cell laminin is either missing or that it is shorter than the α1-chain of laminin-1 (e.g. α4-chain). It appears that no α-chain is necessary for the regeneration of peripheral nerves. This is evident from a study in which a decapeptide from the neurite outgrowth domain of the γ1-chain was coupled with a type I collagen matrix to form a filter. This peptide graft supported nerve regeneration as well as suturation of the rat sciatic nerve (Kauppila *et al.* 1993). Animals that received laminin grafts to repair their sciatic nerves showed significantly lower autotomy scores (numbers of self-mutilated phalanx) compared to the animals with conventional neurorraphy. The thickness of the graft, however, formed a limitation for its use. If a large number of adjacent nerve fascicles were to be separately reconstructed, the graft resulted in a compression damage of the repaired nerves (Kauppila *et al.* 1993). A thick graft could also impair regeneration by preventing the diffusion of factors critical for regeneration.

2.5.3. Attempts to regenerate CNS injuries

The first transplantation studies of nervous tissue were performed as early as in 1863 by using a portion of the optic nerve to regenerate a dog hypoglossal nerve (For references see Dellon and Dellon, 1993). Various types of mature CNS neurons have expressed regenerative capacity of their injured axons when the axons are in contact with peripheral nerve grafts (David and Aguayo, 1981; Munz *et al.*, 1985; Morrow *et al.*, 1993; Decherchi and Gauthier, 1996) and a 20% recovery of the dorsal root fibers into the spinal cord was obtained using living glial cell implants (Kliot, *et al.*, 1990). Cultured Schwann cells were found to promote regeneration of CNS neurons when transplanted into the lesioned adult rat spinal cord (Paino and Bunge, 1991; Li and Raisman, 1994). Recently macrophages, prestimulated with spontaneously regenerating peripheral nerve tissue, have been shown to stimulate optic nerve regeneration in the adult rat (Lazarov-Spiegler *et al.*, 1996). These activated macrophages were also found to enhance the clearance of damaged myelin from the lesion site (Lazarov-Spiegler *et al.*, 1998). Grafts of cultured microglial cells were also shown to induce prominent neurite outgrowth of the lesioned spinal cord of adult rats (Rabchevsky and Streit, 1997).

No therapy for the repair of spinal-cord injuries is presently available (Olson, 1997). Recently, promising results regarding both the histological and functional recovery of CNS injuries in rats has been achieved by chronic infusions of IN-1 antibody, neurotrophin-3 or both (Diener and Bregman, 1994; Schnell *et al.*, 1994; Bregman *et al.*, 1995). The first evidence for true functional regeneration in the adult mammalian spinal cord was reported when the cut rat spinal cord was bridged by peripheral nerves in-between the individual spinal axon tracts and areas of neuronal cell bodies (Cheng *et al.*, 1996). The bridge was further stabilized by FGF-impregnated fibrin glue (Cheng *et al.*, 1996).

Neural transplantation of dopaminergic neurons of human embryos into the brains of Parkinson's disease patients offers a novel therapeutic tool to treat this neurodegenerative disease (Lindvall *et al.*, 1994; Kopyov *et al.*, 1996). Human CNS progenitor cells, maintained in a proliferative state in culture, have been shown to migrate and differentiate into both neurons and astrocytes following intracerebral grafting in rats (Svendsen *et al.*, 1997). Although a low number of tyrosinase hydroxylase-positive neurons (approximately 200) were identified *in vitro*, they could allow functional recovery, because allograft

studies have shown that only 120 dopamine cells are needed to produce functional recovery effects (Brundin *et al.*, 1985). Transplanted pig fetal neural cells have also been grafted in brains of Parkinson's disease patients (Deacon *et al.*, 1997). The implanted pig dopaminergic neurons matured and extended neurites into the human host brain (Deacon *et al.*, 1997). Continuous neurogenesis is shown to occur in the adult mammalian brain, which may allow future development of methods that favour regeneration in the CNS.

AIM OF THE STUDY

Aim of this study was

- to investigate the possible involvement of the neurite outgrowth domain of the γ1-chain of laminin-1 in pathophysiology of human neurodegenerative disorders (I)

- to use the weaver mutant mouse as a model system to investigate the possible role of laminin-1 γ1-chain in neuronal migration defects and in neuronal death (II)

- to develop novel tools for neurorraphy (III)

- to use the F9 cells to develop a novel model system for future studies on neuronal differentiation (IV)

MATERIALS AND METHODS

I

Production of Antisera to Laminin Peptides

Synthetic peptides derived from the C-terminal part of the mouse γ-1-chain (Sasaki and Yamada, 1987) or α1-chain (Tashiro *et al.*, 1989) of laminin-1 were from Multiple Peptide Systems (La Jolla, CA). Rabbits for anti-1533 a and anti-1533 b were immunized with a 30 amino acid peptide (p32; I Fig. 1) and rabbits for anti-1543 were immunized with a 10 amino acid peptide (p20; I Fig. I) conjugated to itself to form a larger polymer (Linder and Robey, 1987). Rabbits for anti-2091 were immunized with a 22 amino acid peptide from the α-chain of laminin-1 (PA-22; I Fig. 1.). The peptide columns used to absorb each antibody were done by coupling the peptides to Sepharose 4B (Pharmacia, Uppsala, Sweden) according to the instructions of the manufacturers.

Characterization of Antipeptide Antisera

The peptides p20 and p32 were labeled with ^{125}I using the Bolton-Hunter reagent and the chloramide T method (Risteli and Risteli, 1987), respectively. The radioimmunotitration (Timpl and Risteli, 1982) and inhibition (Risteli and Risteli, 1987) assays were carried out as described previously. Mouse laminin-1 was purified as described earlier (Timple *et al.*, 1979). Ten micrograms of purified laminin-1 was run in 5% sodium dodecyl sulfate (SDS) gel electrophoresis under reducing conditions and blotted onto nitrocellulose filter as described before (Towbin *et al.*, 1979). The transferred proteins were visualized with 0.2% Ponceau (Sigma, St Luise, MO) in 4% trichloracetic acid and processed for immunostaining using antipeptide antibodies or antibodies to native laminin-1 as described earlier (Liesi and Risteli, 1989). 100 ng of native laminin-1 or 10 ng of each synthetic peptide were dot blotted onto a nitrocellulose filter. The filter was dried and processed as described earlier (Liesi and Risteli, 1989).

Immunostaining of Brain Tissue Sections

Brain tissue samples from Alzheimer's disease and Down's syndrome patients and from normal, age-matched controls were obtained from the National Neurological Research Bank (VAMC Wadsworth Division, Los Angles, CA). All samples were neuropathologically verified and were from either the hippocampus or the frontal cortex of the diseased brains. Brain samples from a total of four different Alzheimer's disease patients and five normal, age-matched controls were analyzed. In addition autopsy material from the hippocampus of a 44-year-old Finnish Down's syndrome patient was

obtained. The deep frozen brain tissue was cut into 10 μm cryostat sections and dried for 2 hours at room temperature. The sections were fixed with either 2% paraformaldehyde or 0.4% p-benzoquinone (Koch-Lights Inc.) in phosphate-buffered saline (PBS) for 15 min at 4°C and processed for immunocytochemistry for laminin or other molecules as described elsewhere (Liesi and Silver 1988). In short, the sections were dehydrated in graded series of alcohols (50%-70%-96%-100%-xylene) for 5 min and rehydrated through the same series to PBS. After rinse in PBS for about 30 min the sections were incubated overnight at 4°C in normal sheep serum (Sigma, St. Louis, MO) and incubated with the primary antisera for 24 hours at 4°C.

The antiserum against mouse laminin-1 (Liesi and Silver 1988) was used at a dilution of 1:2000 and affinity-purified antibodies against the P1 fragment of human laminin-1 (Risteli and Timpl, 1981) at a concentration of 10 μg/ml. These two antibodies were applied as an additional control for the specificity of the staining patterns observed. Antibodies against the synthetic peptides derived from laminin-1 (I) were applied at 1:1000- 1:2000 dilutions. The sections were incubated with the primary antibodies for 24h, briefly washed in PBS, and exposed to sheep antirabbit immunoglobulins coupled to fluorescein isothiocyanate (FITC; Wellcome, Beckenham, UK). In double staining for α-antichymotrypsin or amyloid β protein the sections were further incubated with normal rabbit serum followed by incubation with rabbit antibodies to α-antichymotrypsin (Dakopatts, Copenhagen, Denmark) or rabbit antibodies to the amyloid β protein peptide (Masters *et al*. 1985). The antibodies were diluted 1:500 and 1:1000, respectively. The specificity of this sequential immunostaining procedure for two rabbit antibodies has been confirmed (Liesi *et al*. 1983b). After a brief wash in PBS, the sections were mounted in PBS:glyserol (1:1) and viewed with an Olympus BH2 microscope using epifluorescence and appropriate filter combinations.

Demonstration of mRNA in Brain Tissue

The brain tissue to be analyzed was frozen and ground in liquid nitrogen, solubilized in guanidine isothiocyanate, and layered on a CsCl cushion (Chirgwin *et al.*, 1979). The separated RNA was electrophoresed in formaldehyde agarose gels (Goldberg, 1980) and visualized by ethidium bromide shadowing. After transfer, the RNA was hybridized with oligolabelled cDNA probes to the α1 (Sasaki *et al*.,1988), β1 (Sasaki *et al*., 1987) and γ1 (Sasaki and Yamada, 1987) chains of laminin-1. Shortly, the cDNA probes were

oligolabelled using Random Primed DNA Labelling Kit (Boehringer Mannheimm) and purified in G50 column. The filters were incubated in prehybridization solution (50% deionized formamide, 50mm Hepes, 3×SSC, 5×Denharts, 0.2 mg/ml SS-DNA, 1 mm EDTA, 0.2% SDS, 0.12%NaCl) for one hour at +37°C. After prehybridization SS-DNA (6.4 mg) and oligolabelled cDNA probe was added to the prehybridization solution and filters were further incubated overnight at +37°C. After hybridization filters were washed in 2×SSC with 0.1% SDS at+37°C 15 min and exposed to X-ray film. A mouse α-actin cDNA probe (Minty *et al.*, 1982) served as a control for the quantity of mRNA loaded. The same Northern blot lane was hybridized with each of the four probes in a sequential order. To ensure that washing off of the probe at 60°C in 50% deionized formamide/0.1xSSC/0.1% SDS for 30 min did not wash off the mRNA, the α-chain message was detected first, the γ-chain message second followed by the β-chain and α-actin transcripts.

Immunoblotting of Laminin-1 in Brain Tissue

The frozen brain tissue was transferred into a sterile 50 ml Falcon tube containing 10 ml of lysis buffer (2% Triton-X-100, 0.5 M NaCl, 10 mM Tris HCL, pH 7.4, 1mM PMSF). After homogenization the tissue was dissolved in the lysis buffer and incubated for 30 min at 4°C. The undissolved material was centrifuged at 14 000g for 20 min at 4°C. The supernatant was transferred to a new tube and dissolved in 5 ml Laemmli sample buffer with 0.1 M β-mercaptoethanol. The proteins were analyzed via 5% SDS-gel electrophoresis. After transfer to nitrosellulose (Towbin *et al.*, 1979) the filter was stained with 0.2% Ponceau S in 4% trichloroacetic acid and processed for immunoblotting using antibodies to native laminin-1 as described elsewhere (Liesi and Risteli, 1989).

Polymerase Chain Reaction (PCR) Analysis of the Laminin β Chain mRNA in Brain Tissue

A specific DNA copy of the laminin β1 chain RNA sequence was synthesized using two oligonucleotide primers (B1 and B2) derived from the published sequence of human laminin β1 chain gene (Vuolteenaho *et al.*, 1990). The primer set spanned a region of 287 bp, extending from exon 9 to exon 10. First-strand cDNA synthesis was accomplished by extension with the downstream primer B2 (5´-GGATCTCGGATGTCCCTCTCTGG-3`) in a 20 µl reaction volume containing 1 µg

total RNA, 30 pmol PCR primer, and reverse transcriptase from AMV (Promega) under conditions suggested by the supplier. Fifty percent of the heat-treated first-strand cDNA mixture was then included in 50 μl volume with 1 μM of each of the primers B1 (5´-CATGTGCAGGCATAACACCAAGG-3`) and B2, 0.2mM each of dNTPs, 10 mM Tris-HCL (pH 8.4), 50 mM KCL, 1.5 mM MgCl$_2$, 0.1mg/ml gelatin, and 1.5 U AmpliTag DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT). Forty-five cycles of PCR amplification were performed at 95°C, 55°C and 72°C for 1 min each in a DNA thermal cycler (Techne PHC-2) according to the method of Saiki *et al.* (1985). The double-stranded PCR products resulting from this amplification were fractionated by electrophoresis on a 2% agarose gel followed by elution, extraction with equal volumes of phenol and chloroform, and precipitation with ethanol. One-half of this template was subjected to DNA sequence analysis using the dideoxy chain termination method of Sanger (1977) with Sequenase (U.S. Biochemicals). The sequence was determined in both directions using the PCR primers as sequencing primers. The sequencing reactions were run on 5% polyacrylamide gels containing 7 M urea and were visualized by autoradiography (Konica X-ray film).

II

Weaver Mice

Heterozygous (+/wv) mice carrying the weaver mutation were obtained from the Jackson Laboratories (Bar Harbor, ME) and bred at the colony of the Institute for Basic Research, New York or at the Department of Anatomy, University of Helsinki. The mice were free of mycoplasma, MHV, Sendai, and other common mouse pathogens. They were bred on a B6CBA-A^{W-J}/A wv genetic background and homozygous weaver (wv/wv) or control (+/+) mice were used for experiments 7 to 13 days after birth.

Neuronal Cultures

Cultures of the granule neurons were initiated from cerebella of 7- to 10-day-old homozygous (wv/wv) weaver mice and their normal (+/+) litter mates. The cerebella were aseptically removed and the cells dissociated using a trypsin/DNase-treament as described in detail by Trenkner (1991). One hundred thousand cells were plated on laminin-coated, 22-mm glass coverslips and cultured for 24 hr in serum-free RPMI 1640 culture medium (Gibco, BRL) supplemented with antibiotics and 2 mM glutamine. The cultures were fixed in 2% paraformaldehyde in PBS for 15 min for quantification and

immunostaining experiments. In a subset of experiments cultures were grown in the presence of 100 U/ml of aprotinin (ICN Biomedicals, Costa Mensa, CA) added into the culture medium 1hr after plating. For biochemical studies on proteolytic enzyme activities the cells were cultured overnight, lysed, collected in Laemmli sample buffer without β-mercaptoethanol (LSB-), and run in 3-12 % gradient SDS gels. Quantification of neurite outgrowth was done in six normal cultures, six weaver cultures, and six weaver cultures containing aprotin: The numbers of neurons in six representative fields on each laminin -coated glass coverslip were evaluated using phase-contrast microscopy. The mean numbers of neurons, and of neurons bearing long neurites (>10 times cell soma), were evaluated in each case. Approximately 60 neurons were counted per coverslip. Oneway variant analysis (ANOVA) on the Instat (1.11a) program was used for statistical analysis. The statistical comparisons between each individual group of neurons were performed using the Bonferroni's modified t-test.

Immunocytochemistry

Cerebellar tissues of the normal (+/+) and homozygous (wv/wv) weaver mutant mice were frozen in powdered dry ice and cut to 10-μm cryostat sections. The sections were dried for 2 hr at room temperature, lightly fixed in freshly prepared 0.4% *p*-benzoquinone (Sigma, St. Louis, MO) in PBS, and processed for immunocytochemistry as described in detail by Liesi and Silver (1988). Antibodies against laminin-I (Liesi and Silver, 1988) and its neurite outgrowth domain (anti-1533a and anti-1534 in I) were those used previously, and their specificities were confirmed (I). The anti-1533a was used for *in vivo* studies and the anti-1543 was used for *in vitro* studies. Rabbit antibodies against tissue plasminogen activator (tPA) were those used in earlier studies (Tienari *et al.*, 1991). Antibodies against laminin-1 and its neurite outgrowth domain were applied at 1:2000 and those against tPA at 6 μg/ml for overnight incubations at +4 C. Binding of primary antibodies was detected using sheep anti-rabbit immunoglobulins coupled to FITC (Wellcome, Beckenham, UK). In some experiments sections were immunostained for the glial fibrillary acid protein (GFAP; Bignami and Dahl, 1974a, b) or the L1 antigen using rabbit polyclonal antibodies (Rathjen and Schachner, 1984). The immunostained tissues were viewed and photographed using an Olympus BH2 fluorescence microscope with appropriate filter combinations.

Zymographic Assays of the Normal and Weaver Mouse Cerebellar Tissues in Vivo and in Vitro

Cerebellar tissue or granule neurons on a laminin substratum were collected, lysed in LSB-, and run in 3-12% gradient gels. The gels were washed free of SDS and overlaid with agarose containing casein and plasminogen as described in detail (Tienari *et al.*, 1991).Proteolytic enzyme activities of the cell extracts were monitored by incubating gels with the overlays for 12-24 hr followed by staining of the overlays with amino black to detect the sites for enzyme activity. Human recombinant urokinase (1.0 U/ml uPA; Calbiochem, San Diego, CA) and tissue plasminogen activator (5 ng/ml tPA, American Diagnostica, Greenwich, CT) were used as controls. The specificity of the enzyme reactions was monitored by control experiments in which 100 μg/ml of the neutralizing antibodies against either uPA or tPA (Tienari *et al*. 1991) was added into the agarose overlays to inhibit the functions of the respective enzymes present in the cell or tissue extracts.

Electrophysiology

Resting membrane potentials of the normal and weaver neurons on a laminin substratum were determined using a List EPC-7 patch clamp amplifier. The resting membrane potentials of the cultured cells were measured immediately after entering the whole cell patch configuration as described (Hamill *et al*. 1981). Pipettes were pulled from borosilicate glass and lightly fire polished. Patch pipettes contained (in mM): 140 CsCl, 10 BAPTA, 2 $MgCl_2$, and 10 HEPES (pH 7.2). Experiments were performed at room temperature in the RPMI 1640 culture medium.

III

Animals

Male Wistar rats (Hannover strain; Harlan, Netherlands) were used in all experiments. The rats were 8 months old (380-470 g) and had water and food available *ad libitum*. They were housed in groups of six animals with a light cycle 6.00-18.00 h and a relative humidy of 35-55%. All experiments were apporoved by the Institutional Ethics Committee of the Institute of Biomedicine, University of Helsinki and by the Provincial Goverment of Uusimaa, Finland.

Behavioral Testing of Neuropathic Symptoms

The mechanical withdrawal thresholds of the sciatic nerve areas of the hindpaws of twelve rats were first determined as described earlier (Mansikka and Pertovaara, 1995). Briefly, the rat was standing or walking on a metal grid and the right hindpaws were stimulated with a series of calibrated von Frey monofilaments (Stoelting, USA). The central pads of the paw served as the stimulus site. The monofilaments were applied to the foot pad in series of increasing force until the rat withdrew the limb. The lowest force producing a withdrawal response was considered the threshold. The threshold for each hindpaw was based on three separate measurements. The tests were performed double-blind 1 and 2 months after trauma. The statistical comparisons were performed using Friedman repeated measures of variance on ranks followed by two tailed Mann-Whitney U-test. $P < 0.05$ was considered significant in all statistical comparisons of this series of experiments.

Traumatization and Reconstructive Surgery

The rats (6 in each experimental group) were anesthetized with pentobarbital (50 mg/kg, Orion, Finland) and the right sciatic nerve was exposed under aseptic conditions. The nerve was transected at mid-thigh and either resutured with two perineural 10-0 monofilament sutures or reconnected by using a moistened lens cleaning paper (Illford, U.K.) which was wrapped around the stumps of the transected nerves (Kauppila *et al*. 1993). The anatomical orientation of the nerve stumps was restored by observing the fascicular and vascular anatomy of the transected nerves. After reconstruction the wounds were closed in layers with 3-0 silk sutures.

Scoring of Autotomy

Autotomy of the digits was observed once a week during a period of six months. Autotomy was scored as described earlier (Kauppila and Pertovaara, 1991): Each self-mutilated phalanx represented one score point. Two-tailed Mann-Whitney U-test was used for statistical comparisons. The tests for mechanical withdrawal thresholds were performed one month and two months after nerve resuturation because the regenerated axons reach the mid-thigh and establish their connections to the periphery during the next month (Kauppila *et al*. 1993).

Electrophysiologic Testing of Recovery

Six months after surgery the rats were anesthetized with pentobarbital (50 mg/kg) and reinnervation of the soleus and gastrocnemicus muscles was studied as described earlier (Kauppila *et al*. 1993). The sciatic nerve (both operated and unoperated side) was exposed, transected proximal to the lesion, mounted on a bipolar platinum stimulating electrodes, and covered with liquid paraffin. The hindlimb was immobilized with needles and the achilles tendon was cut and connected to a strain gauge (Grass, U.S.A). The initial load was adjusted to 5 g. The sciatic nerve was stimulated proximal to the trauma site with square wave pulses of 0-01 or 0.1 ms duration and constant voltage of 15 V (stimulator Nihon-Kohden, Japan). Five consecutive stimuli were used for testing at both stimulus durations. The tension resulting in a muscle twitch was recorded after each stimulus and the differences in tension between the control and operated side were compared with the one-sided Students t-test. This test was chosen, because we wanted to detect even the smallest putative differences between the forces of the control (left) side and the trauma (right) side.

Testing of Anatomic Recovery

To evaluate the degree of anatomical recovery the rats were killed with an overdose of pentobarbital,and the muscles dissected out and weighed at the end of the electrophysiological experiments (Kauppila *et al*. 1993). The statistical analysis was carried out using the Student two-tailed t-test.

Immunocytochemistry

The nerves were dissected free, frozen on dry ice and cut in 10 μm cryostat sections. The cryostat sections were fixed in 0.4% p-benzoquinone in PBS for 15 min, washed in PBS, and dehydrated and rehydrated as described earlier (Liesi and Silver, 1998). Mouse monoclonal antibodies against the 200 kDa neurofilament protein (RT97, Boehringer, Germany) were applied at 1:1000 dilutions. After an overnight incubation with the first antibodies the sections were washed in PBS and exposed to goat anti-mouse immunoglobulins coupled to TRITC (Cappel, PA, U.S.A) for 1 h. The immunocytochemistry was viewed and photographed with an Olympus BH-2 microscope with appropriate filter combinations.

IV

Cell Cultures

The cells were those used previously (Wartiovaara *et al.*, 1978; Liesi *et al.*, 1983). The uninduced cells were maintained in plastic Petri dishes (Nunc, Denmark) in 10% fetal calf serum supplemented with RPMI 1640, antibiotics ,and glutamine. For induction studies the cells were trypsinized and plated on 13 mm glass coverslips sterilized by flaming in alcohol. The cells were cultured at a density of 10^5/coverslip in 3% fetal calf serum/RPMI overnight after which RA (Sigma, St.Louis, MO; 10^{-7}) and dibutyryl cAMP (Sigma;10^{-3}) were included into the culture medium. The induction was completed by culturing the cells for 10 days with a media change every three days. The control cultures were plated at the same density but either maintained in 10% fetal calf serum or in 3% fetal calf serum without RA/dbcAMP and fixed after 10 days of cultivation. The cells were fixed in 2% paraformaldehyde in PBS for 15 min and immunostained immediately afterwards. For the RNA isolation studies the cells were plated at an initial cell density of 100 000 on a 9 cm plastic Petri dish either in the presence of 10% fetal calf serum or in 3% fetal calf serum with or without the inducing agents.

Cloning of a F9 Teratocarcinoma Cells

Trypsinized F9 cells were plated at low density (1×10^3) on 10cm bacteriological dishes and picked one by one to the microwells of the tissue culture plastic or to ELISA plates precoated with laminin (100 µg/µl). The cells were grown in 10 % fetal calf serum supplemented with RPMI 1640, antibiotics, and glutamine. Cells from the confluent microwells were transferred into the 3 cm plastic Petri dishes and further expanded onto the 10 cm plastic Petri dishes. To ensure that each clone originated from a single F9 cell the cells from 10 cm dishes were sub-cloned again in single cells as described above, regrown, and stored in 10% DMSO/FCS in liquid nitrogen. The clones were initially tested for their neuronal properties by plating them on 25 mm glass coverslips in 10% FCS supplemented with RPMI 1640, antibiotics, and glutamine. After 48 h, the cloned cells were fixed in methanol and immunostained for the neurofilament triplet proteins and other neuronal marker proteins. Only the clones derived from the laminin-coated microwells were chosen for future studies, because they expressed neuronal properties in a serum free medium (not shown), e.g. the cells had both a neuronal phenotype with neurite like extensions and expression of neurofilaments and other neuronal markers. One of such clones, the clone D9L2, was selected for further studies. The D9L2 clone was plated on 25 mm glass coverslips in a serum free RPMI supplemented with

antibiotics and glutamine. After 48 hrs the cells were fixed and processed for immunocytochemistry.

Northern Analysis

Total RNA of the uninduced (grown in 10% serum), control (grown in 3% serum), and induced F9 cells (grown in 3% serum with RA or with RA and dbcAMP) were isolated as described (Liesi and Risteli, 1989). 10 μg of each RNA was run in formaldehyde agarose gels, blotted onto nitrocellulose and hybridized with oligolabeled cDNA probes for the rat 68 kDa neurofilament protein (Julien *et al.*, 1985), and β-actin (Minty *et al*.,1982) as described (Liesi and Risteli, 1989).

Immunocytochemistry for Neurofilament Proteins

Uninduced F9 teratocarcinoma cells grown in 10 % serum, the control cells in 3% serum, the RA/dbcAMP induced cells in 3% serum, and the D9L2 clone were fixed in 2% paraformaldehyde in PBS for 15 min, washed in PBS, and permeabilized with cold (−20°C) methanol for 5 min. The cells were immunostained using either polyclonal antibodies against neurofilament triplet proteins (Dahl, 1981) or a control antibody absorbed with neurofilaments (Dahl, 1981) as described earlier (Liesi and Risteli, 1989). Monoclonal antibodies against the 68 kDa neurofilament protein (Boehringer, Germany) and monoclonal antibodies against the 200 kDa neurofilament protein (RT97; Boehringer, Germany) were further applied at 5μg/ml concentration. Rabbit anti-NCAM antibodies (Gegelashvili *et al.*, 1993) were diluted 1:500 and mouse monoclonal antibodies against the neuron specific β-tubulin isoform (TUJI; Lee *et al.*, 1990) and were applied at 1 μg/ml concentration. The immunostained cultures were viewed with an Olympus BH2 fluorescence microscope with appropriate filter combinations.

RT-PRC Analysis

170 ng of each isolated RNA was used for one tube RT-PCR (Titan[TM] One Tube RT-PCR System, Boehringer Mannheim) to detect the 68 kDa neurofilament and the 200 kDa neurofilament gene transcripts. The RT-PCR was carried out for 40 min at 56 °C for RT-reaction and denaturation at 94 °C for 4 min followed by 35 cycles of denaturation at 93 °C for 1 min, annealing at 58 °C for 2 min, and elongation at 72 °C for 3 min. The primers used in RT-PCR are listed in IV, Table 1. PCR products of NF 68 kDa were isolated using a Sephaglas Bandprep kit (Pharmacia Biotech) and sequenced by the Sanger method.

RESULTS

I. LAMININ AND ITS NEURITE OUTGROWTH PROMOTING DOMAIN IN THE BRAIN IN ALZHEIMER'S DISEASE AND DOWN'S SYNDROME PATIENTS

1.1. Immunochemical characterization of antibodies against the γ1-chain neurite outgrowth promoting peptides of laminin-1

Three different antibodies against the neurite outgrowth promoting peptide derived from the γ1-chain (Liesi *et al.*, 1989) were produced and their specificities confirmed using dot-blots and radioimmunoassays. Anti-1543 was specific for a 10 amino acid long peptide p20 (1543-1553). Anti-1533a and b recognized a 30 amino acid long peptide p32 (1533-1563) and native and denatured form of laminin-1 Anti-1533b recognize also the p20 peptide. Specificity of anti-1533a for p32 peptide was confirmed by inhibition assays (I, Fig 2c). Inhibition assays for anti-1533b indicated specificity for both peptides.

1.2. Immunocytochemical studies on laminin and its neurite outgrowth peptide in AD brain

Antibodies against mouse EHS-tumor laminin, mainly recognizing the P1 fragment (Ott *et al.*,1982) of laminin-1, detected laminin-1 as punctate extracellular deposits in all senile plaques in brain samples of aged male Down's syndrome patients and in brains of Alzheimer's disease patients. This was verified by double immunocytochemistry for laminin-1 and Aβ or α-chymotrypsin (I, Fig 4A,B). Some glial cells and their processes were also weakly immunoreactive for laminin-1. In normal control brains capillaries were the only structures immunoreactive for laminin-1. Antibodies against the human P1 fragment of laminin-1(Risteli and Timpl, 1981) showed a similar distribution, but gave a stronger positive signal of the glial elements (I, Fig, 4C,D). Absorption of the laminin-1 antibodies by passing them through a laminin-1 column abolished immunoreactivity of the plaques and glial fibers (I, Fig. 4E,F). Using antibodies against the human laminin-1 P1 fragment laminin-1 immunoreactivity was detected around some capillaries in control brain tissue as well as in Alzheimer's disease and Down's syndrome brains.

Antibodies against synthetic peptides, derived from the C-terminal domain of the γ1-chain (Sasaki and Yamada, 1987) of mouse laminin-1 (anti-1543 against the decapeptide; anti-1533a and anti-1533b against the 30-amino acid peptide, I), showed no punctate immunoreactivity in the plaque regions (I, Fig. 4A,B). Instead the glial cells and their fibers were immunoreactive for these peptide antibodies in the diseased (I, Fig. 4G), but not in normal control brains. This immunoreactivity was abolished by preabsorption of the antibodies through a peptide column. (I, Fig. 4H). An antibody against a neurite outgrowth domain of the α1-chain of laminin-1 stained only the capillary basement membranes. Peptide antibodies that recognized the 10-amino-acid peptide p20 (anti-1543 and anti 1533b) showed binding of this peptide antigen as fine extracellular punctate deposits in the affected Down's syndrome brain tissue (I, Fig. 5D). The deposits of this peptide antigen were also found in the plaque areas, but there was no specific correlation with the plaques. Similar fine punctate deposits of the peptide antigen are relevant by these antisera in all tAlzheimer's brain tissue investigated. This immunoreactivity was absent in normal control brains and was abolished in tAlzheimer's disease brains, if the antibodies were preabsorbed with the corresponding peptide conjugated to Sepharose.

1.3. Demonstration of laminin-1 mRNA in Alzheimer's disease brain

Northern analysis showed a differential expression of the mRNAs for the three laminin-1 polypeptide chains in Alzheimer's disease and normal control brains (I, Fig.6). The mRNA for the α1-chain was expressed in neither case (I, Fig.6). The 8.2 kb γ1-chain transcript of laminin-1 was present both in control and Alzheimer's disease brains, but the expression was increased 10-fold in Alzheimer's disease brain tissue compared to the normal control brain tissue (I, Fig.6). The 5.6 kb β1-chain transcript was detectable only in Alzheimer's disease brain tissue (I, Fig.6). As Northern blots showed no β1-chain transcripts in control brains, a more sensitive RT-PCR was performed on normal and Alzheimer's disease brain tissues. Direct sequencing of the RT-PCR products showed that the β1-chain transcripts were present in both Alzheimer's disease and control brain tissues.

1.4. Immunoblotting experiments on Alzheimer's disease brain tissue

Immunoblots of tissue extract of Alzheimer's disease brain tissue revealed no expression of the laminin α1-chain, although both β1- and γ1-chains were seen using antibodies against the native laminin-1 molecule (I, Fig.7). In normal control brains the expression of laminin α1-chain or β1- and γ1-chains was not detectable (I, Fig.6). The protein staining showed equal amounts of samples being loaded in the gels. These experiments together with Northern analysis indicated that in the Alzheimer's disease brain tissue the expression of laminin-1 is elevated compared to the normal brain tissue.

II. INCREASED PROTEOLYTIC ACTIVITY OF THE GRANULE NEURONS MAY CONTRIBUTE TO NEURONAL DEATH IN THE WEAVER MOUSE CEREBELLUM

2.1. *In vitro* studies on neuronal migration and death in the weaver cerebellum

Normal wild-type granule neurons (+/+) extended long neurites on a laminin-1 substratum (II, Table 1,Fig.1A) and deposited laminin around themselves (II, Fig.1B). The homozygous weaver (wv/wv) granule cells showed impaired neurite outgrowth on a laminin-1 substratum (II, Table 1,Fig.1B). The weaver neurons degraded laminin-1 from their substratum (II, Fig.1D). Polyclonal antibodies against the neurite outgrowth domain of the γ1-chain of laminin-1 showed binding of this antigen along the surfaces of the weaver granule neurons (II, Fig.2A), whereas wild type granule cells did not bind this antigen (II, Fig.2B). A serine protease inhibitor, aprotinin, promoted the survival of weaver granule neurons on a laminin substratum and restored their neurite outgrowth to the level of normal granule neurons (II, Fig.4). After 12 hours in culture patch clamp studies indicated that normal neurons had resting membrane potentials (RMPs) of -61.2mV +/- 2.8, whereas weaver neurons had RMPs of only -37.7 mV +/-3.4 (II, Table 2.) Aprotinin restored the RMPs of the weaver granule neurons to normal levels (-58.6mV +/-3.7). In zymographic assays normal granule neurons did not secrete detectable amounts of urokinase or tissue plasminogen activator *in vitro* (II, Fig.3A), whereas the weaver granule cell secreted tissue plasminogen activator but no urokinase (II, Fig.3B).

2.2. *In vivo* studies on neuronal migration and death in the weaver cerebellum

In the normal wild-type mouse the external granule cell layer of the cerebellum was L1-antigen positive, and the white matter of the cerebellum showed some L1-immunoreactivity (II, Fig.5A). In the weaver mouse the external granule cell layer was completely devoid of L1-antigen and only moderate L1-immunoreactivity was detected in the white matter (II, Fig.5B). Immunocytochemistry for GFAP showed that Bergmann glial fibers were highly immunoreactive for GFAP in the weaver cerebellum (II, Fig.5D), whereas the glial cells of the normal cerebellum were weakly immunoreactive for this antigen (II, Fig.5C).

Using a laminin-1 antibody that recognizes native laminin we detected an overall increase in the production of laminin-1 in the weaver cerebellum. In the normal and weaver cerebella the Purkinje cell layer showed the highest levels of laminin expression (II, Fig.6A), but in the weaver cerebellum the Purkinje cell layer and the external granule cell layer expressed higher amounts of laminin than in the normal cerebellum (II, Fig.6B). Similarily, an antibody against the γ1-chain of laminin-1, anti-1533a, that recognizes the γ1-chain of the native laminin-1 molecule showed an overall increase in the laminin-1 γ1-chain expression in the weaver cerebellum (II, Fig.7B,D) compared to the normal cerebellum (II, Fig.7A,C). In the normal cerebellum the γ1-chain antigen was detected in the Purkinje cells. In the external granule cells there was only weak immunoreactivity for this antigen. In the weaver cerebellum the immunoreactivity for the γ1-chain was present in Purkinje cells, external granule cells, and in the molecular layer, as well as glial fibers. The weaver granule cells in the external granule cell and molecular layers showed intense immunoreactivity for the γ1-chain antigen (II, Fig.7D).

Immunocytochemical localization of tissue plasminogen activator demonstrated a coexpression with the γ1-chain immunoreactivity (compare II Figs. 7D and H). In the weaver cerebellum tPA showed an overall increase, but the distribution of tPA in the weaver cerebellum was similar to that in the normal cerebellum (compare II Figs. 7E,G and 7F,H). In the normal cerebellum tPA was expressed in fibers extending through the external granule cell layer, in the molecular layer, and in the Purkinje cell layer. In the weaver cerebellum the external granule cell, molecular, and Purkinje cell layers showed the strongest tPA-immunoreactivity. The upper parts of the external granule cell layer contained tPA immunoreactive glia-like fibers,and diffusely immunostained the immature

granule cells. An increase in the weaver tPA activity, consistent with the immunocytochemistry, was also verified by zymographic assays on cerebellar tissues of normal and weaver mutant mice. At P7 there was no difference in the expression of tPA between normal and weaver cerebellar tissues. However, at P13 the weaver cerebellum showed a 10-fold increase in tPA activity compared to the normal cerebellum.

III USE OF PAPER FOR TREAMENT OF A PERIPHERAL NERVE TRAUMA IN THE RAT

3.1. Immunocytochemical analysis of nerve regeneration

The 200 kDa NF expression was studied in sagittal sections of the regenerating rat sciatic nerve 6 months after reconstruction. When cellulose was used for regeneration antibodies against the 200 kDa NF protein showed neurofilament expression in nerve fibres at the distal tip of the injured nerve (III, Fig. 2C). This 200 kDa NF expression was comparable to that seen if the sciatic nerve was reconstructed using suturation (III, 2A). In the middle of the cellulose reconstructed nerve the 200 kDa NF protein was localized as well-organized nerve bundles (III, Fig. 2D) that appeared similar to the bundles of the sutured nerve (III, Fig. 2B). Thus, the 200 kDa NF positive nerve fibres had regenerated through the entire graft. Compared to suturation the cellulose treatment induced a fibrous scar around the site of injury, whereas no scar developed in-between the transected nerve stumps. These results indicate that cellulose grafts promoted neurite outgrowth through the injury zone.

3.2. Twitch tensions of the muscles after treatment

Twitch tensions of the muscles produced by electrical stimulation using either 0.01 ms or 0.1 ms single stimuli were not statistically different between the paper-grafted side and the uninjured control side (III, Fig. 1). If the nerve repair was performed using ordinary neurorraphy, the twitch tensions were significantly reduced on the injury side compared to the uninjured control side (III, Fig.1). Furthermore, the proportional muscle mass, the muscle mass of the trauma side compared to the control side, was significantly greater when the reconstruction was performed using paper compared to the ordinary neurorraphy.

3.3. The scores and rate of autotomy

The autotomy scores and the rates of autotomy were compared between paper reconstruction and neurorraphy. The incidence of autotomy was 50% in both groups. The latency of onset of autotomy was 2.3±0.8 weeks (mean±S.E.M.) in the neurorraphy group and 2.7±0.4 weeks in the paper-treated group. The sutured and cellulose-treated groups did not differ significantly from each other regarding the time within which the maximal autotomy scores were reached. The mean times required were 2.7±1.1 weeks in the sutured group and 3.6±1.1 weeks in the cellulose-treated group. The final autotomy scores were 4.2±2.1 weeks for the suturation group, and 4.0±2.0 weeks for the paper-treated group.

IV NEUROFILAMENT PROTEINS ARE CONSTITUTIVELY EXPRESSED IN F9 TERATOCARCINOMA CELLS

4.1. Immunocytochemical analysis of induced F9 cells

Polyclonal antibodies against the neurofilament triplet protein were used to demonstrate that neurofilament proteins were expressed in the uninduced F9 cells grown in 10% fetal calf serum. Under these culture conditions the cells grew as embryonal bodies in which the neurofilaments were seen as short filamentous accumulations within the cell explants (IV, Fig. 2A). When antibodies against the neurofilament triplet proteins were absorbed with purified neurofilaments the filamentous immunoreactivity of the embryonal bodies was abolished (IV, Fig. 2C). Monoclonal antibodies against the 68 kDa neurofilament protein revealed similar filamentous accumulations in the uniduced F9 cell cultures (IV, Fig.2B). A prolonged (10 days *in vitro*) cultivation of the F9 cells in 3% serum in the presence of RA and cAMP induced a neuronal phenotype with extensive neurite outgrowth and spreading of the F9 cells from the aggregates. Importantly, the F9 cells with a neuronal phenotype expressed the neurofilament triplet proteins (IV, Fig. 2D). Double immunocytochemistry for the 200 kDa neurofilament protein confirmed that the neuronal phenotype in these cultures was accompanied by expression of this mature type neurofilament protein (IV, Fig. 2E). These NF-positive neuron-like cells grew either as small clusters on top of the undifferentiated F9 cells, or were attached to the glass surface and sent out long neurites.(IV, Fig. 2E). In addition to neurofilament proteins (Figs. 2-3) the uninduced F9 cells also expressed other proteins involved in neuronal maturation, such

as N-CAM and TUJI (IV, Fig.3). The control cells (in 3% serum without RA and cAMP) failed to express immunocytochemically detectable neurofilament proteins (Fig. 3), but expressed both N-CAM and TUJI (Fig. 3).

4.2. Northern blot analysis of the induced F9 cells

Northern blot analysis showed that the 3.5 and 2.3 kb transcripts of the 68 kDa neurofilament protein gene (IV, Fig. I.) were expressed at the same level in the uninduced F9 cells cultured in 10% fetal calf serum and in the RA/cAMP induced F9 cells cultured in 3% serum (IV, Fig. I.). The uninduced F9 cells in 3% fetal calf serum did not express the 68 kDa neurofilament protein gene transcripts at detectable levels (IV, Fig. I.). Equal quantities of mRNA were loaded, as shown by ethidium bromide shadowing of each loaded mRNA. The mRNA levels were further evaluated by demonstration of β-actin mRNA levels in each sample using a cDNA probe for human β-actin. Each lane showed one sharp undegraded actin transcript at approximately 1.7 kb level (IV, Fig. I.). Densitometric scanning of each lane indicated that the levels of actin transcription were roughly comparable -between all samples.

4.3. RT-PCR analysis

RT–PCR analysis of both the 68 and 200 kDa NF gene transcripts further confirmed that the NF genes were constitutively expressed in the F9 cells. The 640 bp PCR product of the 200 kDa NF was expressed in uninduced (grown in 10% serum), induced (grown in 3% serum with RA or RA/dbcAMP) and control (grown in 3% serum) cultures of the F9 cells (IV, Fig 4). The 419 bp transcript of the 68 NF was also expressed in all conditions mentioned above. Sequencing of the 419 bp PCR products from all culture conditions verified that the 68 kDa NF gene products were identical and 98% similar to the cloned mouse NF 68 kDa gene.

4.4. Cloning of a neuronal F9 cell line

Single cells were picked from heterogeneous populations of F9 cells and grown in 10 % serum. The clones obtained were tested for their neuronal properties in a 24 hr assay in a serum free medium. One clone, D9L2, expressed both neurofilament triplet proteins, the 200 kDa neurofilament protein and TUJI (IV, Fig. 3 i-l). Thus, we conclude having successfully cloned a F9 cell line with neuronal properties.

DISCUSSION

The results of this thesis indicate that laminin-1 and its neurite outgrowth promoting γl-chain peptide (Liesi *et al.,* 1989) may be involved in neurodegenerative processes in Alzheimer's disease (I) and in the weaver mutant mouse (II). The results also show that thin grafts of paper may serve as potential carriers of laminin-1 and its neurite outgrowth promoting γl-chain peptide in attempts to regenerate peripheral nerves (III). Lastly, the present results indicate that laminin can be successfully used to subclone a novel neuronal cell line of the F9-teratocarcinoma cells (IV). This cell line may be used as a simplified model system to study the molecular mechanisms of neuronal differentiation.

I. Laminin and its neurite outgrowth-promoting domain in the brain in Alzheimer's disease and Down's syndrome patients

Even though laminin-1 (i.e. the isoform of laminins that is best characterized for its function in the nervous tissue) is generally known to promote neurite outgrowth and regeneration in both the CNS and the PNS (Liesi, 1990), results from this laboratory and other groups have merged to suggest that laminin-1 and its other isoforms may act in soluble form (Liesi *et al.*, 1989; Colamarino and Tessier-Lavigne, 1995) and may have a dual neurotrophic-neurotoxic function (Liesi *et al.*, 1989). An 11 amino acid long peptide derived from Aβ, the major constituent of the Alzheimer's plaques (Masters *et al.*, 1985; Selkoe *et al.*, 1986), was also shown to have a dual neurotrophic/neurotoxic effect on primary neurons (Yankner *et al.*, 1990b). This similarity led us to investigate whether the γl-chain of laminin-1 is present in AD and Down's syndrome brains and could therefore participate in the neuronal death mechanism in these disorders.

We found that the immunocytochemical distribution of laminin-1 served as a reliable marker for both senile plaques and pre-plaques in Alzheimer's disease (I); such a highly specific expression of laminin-1 in Alzheimer's disease and Down's syndrome brains was a novel finding. Previously, laminin-1 was demonstrated in brains of Alzheimer's disease patients in areas surrounding capillaries (Snow *et al.*, 1990) and it was thought to leak through the capillaries (Perlmutter and Chiu, 1990). However, accumulation of laminin-1 nearby the capillaries in AD brains may not be essential for pathophysiology of the disease, because similar accumulations were also detected in normal brain tissue (I). In contrast, normal control brains showed no expression of the punctate deposits of

laminin-1 (I), which further supported the view point that deposition of laminin-1 in the plaque areas could play a role in pathophysiology of Alzheimer's disease. E.g. the punctate deposits of laminin-1 in AD plaques could attempt to enhance neurite outgrowth and be responsible for sprouting events shown to take place in Alzheimer's brains (Scheibel and Tomiyasu, 1978; Geddes *et al.*, 1986). This point of view is feasibly based on the localization of similar punctate deposits of laminin-1 along growing axon tracts during embryonic brain development (Cohen *et al.*, 1987; Liesi and Silver, 1988; Letourneau *et al.*, 1988).

Antibodies against the γ1-chain neurite outgrowth promoting domain did not localize in as large punctate deposits in the plaques (I), which might indicate that laminin-1 was deposited in the plaque regions in such a conformation that the antigenic epitopes recognized by the peptide antibodies could be hidden or blocked. E.g. HSPGs, also present in the plaque regions (Snow *et al.*, 1990), could bind to the heparin binding domains of laminin-1 (Engel, 1991) and prevent the binding of peptide antibodies to the neurite outgrowth domain of the γ1-chain close to the heparin binding site. Alternatively, the neurite outgrowth domain of the γ1-chain might have been degraded in the plaque areas and could not be recognized by the antibodies. The C-terminal parts of laminin are known to be more sensitive to proteolysis than other parts of the molecule (Ott *et al.*, 1982). Both the plaques and the reactive astrocytes of the Alzheimer's brain are rich in lysosomal proteinases, such as cathepsins B and D (Cataldo *et al.*, 1990) and these enzymes could degrade laminin (Heck *et al.*, 1990; Steadman *et al.*, 1993; Buck *et al.*, 1992; Guinec *et al.*, 1993). If the neurite outgrowth domain of the γ1-chain has been degraded, however, sprouting in the plaques could also be due to the growth factor-like properties of laminin-1 (Panayotou *et al.*, 1989) or other growth factors in the plaque regions (Birecree *et al.*, 1988; Stopa *et al.*, 1990; Fenton *et al.*, 1998).

Instead of a localization as large punctate deposits in the plaques, the γ1-chain peptide antibodies recognized fine punctate deposits of the neurite outgrowth domain in AD brain tissue (I). These fine punctate deposits were detected by antibodies that recognized a 10 amino acid neurite outgrowth domain of the γ1-chain but not native laminin (I). This result indicates that proteolytic degradation of laminin-1 into smaller peptides, antigenically similar to the peptides used to produce the γ1-chain specific antibodies, might occur in the Alzheimer's brain. The fact that glial cells in Alzheimer's disease and

in Down's syndrome brains were strongly immunoreactive for the γ1-chain peptide while native laminin-1 antibodies showed only weak staining of glial cells suggests the glial laminin was rich in the γ1-chain or had antigenic epitopes needed for recognition of the γ1-chain better exposed.

A role for laminin-1 in AD is further suggested by the fact that the IKVAV-peptide from the α1-chain of laminin-1 has been shown to bind APP (Kibbey *et al.*, 1993). This binding may facilitate the deposition of APP and amyloid in plaques (Kibbey *et al.*, 1993). We failed to detect mRNA for the α1-chain of laminin-1 in AD or control brains (I). Therefore, if the IKVAV sequence exists in plaques, there must be an uncharacterized α-chain or some other neurite outgrowth promoting protein carrying the IKVAV sequence. Recent studies have shown that laminin reduces the fibril formation of amyloid-β-peptide (Aβ1-42) *in vitro* (Bronfman *et al.*, 1996a, 1996b, 1998; Monji *et al.*, 1998a, 1998b; Drouet *et al.*, 1999) and modulates the biogenesis of APP (Monning *et al.*, 1995; Coulson *et al.*, 1997). These results indicate that laminin-1 and its γ1-chain may have a direct interaction with APP and that this interaction may be have importance for the pathophysiology of Alzheimer's disease.

The induction of both laminin-1 and APP occurs in reactive astrocytes of the injured adult rodent brain (Liesi *et al.*, 1984; Siman *et al.*, 1989). Here we report an approximately 10-fold increase in laminin-1 γ1-chain expression in AD brains compared to control brains (I). The increased expression of laminin-1 and its γ1-chain may be due to the response of the diseased brain to the tissue injury occuring in AD brains. Factors involved in the gene regulation for the expression of laminin-1 are largely unknown. However, expression of the γ1-chain of laminin-1 is known to be induced by interleukin-1 beta (Richardson *et al.*, 1995) or Sp-1 transcription factors (Lietard *et al.*, 1997). Both interleukin-1 beta and Sp-1 are induced by trauma (Giulian and Lachman, 1985; Pearson *et al.*, 1999; Feng *et al.*, 1999). The trauma-induced over-expression of both APP and laminin-1 together with increased proteolysis could release Aβ and laminin-1 γ1-chain peptides in the AD brain tissue. As the γ1-chain peptide of laminin-1 is neurotoxic at high concentrations *in vitro* (Liesi *et al.*, 1989), its binding and accumulation in the AD brain tissue may produce a synergistic neurotoxic effect together with Aβ peptide. This neurotoxity can be further enhanced by the release of excitatory neurotransmitters (Koh *et al.*, 1990) and growth factors (Yankner *et al.*, 1990a,b; Kowall *et al.*, 1991). Thus, we

hypothesize that laminin-1 synthesis is initially induced as tissue response to trauma with no direct link to the disease. However, laminin-1 may be involved in neuronal death in both AD and Down's syndrome via its interaction with APP (Narindrasorasak *et al.*, 1992) and its toxic peptides as well as via accumulation of the neurotoxic γ1-chain peptides in plaques and the brain tissue.

II Increased proteolytic activity of the granule neurons may contribute to neuronal death in the weaver mouse cerebellum

Increased expression of the γ1-chain neurite outgrowth peptide (Liesi *et al.*, 1989) in AD brain tissue (I) suggested that this peptide may be involved in neuronal death. We used the weaver mutant mouse, an animal model of neuronal degeneration, to study further the possible involvement of the γ1-chain peptide in neuronal death. We found that expression of laminin-1, and its γ1-chain neurite outgrowth domain were elevated in the cerebellum of the weaver mutant mouse (II). Expression of tissue plasminogen activator (tPA) in the weaver cerebellum was also high compared to the normal cerebellum (II). As tPA co-localized with laminin-1 in the weaver cerebellum (II), increased expression of both tPA and laminin-1 and its γ1-chain may result in increased proteolytic cleavage of laminin-1 and its γ1-chain. Thus, toxic amounts of peptides derived from the γ1-chain neurite outgrowth domain might accumulate in the weaver cerebellum and result in massive neuronal death. This point of view is supported by the fact that weaver granule neurons degrade their laminin-1 substratum (II) and bind increased amounts of the neurite outgrowth domain of the γ1-chain (II). Importantly, weaver neurons can be rescued by a serine protease inhibitor aprotinin (II) or by antibodies against the γ1-chain peptide (Liesi and Wright, 1996). As both the RMPs and the neurite outgrowth potential of weaver granule neurons can be rescued by aprotinin (II), increased proteolysis may be one of the primary defects in the weaver mouse cerebellum.

The role of tPA in neuronal degeneration and degradation of laminin has been demonstrated by the fact that mice deficient for tPA or plasminogen are resistant to neuronal degeneration (Tsirka *et al.*, 1997), and that degradation of laminin by tPA proteolysis is shown to preceed neuronal death after an injection of excitotoxin (Chen and Strickland, 1997; Nagai, *et al.*, 1999). Recent results by Mecenas *et al.* (1997)

contradict these data and show that neurons in tPA -/- homozygous weaver mice are not rescued. It is not currently known why Mecenas *et al*. failed to rescue the weaver neurons, but it is possible that the complete lack of tPA in the wv/wv-tPA-/tPA- mice opposed to a reduction of tPA-proteolysis by aprotinin (II) is generally harmful to neurons, especially since tPA induced proteolysis is know to be essential for neuronal migration (Kalderon, 1982; Seeds *et al*., 1990).

In addition to the abnormal laminin-1 expression and proteolytic activity of the weaver neurons (II), additional molecular mechanisms may impair neuronal migration in the weaver mouse cerebellum. A point mutation of the GIRK2 potassium channel gene has been proposed to be responsible for the weaver phenotype (Patil *et al.*, 1995; Slesinger *et al.*, 1996). However, the importance of the GIRK2 gene as a weaver gene is fading because electrophysiological experiments have failed to detect functional GIRK2 channels in the cultured weaver granule neurons (Mjaatved *et al.*, 1995; Surmeier *et al.*, 1996). Furthermore, the GIRK2 knockout mice have normal cerebella (Signorini *et al*., 1997), which indicates that the GIRK2 channel is not essential for early postnatal development of the cerebellum and cannot therefore be the weaver gene. Rescue of the weaver granule neurons from death by verapamil a L-type calcium channel blocker (Liesi and Wright, 1996), or other means that reduce the levels of intracytoplasmic calcium (Liesi *et al.*, 1997) strongly suggest that the weaver gene action is mediated by calcium-dependent mechanisms. The Weaver granule neurons fail to express functional N-methyl-D-aspartate (NMDA) receptors (Liesi and Wright, 1996), which may be due to the fact that the $\varepsilon 2$ subunit is absent in the weaver cerebellum (Liesi *et al.*, 1999). The $\varepsilon 2$ subunit is induced in the granule neurons after rescue with verapamil (Liesi and Wright, 1996; Liesi *et al.*, 1999), which suggests that the down regulation of NMDA receptors may be a protective measure to reduce calcium entry into weaver granule neurons via functional NMDA-receptors. As NMDA receptors have been shown to play a role in neuronal migration (Komuro and Rakic, 1992, 1993), the lack of NMDA-receptor function may result in the expression of the weaver phenotype (Liesi and Wright, 1996; Liesi *et al.*, 1999). The role of NMDA receptors in the expression of the weaver phenotype is further emphasized by results that blocking of the NMDA receptor function in the homozygous weaver mice by eliminating the $\zeta 1$-subunit rescues the weaver granule neurons from death (Jensen *et al.*, 1999).

III Use of cellulose for treatment of a peripheral nerve trauma in the rat

Laminin-1 and its γ1-chain domain support axon growth of CNS neurons (Matsuzawa *et al.*, 1996; 1997). Laminin-1 is known to associate with the regenerating CNS (Liesi 1985b) and peripheral nerves have been shown to grow along the laminin-rich basement membranes (Ide *et al.*, 1983). Thus, laminin-1 grafts have been used by several laboratories in attempts to repair peripheral nerve injuries (Madison *et al.*, 1985; 1987; Bailey *et al.*, 1993; Tong *et al.*, 1994; Labrador *et al.*, 1998 Kauppila *et al.*, 1993). Laminin-1 or its γ1-chain neurite outgrowth promoting peptide (Liesi *et al.*, 1989) coupled with a type-I-collagen have been used to support neuronal regeneration *in vivo* (Kauppila *et al.*, 1993). These grafts supported regeneration comparable to that achieved by suturation (Kauppila *et al.*, 1993). The main limitation for the use of such laminin-1 grafts was the thickness, which made these grafts difficult to handle and caused compression of damaged nerves. As laminin-1 and its γ1-chain neurite outgrowth peptide were found effective in peripheral nerve regeneration (Kauppila *et al*., 1993), the development of more suitable graft materials is needed.

We therefore tested thin cellulose grafts for their ability to regenerate peripheral nerves. We applied cellulose because it has been successfully used for the treatment of burns as well as in otolaryngology in humans (Palva, 1982; Hazarika, 1985) and could be immediately applied to human nerve surgery. The use of cellulose grafts was more feasible than using collagen-I grafts, since proteins could be covalently coupled to cellulose. Thin sheets of cellulose were used to reconstruct severed peripheral nerves in rats (III). Cellulose was sticky and allowed good positioning of the severed nerves, but provoked a stronger foreign body reaction inflammation than resuturation (III). When cellulose grafts were used in restorative surgery the twitch-induced forces of the muscles between the operated and control sides were identical (III), which was an unexpected result. In sutured animals the twitch-induced forces of muscles decreased as compared to control side. The latter result is consistent with earlier studies by Brunetti *et al.* (1985) and Kauppila *et al.* (1993). Also macroanatomical measurements of the muscle mass favor the hypothesis that cellulose around the trauma site may favor neuroregeneration. In fact, the proportional muscle mass had a tendency to increase when cellulose grafting was used for reconstruction. Increase in the proportional muscle mass is considered as a measure of nerve regeneration (Kauppila *et al.*, 1993; Kauppila, 1994; Greensmith *et al.*,

1995). Thus, our results indicate that nerve regeneration improved using cellulose grafts compared to conventional suturation.

Thin cellulose grafts were found to induce scar formation around the trauma site (III), whereas scar formation was minimal if reconstruction was made by suturation. Inflammation near the trauma site is known to promote regeneration of both rat dorsal root and sciatic nerve injuries (Lu and Ricahardson, 1991; Dahlin, 1992), which suggests that scar-formation may be one of the factors in cellulose grafting that supports regeneration.

Cellulose grafting was as efficient as suturation in preventing dyesthesias induced by self-mutilation. The low autotomy scores of both cellulose grafting and suturation indicated that cellulose grafting effectively supported sensory regeneration of the denervated paws. This could be concluded, since the high incidence of autotomy correlates with poor recovery (Kauppila, 1994) and autotomy is known to disappear when regenerating axons form their connections (Wall *et al.*, 1979; Kauppila *et al.*, 1993; DeLeo *et al.*, 1994; Kauppila, 1994).

The present results suggest that cellulose grafts may be used to repair peripheral nerves. However, additional research will be required before this technique will be clinically applicable. This is due to the fact that rats have only few fascicles in their sciatic nerves and therefore the repair of rat nerves differs from a normal clinical situation in which several adjacent facicles are damaged and need to be rejoined. Scar formation that occurs in cellulose grafting may result in intraneural fibrosis in humans and hamper regeneration, although regeneration of rat neurons occurred successfully.

IV Neurofilament proteins are constitutively expressed in F9 teratocarcinoma cells

Neuronal differentiation has been shown to be under a negative control, i.e. cells will become neurons if they do not receive inductive signals to become other cell types (Hemmati-Brivanlou and Melton, 1997). Bone morphogenetic protein (BMP) is one of the recently identified factors involved in initial neuronal induction (Wilson and Hemmati-Brivanlou, 1995). Binding of neuronal inducers (such as noggin, follistatin, and chordin) to BMP inactivates BMP and results in expression of several transcription

factors that promote the neuronal lineage (Sasai, 1998). Until now, neurogenesis of mammals has been thought to occur during the embryonic and early postnatal period. However, recent research has shown that the adult mammalian brain has neural stem cells that can give rise to both neurons and glial cells (Reynolds and Weiss, 1992, 1996; Lois and Alvarez-Buylla, 1993; Morshead *et al.*, 1994; Palmer *et al.*, 1997; Johansson *et al.*,1999; Chiasson *et al.*, 1999). These adult stem cells are very similar to those present in the embryonic brain and similar mechanisms are therefore thought to induce their differentiation into various cell types (Johe *et al.*, 1996; Palmer *et al.*, 1997; Johansson *et al.*,1999; Chiasson *et al.*, 1999).

Apart from normal stem cells neuronal differentiation has been studied extensively using teratocarcinoma cell lines (Pleiffer *et al.*, 1981, McBurney *et al.*, 1982, Levine and Flynn 1986, Kubo 1989; Kuff and Fewell, 1980, Liesi *et al.*, 1983). As an approach to the molecular mechanisms of neuronal differentiation we used F9 teratocarcinoma cells as a model system (IV). We chose this cell line, because Liesi *et al.* (1983) have previously shown that the F9 cells can choose a neuronal lineage when exposed to RA/dbcAMP under serum deprivation. Our studies confirmed these results and also showed that the uninduced F9 cells, grown in 10 % serum, expressed both the 68 kDa neurofilament gene transcripts and protein without having a neuronal phenotype (IV). Even without the neuronal phenotype these cells expressed additional neuronal markers, such as the neuron specific tubulin III isoform (TUJI) and N-CAM. RT-PCR studies showed that both the 68 kDa and the 200 kDa neurofilament (NF) gene transcripts were constitutively expressed by F9 cells (IV). The present results confirm and expand the earlier results by Liesi *et al.* (1983). We show here that RA/dbcAMP treatment of the F9 cells is not required for their expression of neurofilament genes and proteins (IV). However, the expression of a neuronal phenotype by the F9 cells appeared to depend on serum deprivation and RA/dbcAMP stimulation (IV).

Even though the neuronal differential potential of F9 cells has been published by several laboratories, contradictory results have also appeared. The F9 cells have also been found not to express NF-proteins (Tienari *et al.*, 1987). This controversy is not presently understood, but it is possible that laboratories that failed to induce/demonstrate neurofilament expression of the F9 cells may have used a cell line in which the non-

neuronal subpopulation of F9 cells might have been selected over the neuronally differentiating one.

This view is feasible, because we verified that only a subpopulation of F9 cells developed into neuron-like cells under serum deprivation and exposure to RA/dbcAMP (IV). Thus, the F9 cells could not be used in biochemical or molecular studies on neuronal differentiation. In order to solve this problem we subcloned the F9 cells on a laminin-1 substratum by using a single parent cell isolation technique. Using this technique we obtained a homogenous, neuronally differentiating D9L2 cell line that 1) expressed the 200 kDa NF protein and 2) had a neuronal phenotype on the regular tissue culture plastic in a serum free medium without RA and dbcAMP. Laminin-1 was chosen as an initial growth substratum of the isolated cells, because earlier studies indicate that laminin-1 promotes neuronal of several types of neurons, such as early neuroepithelial cells (Heaton and Swanson, 1988; Frade *et al.*, 1996), embryonic hippocampal neurons (Lein *et al.*, 1992), sympathetic neurons (Chu and Tolkovsky, 1994), and enteric neurons (Chalazonitis *et al.*, 1997). Our results indicate that laminin-1 indeed is a favored substratum for the cloning of a neuronal D9L2 cell line. This cell line is currently being used in our laboratory to study the molecular mechanisms of neuronal differentiation.

SUMMARY AND CONCLUSIONS

In this Thesis, I have studied the potential role of laminin-1 and its γ1-chain neurite outgrowth domain in Alzheimer's disease and in the weaver mutant mouse model. Based on previous results on the role of laminin-1 I have tested cellulose as a suitable graft material in the repair of neuronal injuries and used laminin-1 to develop a neuronal F9 cell line for future studies on neuronal differentiation.

We wanted to study the expression of laminin-1 and its γ1-chain peptide in Alzheimer's disease and Down's syndrome brains, because both the neurite outgrowth domain of the γ1-chain of laminin-1 and the Aβ-peptide were shown to have a dual concentration-dependent neurotrophic/neurotoxic effect. We found that laminin-1 and the neurite outgrowth promoting domain of its γ1-chain accumulate in Alzheimer's disease and Down's syndrome brains, but not in normal control brains. The punctate deposits of laminin localize in the Alzheimer's plaques and antibodies against the neurite outgrowth promoting domain of the γ1-chain detect both the extracellular γ1-chain deposits and glial cells in the diseased brains, but not in healthy control brains. These results suggest that deposition of laminin-1 in plaques, and its γ1-chain in the astrocytes and Alzheimer brain tissue may either promote sprouting of the affected neurons or contribute to the neurotoxic mechanisms that cause neuronal death in Alzheimer's disease.

Our results on the increased expression of laminin-1 and its γ1-chain neurite outgrowth domain in Alzheimer's disease and Down syndrome brains implied that laminin and its γ1-chain may be involved in neuronal death mechanisms. To study this in detail we used weaver mutant mice as an experimental model system for neuronal migration defects and neuronal degeneration. We found that the weaver mouse cerebellum shows an increased expression of laminin-1, and its γ1-chain. Increased proteolytic activity in the weaver cerebellum may lead to degradation of laminin-1, and accumulation of the neurite outgrowth domain on the surfaces of the weaver granule cells. This may result in accumulation of neurotoxic peptides that provoke the death of weaver neurons.

The role of laminin-1 in peripheral nerve regeneration has been verified in an earlier study, but suitable graft materials have not yet been found for the coupling of laminin-1 and its biologically active peptides. Therefore, we tested cellulose as a graft material to

be coupled with laminin or synthetic peptides with a neurite outgrowth promoting activity. We found that cellulose grafts induced more fibrous scarring around the transection site compared to the microsurgical neurorraphy. However, this scarring did not impair functional recovery or cause signs of neuropathic pain. Thus, our results indicate that cellulose may be a potentially useful material in the repair of peripheral nerves.

Laminin-1 is known to promote neuronal differentiation. Therefore, we used laminin-1 to subclone a novel neuronal cell line of the F9 teratocarcinoma cells. This was necessary, because we found that only a subpopulation of F9 cells expressed neuronal markers and could be differentiated into cells with a neuronal phenotype. In the course of our study, we found that the undifferentiated F9 cells constitutively expressed both the 68 kDa and 200 kDa neurofilament transcripts and proteins as well as other neuronal marker proteins. These results indicate that RA and dbcAMP are not necessary for neurofilament gene expression in F9 cells. Instead, they may be required for the expression of a neuronal phenotype by the F9 cells.

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