expression in the lesioned CNS, regulation by bFGF, and their role during brain development

two members of the L1 family

Author(s): Lang, Doris

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L1 AND CHL1

Two Members of the L1 Family: Expression in the Lesioned CNS, Regulation by bFGF, and their Role during Brain Development

A dissertation submitted to the
Swiss Federal Institute of Technology Zürich
for the degree of

Doctor of Natural Sciences

presented by

Doris Lang
M.A., University of Colorado, USA

born May 15, 1964
citizen of Obererlinsbach SO, Switzerland

accepted on the recommendation of
Prof. M.E. Schwab, referee
Prof. M. Schachner, co-referee
Prof. H.-P. Lipp, co-referee
2000
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L1 and the close homolog of L1 (CHL1) are two members of the L1 family of cell recognition molecules believed to play critical roles in the developing, adult, and lesioned nervous tissue. L1 is implicated in such diverse processes as migration of nerve cells, elongation, fasciculation and pathfinding of axons, myelination in the peripheral nervous system (PNS), and synaptic plasticity. Although a detailed functional characterization of CHL1 has still to be performed, CHL1 has been demonstrated to support neurite elongation in vitro. Experimentally manipulated and genetically modified mouse mutants were analyzed to obtain further insights into the functional roles of both proteins in vivo.

Lack of axonal regeneration in the mammalian central nervous system (CNS) has been related to inhibitory molecules which are expressed by glial cells and which prevent axonal regrowth. The paucity of molecules with neurite-growth promoting properties on cell surfaces of differentiated CNS glial cells may also explain the inability of the adult mammalian CNS to regenerate injured axons. To test the latter hypothesis, axonal regeneration was studied in the optic nerve of a transgenic mouse ectopically expressing L1 in astrocytes under regulatory sequences of the gene for the glial fibrillary acidic protein (GFAP). Different transgenic mouse lines were cross-bred to increase levels of transgene expression. Exogeneous basic fibroblast growth factor (bFGF), known to increase GFAP expression, was applied to further increase transgene expression. Increased expression of transgenic L1 mRNA and protein was found in retinal Müller cells. Despite the fact that glial cells expressed high levels of the L1 transgene, regrowth of injured retinal ganglion cell axons in transgenic mice was not improved when compared with wild-type animals. Thus, high levels of the L1 transgene present on CNS glial cells are not sufficient to override the inhibitory properties of adult CNS tissue.

A critical role of L1 for normal brain development is indicated by the fact that mutations in the L1 gene cause a severe neurological disease, termed CRASH. Mutations in the L1 gene lead to severe malformations of the brain. The phenotype of CRASH patients is complex and variable, both within and between families. Typical defects associated with CRASH syndrome include: corpus callosum hypoplasia, mental retardation, adducted thumbs, spastic paraplegia, and/or hydrocephalus. L1-deficient mice have been generated to establish an animal model for CRASH syndrome. Mutant mice revealed a
phenotype reminiscent to that of CRASH patients. The variability of the phenotype was low, with the only exception of hydrocephalus. Depending on the genetic background, mice either developed an apparently normal ventricular system, significantly enlarged lateral ventricles, or severe macrocephalus.

In contrast to affected male patients, heterozygous females are considered to be healthy. The L1 gene is located on the X-chromosome, and in females one X-chromosome is inactivated at random early during neural development. The nervous system of carrier females should thus consist of a mosaic of cells expressing either wild-type or mutated L1. Analysis of heterozygous female mice revealed an apparently normal development of the brain, and a mosaic of cells expressing either wild-type or mutant L1. Observations demonstrate that, on average, 50% of all normally L1-positive cells have to express wild-type L1 to allow normal brain development.

CHL1 (close homolog of L1) is a recently identified cell recognition molecule of the L1 family expressed in nervous tissue. CHL1 mediates adhesion by heterophilic interactions, and supports neurite elongation in vitro. To obtain first insights into possible functions of CHL1 in vivo, its expression was studied in the lesioned optic nerve. Expression of CHL1 was strongly upregulated by optic nerve glial cells in response to an optic nerve crush, both at the mRNA and the protein level. Furthermore, application of exogenous bFGF also induced up-regulation of CHL1 by CNS glial cells. Observations demonstrate that CHL1 is a novel marker for reactive astrogliosis, and suggest important functional roles of CHL1 in the lesioned CNS.
ZUSAMMENFASSUNG


Die Unfähigkeit zur axonalen Regeneration im zentralen Nervensystem (ZNS) der Säuger konnte auf inhibitorische Moleküle zurückgeführt werden, die von glialen Zellen exprimiert werden und die die Regeneration von Axonen verhindern. Die geringe Zahl von Oberflächen-Molekülen auf differenzierten Gliazellen im ZNS mit Eigenschaften, die das Neuritenwachstum fördern, könnte ebenfalls die Unfähigkeit zur Regeneration im adulten Säuger-ZNS erklären. Um diese Hypothese zu testen, wurde die axonale Regeneration im optischen Nerv von transgenen Mäusen studiert, welche L1 ektopisch in Astrozyten unter regulatorischen Sequenzen des "glial fibrillary acidic protein" (GFAP) exprimieren. Verschiedene transgene Mauslinien wurden untereinander gekreuzt, um die Expression des transgenen L1 zu erhöhen. Exogener "basic fibroblast growth factor" (bFGF), welcher die GFAP Expression erhöht, wurde appliziert, um die Expression des Transgenes weiter zu erhöhen. Eine erhöhte Expression des Transgens auf mRNA- und Protein-Niveau konnte in den retinalen Müller-Zellen gefunden werden. Trotz der Tatsache, dass eine erhöhte Menge an transgenem L1 in Gliazellen des ZNS exprimiert wurde, regenerierten die Axone der retinaler Ganglienzellen in den transgenen Mäusen nicht besser als in Wildtyp-Mäusen. Folglich sind erhöhte Mengen an transgenem L1 auf Gliazellen im ZNS nicht ausreichend, um die inhibitorischen Eigenschaften des adulten ZNS Gewebes zu überwiegen.

Dass L1 eine bedeutende Rolle bei der Entwicklung des Gehirns spielt, wird durch die Tatsache deutlich, dass Mutationen im L1-Gen gravierende Missbildungen im Gehirn verursachen. Diese Missbildungen werden unter der


1. **INTRODUCTION**

1.1 **Cell-Cell and Cell-Matrix Adhesion**

The development of multicellular structures is a complex process including differentiation of cells, tissue formation, and coordinated interactions between cells. These processes require direct contact among cells and/or between cells and their environment (e.g., the extracellular matrix), in order to facilitate information flow from one cell to the other. The most complex structure in mammals is the brain that contains about $10^{12}$ neurons. Many of these cells receive information from and project to several thousand other nerve cells. During development guidance cues help cells to find their paths during migration and help axons to grow to the correct targets. Finally cells differentiate to adopt specific functional properties. Once the development of the nervous system is completed, the adult brain does not remain static. Glial cells, and in a few brain regions (i.e., olfactory bulb and hippocampus) also nerve cells, are generated in the adult mammalian system. Although the number of newly generated neural cells is low in the adult, they become integrated into existing circuits, indicative for a high degree of plasticity of nervous tissue. Moreover, differentiated nerve cells still have the capacity to change synaptic contacts to facilitate memory formation, to learn tasks, or to react to an injury.

Within the last two decades a considerable progress has been made in understanding signaling events between and within neural cells and the possible functions of many different molecules involved in these events. At the molecular level, these molecules can be grouped into several classes of receptors and ligands such as the EPH family of receptor tyrosine kinases and their ligands (Brambilla and Klein, 1995; Sefton and Nieto, 1997; Zisch and Pasquale, 1997), the receptor phosphatases (Brady-Kalnay and Tonks, 1995), the netrins (diffusible matrix-like molecules; Colamarino and Tessier-Lavigne, 1995; Keino-Masu et al., 1996; Winberg et al., 1998), the collapsins or semaphorins (Kolodkin, 1996; Mark et al., 1997; Tessier-Lavigne and Goodman, 1996; Winberg et al., 1998), the integrins and their receptors (Clark and Brugge, 1995; Hynes, 1992; Reichardt and Tomaselli, 1991), and the immunoglobulin superfamily of cell recognition molecules (Brümmendorf and Rathjen, 1995; Chothia and Jones, 1997; Kadmon and Altevogt, 1997; Walsh and Doherty, 1997). The adhesion molecules may further be grouped into substrate adhesion molecules (SAMs) and cell adhesion/recognition molecules (CAMs). Most SAMs are found among
the integrin family of heterologous glycoprotein dimers (Kadmon and Altevogt, 1997).

1.2 Families of Cell Recognition Molecules

During the last decade an enormous number of novel proteins were identified that are involved in cell-cell and/or cell-matrix interaction. Analysis of their sequences indicated that many proteins evolved from common precursors and were the result of divergent evolution. A duplication and diversification of genes resulted in the generation of large protein families. Proteins with structural similarities were grouped into families and superfamilies (Dayhoff et al., 1983).

The cell recognition molecules have various specific functions. For instance, they mediate cell-cell binding, cell-migration on specific substrates, axonal pathfinding and fasciculation, or myelination of axons. Cell recognition molecules are cell-surface molecules that bind to itself (homophilic interaction) or to other molecules (heterophilic interaction) that are present on the same membrane (cis interaction), on an opposing membrane (trans interaction), or to molecules of the extracellular matrix (Brümmendorf and Rathjen, 1995; Walsh and Doherty, 1997).

Several different classifications of the CAMs are proposed (for example according to their dependence on calcium ions, their structure, their function, etc.). One classification of cell adhesion/recognition molecules (CAMs) subdivides the molecules into the following families: immunoglobulin superfamily (IgSF), integrins (Clark and Brugge, 1995; Hynes, 1992; Reichardt and Tomaselli, 1991), tenasin gene family (Chiquet-Ehrismann et al., 1994; Erickson, 1994; Faissner, 1997; Faissner et al., 1995), fibronectins (Chothia and Jones, 1997; Romberger, 1997), cadherins (Chothia and Jones, 1997; Takeichi, 1988), and lectin-related selectins (Crockett Torabi, 1998; Tedder et al., 1995). The focus here will be mainly on the members of the immunoglobulin superfamily. The other families of cell adhesion molecules are only briefly described.

1.2.1 The Immunoglobulin Superfamily

The Ig superfamily is further classified into subgroups. The IgSF includes, for instance, the immunoglobulins themselves, the T cell receptors, the B cell receptors, the major histocompatibility complex (MHC) antigens, the T cell subset antigens (e.g., CD4, CD8 α and β chains), the Ig receptors, and the cytokine receptors (Brümmendorf and Rathjen, 1995). Structures and sequences
of the IgSF members are manifold. Sequence similarity between these members is often not more than 10-30%, but domain arrangements and specific sequence stretches are remarkably conserved (Brümmendorf and Rathjen, 1995).

1.2.1.1 Structure and Expression of Members of the IgSF

Despite the low sequence similarity, the molecular structures of these proteins can be grouped into related motifs like the immunoglobulin-like domains and the fibronectin type III repeats. Proteins with a specific motif often have conformational similarities. The Ig-like domain, for example, includes a stretch of 70-110 amino acids (aa) with two characteristic cysteine residues, about 55 - 75 aa apart from each other. A highly conserved tryptophan can be found about 10 - 15 aa downstream of the first cysteine. The Ig-like domains are classified into V, C1, C2, and I sets. The second motif, the fibronectin type III (FNIII) repeats are present in most IgSF members (Main et al., 1992). The FNIII repeats are structurally related to fibronectin and include a stretch of about 90 aa. The number of FNIII and Ig motifs usually varies between different members of the IgSF. Most often, FNIII repeats are found downstream of Ig-like domains. Conformational structures and folding patterns are reviewed by Brümmendorf and Rathjen (1995), Chothia and Jones (1997), Harpaz and Chothia (1994), and Walsh and Doherty (1997). A list of neural IgSF proteins is given in Table 1 (see reviews: Brümmendorf and Rathjen, 1995; Hortsch, 1996) and a selection of structural organizations of neural IgSF proteins are displayed in Fig. 1 (Brümmendorf and Rathjen, 1995; Hortsch, 1996).
Table 1: A selection of vertebrate and invertebrate IgSF proteins

<table>
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<tr>
<th>Protein (Synonymous names in italic)</th>
<th>MW (kDa)</th>
<th>Species</th>
<th>Expression</th>
<th>Binding Partners</th>
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<tr>
<td>BIG-1/PANG</td>
<td>150</td>
<td>Mouse, Rat</td>
<td>low in embryonic brain, high in adult brain, in subsets of neurons: granule cell layer of the olfactory bulb and dentate gyrus, neurons in superficial layers of cerebral cortex, cells in thalamic and amygdaloid nuclei, Purkinje cell layer of cerebellum; in N2A neuroblastoma cell line (1, 2)</td>
<td></td>
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<tr>
<td>CHL1</td>
<td>185, 165, 125</td>
<td>Mouse</td>
<td>embryonic and adult brain, in CNS and PNS, on neurons and glia (3, 4)</td>
<td></td>
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<tr>
<td>DCC</td>
<td>?</td>
<td>Human</td>
<td>in brain highest concentration, lower in other tissues, upregulation in PC12 cells with NGF treatment (5, 6)</td>
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<tr>
<td>F11</td>
<td>135</td>
<td>Human</td>
<td>in adult brain, highest in cerebellum molecular layer and frontal cortex, dentate gyrus of hippocampus, parallel fibers, chick retina (7, 8, 9, 10)</td>
<td>L1 (Ng-CAM) (39), NrCAM (40, 41), tenascin-R (39, 42), tenascin-C (43, 44)</td>
</tr>
<tr>
<td>L1 Neuro-1 antigen, Gp135, Contactin F3, F3/11 Contactin</td>
<td>200, 180, 140, 80 190, 180, 130 200-230 210, 190, 135, 80</td>
<td>Mouse, Human, Rat, Chick</td>
<td>adult cerebellum and hippocampus, fetal brain (cerebellar granule cells, Purkinje cell axons, cerebral cortex, olfactory axons, migrating neurons in developing neocortex), nonmyelinated fasciculated axons, premature Schwann cells, retinal ganglion cell axons in optic chiasm, embryonic spinal cord, concentrated on neurites, chick motor axons (11, 12, 13, 14, 15, 16, 17, 18)</td>
<td>homophilic (45), neurocan (46), phosphacan (46), NCAM (47, 48), TAG1 (49), F11 (40) ankyrin (50)</td>
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<tr>
<td>MAG</td>
<td>90-110</td>
<td>Human, Mouse, Rat, Chick</td>
<td>on oligodendrocytes and Schwann cells, developing retinal Müller cells, on chick small sensory neurons in dorsal root ganglia (15, 19, 20, 21)</td>
<td>collagens (51, 52), heparin (52)</td>
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<tr>
<td>Protein (Synonymous names in italic)</td>
<td>MW (kDa)</td>
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<td>NCAM (CD56, leu-19, 5B4-CAM, D2-CAM, C1H3-antigen)</td>
<td>160-180, 130-140, 110-125</td>
<td>Human, Bovine, Mouse, Rat, Xenopus, Chick</td>
<td>embryonic and adult brain, retina, developing neural tube, olfactory system, striatum, postnatal hippocampal neurons and glia, postnatal DRGs, differentiated cerebellar and retinal neurons, premature and nonmyelinating Schwann cells, mature sensory neurons, growth cones of CNS neurons, on cell bodies, dendrites and axons (in vitro) (12, 15, 24, 25, 26)</td>
<td>homophilic (53), heparin (54, 55, 56), neurocan (46), phosphacan (46), L1 (47), collagens (51, 57)</td>
</tr>
<tr>
<td>Neurofascin (Ankyrin-binding glycoprotein)</td>
<td>185, 155, 140, 185, 160, 110-135</td>
<td>Rat, Chick</td>
<td>in cerebellar molecular layer: on Purkinje cells and granule cells, at nodes of Ranvier of PNS axons, adult brain, in chick spinal cord dorsal and ventral funiculi (27, 28, 29)</td>
<td>ankyrin (50, 58), NrCAM (41)</td>
</tr>
<tr>
<td>Neuroglian</td>
<td>167</td>
<td>Grasshopper, Drosophila</td>
<td>in Drosophila embryo: glia cells and neurons (PNS + CNS), long isof orm restricted to CNS, PNS neurons, photoreceptor cells and their axons, eye imaginal disk, major PNS sensory pathways (30, 31)</td>
<td>homophilic (59, 60)</td>
</tr>
<tr>
<td>Nr-CAM (Bravo)</td>
<td>130-140, 60-80</td>
<td>Chick</td>
<td>on axonal tracts, CNS and PNS neurons, spinal cord floor plate (neurogenesis), cerebellar granule and Purkinje cells, retinal Müller cell processes and endfeet, retinal ganglion cell axons (in vitro) (32, 33, 34)</td>
<td>homophilic (60), ankyrin (50, 61), axonin-1 (62, 63), F11 (40), neurofascin (64), fibroblasts (60)</td>
</tr>
<tr>
<td>P0 (MPZ)</td>
<td>29-31</td>
<td>Human, Bovine, Mouse, Rat, Chick, Shark</td>
<td>only in PNS myelin, only on myelinating Schwann cells, on chick neural crest cells, in shark CNS myelin (35, 36)</td>
<td>homophilic (65)</td>
</tr>
</tbody>
</table>
Table 1: A selection of vertebrate and invertebrate IgSF proteins

<table>
<thead>
<tr>
<th>Protein (Synonymous names in <em>italic</em>)</th>
<th>MW (kDa)</th>
<th>Species</th>
<th>Expression</th>
<th>Binding Partners</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAG-1 TAG-I TAX-1 SNAP Axonin-1, SC2</td>
<td>132-140</td>
<td>Human</td>
<td>Human adult cerebellum, rodant developing brain, differentiating neurons, developing spinal and cranial nerves, corticothalamic projections, subsets of fasciculating CNS axon tracts, cerebellar molecular layer, corpus callosum, anterior and hippocampal commissure, several levels of brainstem, cell surface of immature neurons, later in development only on axons (37, 38)</td>
<td>homophilic (66), L1 (37, 49, 67, 68, 69), NrCAM (62), tenascin-C (70), neurocan (70), phosphacan (70)</td>
</tr>
</tbody>
</table>

References:

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69. Stoeckli et al., 1996
70. Milev et al., 1996
Figure 1: A selection of neural immunoglobulin superfamily cell adhesion molecules. A subset of neural vertebrate and invertebrate CAMs are displayed in a schematic representation. The N-terminal is on top and the C-terminal at the bottom of the drawing. The different domains are specified in the box on top of the scheme. PSA indicates polysialic acid and PAT stands for a proline-, alanine- and threonine-rich domain. Most of the structural representations are adapted from Brümmendorf and Rathjen (1995).
1.2.1.2 Evolution of members of the IgSF

Although the sequence similarity between members of the IgSF may be low, some strikingly conserved regions allow to determine their phylogenetical relationship. A phylogenetic tree of members of the IgSF in vertebrates is shown in Fig. 2 (Brümmendorf and Rathjen, 1995).

**Figure 2:** *Vertebrate immunoglobulin superfamily of cell adhesion molecules.* Proteins were grouped according to their sequence similarity in their extracellular domain. Relationships that could not be determined definitely are indicated by broken lines. (c - chick, h - human, m - mouse; adapted from Brümmendorf and Rathjen, 1995).
1.2.2 Integrins

Integrins are described as a widely expressed family of cell surface receptors belonging to the group of cell adhesion molecules. The major roles of integrins include the facilitation of attachment to the extracellular matrix (ECM), mediation of cell adhesion, and adhesion-dependent intracellular signaling. The integrins are involved in the regulation of a variety of cellular functions such as embryonic development, immune response, tumor cell growth and metastasis, programmed cell death, hemostasis, leukocyte homing and activation, bone resorption, clot retraction, and the response of cells to mechanical stress (Clark and Brugge, 1995).

Integrins require for their interaction with ligands the presence of Ca\(^{2+}\) and Mg\(^{2+}\) ions. Integrins are heterodimeric cell surface glycoproteins. Their structure comprises one of 8 β subunits and one or more α subunits (out of 16 different subunits). So far more than 20 different αβ heterodimers are known. Both subunits are membrane-spanning molecules.

The different integrin members facilitate both inside-out signaling and outside-in signaling. Binding to ligands initiates the flow of information into the cell (outside-in), whereas inside-out signaling results in a conformational change of the integrin extracellular domain to either an active (adhesive) or inactive state. Inside-out events can be induced by the interaction of the cytoskeletal domains with cytoskeletal proteins that include α-actinin, vinculin, talin, paxilin, and tensin. The latter five proteins are further linked to actin or act as substrates for tyrosine kinases (Chothia and Jones, 1997; Clark and Brugge, 1995). Many binding partners of integrins have been described that include ECM components (e.g., fibronectin, collagens, and laminin) and other CAMs (e.g., vascular CAM-1 [VCAM-1] or intracellular CAMs [ICAMs]). More detailed information can be found in Chothia and Jones (1997), Clark and Brugge (1995), Hynes (1992), and Sastry and Horwitz (1993).

1.2.3 Tenascin Family

The tenascin members belong to the group of extracellular matrix (ECM) glycoproteins. So far, five members of the tenascin family have been identified, namely tenascin-C (TN-C), tenascin-R (TN-R), tenascin-W (TN-W), tenascin-X (TN-X), and tenascin-Y (TN-Y), each of which is encoded by a different gene. These proteins comprise a cysteine-rich amino terminal region followed by a variable number of EGF domains and FNIII repeats, and a fibrinogen-like
domain at the carboxy terminus. Tenascins contain FNIII repeats that can be alternatively spliced to form various isoforms. The molecular weight of TN-C ranges between 190 kDa and 320 kDa and of TN-R between 160 kDa and 180 kDa. Tenascins assemble into multimers via disulphide bridges with six linked molecules for TN-C and 2 to 4 linked molecules for TN-R. Tenascin family members are conserved in all vertebrate species. For more detailed information, see Bartsch (1996a), Chiquet-Ehrismann et al. (1994), Erickson (1994), Erickson (1997), Faissner (1997), Faissner et al. (1995), Schachner et al. (1994), and Weber et al. (1998).

Tenascins have been implicated to play a critical role during the development of neural tissues in that they either promote or inhibit cell adhesion depending on the partner cell and how the molecules are presented to the cells. Members of the tenascin family are often expressed early in development. For instance, TN-C is first found during gastrulation and later in distinct areas of the CNS. So far only TN-C and TN-R are expressed in the developing, adult, and lesioned CNS tissue. TN-C and TN-R are mainly expressed by astrocytes and oligodendrocytes, respectively, but both molecules are also expressed by a few nerve cell types (Bartsch, 1996a; Bartsch et al., 1994; Faissner, 1997; Fitch and Silver, 1997; Schachner et al., 1994; Wintergerst et al., 1997). TN-C has been implicated to control cell proliferation, neuronal migration, segregation of neuronal assemblies, and promotion or inhibition of neurite outgrowth. Similarly to TN-C, TN-R promotes or inhibits neurite outgrowth depending on neuronal cell types and how the molecule is presented to cells (i.e., as a homogeneous substrate or as a sharp substrate boundary). A possible role of TN-C or TN-R to promote or inhibit axonal regrowth in lesioned nervous tissue is indicated by the fact that expression of both molecules may be up-regulated after injury (for a detailed discussion, see Bartsch, 1996a). Despite the fact that in vitro studies have demonstrated that TN-C and TN-R mediate a variety of important cell-matrix interactions, knock out mutants for these proteins show either no (Forsberg et al., 1996; Saga et al., 1992) or only subtle phenotypes (Weber et al., 1998).

1.2.4 Fibronectins

A common structure of the IgSF is the FNIII repeat which is the major motif of the fibronectins. Three other types of structural motifs are present in fibronectins. These four motifs are designated as type I, II, III, and V. The fibronectin protein is a very flexible, pliable molecule, soluble or incorporated in various extracellular matrices. Fibronectin is translated from a single gene.
Alternative splicing of pre-mRNA gives rise to several isoforms of 450-500 kDa in size that are involved in cell adhesion, migration and proliferation.

Beside their major binding partner, the integrins, other binding partners are also known such as fibrin, collagen, and heparin. Fibronectins play a role during embryonic differentiation, affect cell morphology and cell migration, and are involved in tissue repair and thrombosis. Interaction of fibronectin with kinases and cytoskeletal proteins may also stimulate signal transduction pathways. More informations on fibronectins are reviewed in: Chothia and Jones, 1997; Poulouin et al., 1997; Romberger, 1997.

1.2.5 Cadherins

Cadherins play a major role during embryonic morphogenesis in that they are involved, for instance, in axon outgrowth, navigation, fasciculation, target recognition, and synaptogenesis. Cadherins are widely expressed in the developing and mature CNS. Several subtypes of cadherins are presently described (Redies, 1997). They form stable contacts between cells of solid tissues and in various kinds of epithelia. Cadherins have been identified in a wide variety of species, from nematodes to humans.

Cadherins are classified into at least four subclasses, i.e., type I and II classic cadherins, protocadherins, desmosomal cadherins, and other cadherins. Solely in the brain, at least twelve different cadherins have been described and each of them shows a distinct expression pattern. Cadherins are expressed in neurons and glial cells (Redies, 1997). The molecular structure of cadherins include an extracellular domain with five homolog motifs of about 110 aa each, a single transmembrane domain, and a cytoplasmic tail. The five extracellular motifs are remarkably similar to the immunoglobulin C1 structures in the folding and secondary-structure topology. The cadherins are anchored to the cytoskeleton through the interaction with catenins. Cadherins belong to the Ca^{2+}-dependent CAMs. The proteins form dimers only in the presence of Ca^{2+}, whereas the absence of Ca^{2+} leads to a fast degradation of the protein by proteases. Each dimer is able to bind two other dimers on the opposing membrane and thus, can form a highly stable zipper-like structure. Adhesion between dimers is also facilitated in the presence of Ca^{2+} (Chothia and Jones, 1997). Beside the homophilic binding, cadherins are also able to bind to other partners such as integrins. Detailed structural and functional information is given in reviews of Chothia and Jones, 1997; Fields and Itoh, 1996; Leahy, 1997; Redies, 1997; Takeichi, 1988.
1.2.6 Lectin-related Selectins

Selectins belong to the family of cell adhesion molecules and are also cell surface molecules. Their expression is solely restricted to the vascular system. Selectins enable the attachment of leukocytes, neutrophils, and monocytes to the vascular endothelium allowing these cells to roll along the venular wall. Interaction with cell adhesion molecules such as integrins and IgSF members ceases the leukocyte rolling and implements stable adhesion between the leukocytes and the vascular wall. These interactions are essential for the immune system as a surveillance tool and for invasion at sites of inflammation. Selectins are further involved in the initiation of intracellular signaling pathways and in regulation of cell-cell interactions with monocytes, lymphocytes, platelets, and endothelial cells (Crockett Torabi, 1998; Tedder et al., 1995).

The best known family members are three closely related cell-surface molecules, e.g., L-selectin, E-selectin, and P-selectin. These selectins have characteristic extracellular regions composed of an amino-terminal lectin domain that binds a carbohydrate ligand, an epidermal growth factor-like domain, and two to nine short repeat units homologous to domains found in complement binding proteins (Tedder et al., 1995).

1.3 Cell Recognition Molecules in the Developing Nervous System

The development of the nervous system involves complex cell-cell and cell-matrix interactions where cell migration and axon growth and guidance play an important role. The families of cell surface proteins are major contributors in recognition, adhesion, inhibition, and repulsion of cells and growth cones. One family of these proteins, the cell adhesion molecules (CAMs), is involved in the control of cell-cell interactions during development by regulating neuronal adhesion, migration, neurite outgrowth, fasciculation, synaptogenesis, and intracellular signaling. Whereas members of the family of CAMs are mainly involved in positive cues for growth and guidance, members of families including semaphorins and EPH receptor tyrosine kinases are involved in negative cues. Here I will focus mainly on the role of cell recognition molecules during the formation of the nervous system.
1.3.1 Migration of Neural Cells

Cell migration is one of the crucial mechanisms during development of the nervous system in multicellular organisms. A key event for the development of the nervous system is the formation of the neural tube and the neural crest where the former gives rise to the CNS (brain and spinal cord) while the latter gives rise to the PNS. Transcription factors, growth factors, proteases, tyrosine kinases, proteoglycans, and CAMs play an important role during this cell migration. Amazing is the fact that CAMs increase cell adhesion and axon fasciculation, but also promote cell motility, and neurite outgrowth during development.

The migration of granule cells from the external to the internal granular layer of the developing cerebellar cortex is a model system for the analysis of nerve cell migration. In the early eighties, L1 has been described to be involved in the migration of postmitotic granule cells from the external granular layer (EGL) through the molecular layer (ML) into the internal granular layer (IGL) of the cerebellum of mice (Lindner et al., 1983). Similar results were found in the chick cerebellum, where antibodies against Ng-CAM (the chick homolog of L1) arrested granule cell migration in the EGL, and where antibodies against cytotactin (the chick homolog of tenascin-C) blocked granule cell movement in the ML (Chuong, 1990; Chuong et al., 1987). The role of L1 (Ng-CAM) for cell migration is described in more detail in Chapter 1.5.3.2. NCAM-PSA (the so-called "embryonic" form of NCAM that is rich in polysialic acid) is uniformly expressed in the developing cerebellar cortex (e.g., Brümmendorf and Rathjen, 1995; Chuong, 1990; Chuong et al., 1987; Daniloff et al., 1986; Hekmat et al., 1990; Persohn and Schachner, 1987), but antibodies against NCAM show only a mild effect on granule cell migration (Chuong et al., 1987). The PSA-rich NCAM disappears once cell migration in the cerebellar cortex is complete (Chuong, 1990). Therefore, PSA may play a role in cell migration, as has been demonstrated for the migration of neuronal precursor cells to the olfactory bulb (NCAM-deficient mice have a reduced size of the olfactory bulb; Ono et al., 1994; Ono et al., 1997). Adhesion molecule on glia (AMOG), an integral cell surface glycoprotein of 45-50-kD molecular weight identical to the β2 subunit of the Na,K-ATPase (Gloor et al., 1990), has also been shown to participate in granule cell migration; antibodies against AMOG strongly inhibited granule cell migration in cerebellar explant cultures from mice (Antonicek et al., 1987).

Another well studied model is the migration of oligodendrocyte-type 2 astrocyte (O-2A) precursor cells arising from the subventricular zone. It has been
demonstrated, for instance, that migration of O-2A progenitors in vitro is highly dependent on PSA-rich NCAM, since removal of PSA completely blocked migration of these highly motile cells (Wang et al., 1994). Tenascin-C has also been implicated to control migration of O-2A progenitors. Kiernan and colleagues (1993) have demonstrated that high tenascin-C concentrations inhibited migration of these cells, whereas low concentrations showed no such an effect. Inhibition or facilitation of migration of different cell types is mediated by distinct fibronectin type III domains of the tenascin-C molecule, where repeats 1, 4, and 5 inhibit migration and repeats 2 and 3 facilitate migration (Husmann et al., 1992; Wehrle-Haller and Chiquet, 1993). Tenascin-C antibodies perturbed cephalic neural crest cell migration in the chicken embryo (Chuong, 1990). Further, tenascin-C has been hypothesized to establish a barrier for migratory O-2A progenitors at the lamina cribrosa in the optic nerve (Bartsch et al., 1992; Bartsch et al., 1994). Another mechanism of modulating cell migration is exemplified by chondroitin sulfate proteoglycans (e.g., neurocan, phosphacan) that are able to bind to Ng-CAM and NCAM and therefore inhibit cell adhesion (Grumet et al., 1993a). In CAM-poor regions, chondroitin sulfate proteoglycans (neurocan, phosphacan, versican, aggregan, and NG2 proteoglycan) may act similar to tenascin-C, namely as barriers to cell migration (Grumet et al., 1996).

During the very early stages of development, i.e. gastrulation, neurulation, and neural crest migration, other molecules such as integrins, cadherins, and fibronectins play an important role. Antibodies against fibronectin and β1-integrin inhibit migration of mesodermal cells and gastrulation in amphibia and birds (Hynes and Lander, 1992). Moreover, antibodies against integrins also block neural crest cell migration. At the time of cell movement NCAM (PSA-poor) and N-cadherin are down-regulated and become re-expressed at the time of aggregation (Barami et al., 1994; Hynes and Lander, 1992; Persohn and Schachner, 1987; Takeichi, 1988). Down-regulation of these two molecules during the migratory period may facilitate migration in that adhesion is decreased.

Recent evidence elucidates the role of other molecules in cell migration in the nervous systems such as the mouse reelin and the human KAL (Goodman, 1996). The ephrine family of tyrosine kinase receptors (Sefton and Nieto, 1997; Zisch and Pasquale, 1997) and the semaphorins (Mark et al., 1997) act in a repulsive manner. In summary, a tremendous progress has been made in identifying functionally important molecules and their receptors. However, it seems likely that only a fraction of molecules involved in cell migration has been identified so far.
1.3.2 Outgrowth, Guidance, and Fasciculation of Axons

Cell surface proteins such as the CAMs and soluble molecules are also involved in the outgrowth, guidance, and fasciculation of axons in the developing nervous system. These molecules act either in an attractive or repulsive manner. They determine cell polarity (axon, dendrite), elongation of axons, fasciculation of extending axons, pathway decisions of the growing axon, and target recognition. The establishment of correct and functional synapses terminates the pathfinding process of an axon (Goodman, 1996).

The outgrowth, guidance and fasciculation of axons is controlled by a variety of molecules of different families such as the IgSF (e.g., Ig CAMs, cadherins, integrins, laminins, tenascins), netrins, semaphorins, ephrine receptor tyrosine kinases and its ligands (see chapter 1.1; Goodman, 1996; Walsh and Doherty, 1997). The functional roles of NCAM (neural cell adhesion molecule), L1, DCC (deleted in colorectal carcinoma), and MAG (myelin-associated glycoprotein) in these processes have been studied extensively, but also Nr-CAM (Ng-CAM related cell adhesion molecule), neurofascin, and the Drosophila neuroglial are, among many other proteins, involved in outgrowth, fasciculation, and pathfinding of axons (Fields and Itoh, 1996; Goodman, 1996).

NCAM (and its invertebrate homolog fasciclin II) is expressed on cell surfaces of neurons, glial cells, and muscle fibers. The gene product of NCAM is alternatively spliced and gives rise to three major isoforms with molecular weights of 180, 140, and 120 kDa. It is expressed as a transmembrane or a GPI anchored molecule and may be associated with polysialic acid (PSA). NCAM associated with PSA is implicated in cell migration, axon outgrowth and plasticity. During development of the nervous system, PSA is gradually lost, and NCAM loses its ability to promote neurite outgrowth. Loss of PSA further results in a decreased migratory capacity of NCAM-positive neurons, in pathfinding errors by motoneurons, in a decrease of fascicle size of retinal ganglion cell axons, and in impaired LTP in hippocampal slices (reviewed in Walsh and Doherty, 1997). NCAM knockout mice revealed a decrease in the density of mossy fibers and pathfinding errors of these axons in the hippocampus, a smaller olfactory bulb, and reduced spatial learning ability (Cremer et al., 1994). The variable alternatively spliced exon (VASE), a 10-amino acid stretch in the fourth Ig domain, inhibits axonal outgrowth. The relative abundance of NCAM isoforms that include the VASE increases with development, but not in areas where synaptic plasticity takes place (Doherty and Walsh, 1994; Walsh and Doherty, 1997).
Besides NCAM, also L1 plays an important role during the development of the nervous system. Mutations of L1 in humans lead to a severe disease known as CRASH syndrome (corpus callosum hypoplasia, retardation, adducted thumbs, spastic paraplegia, and hydrocephalus; Fransen et al., 1995). In CRASH patients or L1 mutant mice, axon tracts such as the corpus callosum or the corticospinal tract may be decreased in size or may be completely absent (Cohen et al., 1997; Dahme et al., 1997; Fransen et al., 1995; Wong et al., 1995b). In fact, the majority of corticospinal tract fibers of L1-deficient mice failed to cross the midline in the medulla from the ventral pyramids in the medulla to the contralateral dorsal columns of the spinal cord (Cohen et al., 1997). However, no other axon pathfinding errors were found (e.g., corpus callosum, optic chiasm and spinal commissural projection; Cohen et al., 1997). Interestingly, other members of the L1 family are also implicated in axon pathfinding. A proper axonal crossing of commissural axons at the floor plate requires an interaction between axonin-1 and Nr-CAM, a close relative of L1 (reviewed in Walsh and Doherty, 1997). L1 is also implicated in axon fasciculation, myelination, cell migration, and synaptic plasticity (Fields and Itoh, 1996; Hortsch, 1996). A more detailed discussion about L1 follows in chapter 1.5.

Another member of the IgSF implicated in neurite outgrowth, is the DCC (deleted in colorectal carcinoma) protein. Its structure is shown in Fig. 1. DCC is widely expressed in the nervous system and in vitro studies have demonstrated that DCC promotes neurite outgrowth from PC12 cells. DCC is expressed by commissural neurons and is able to bind netrins. Interestingly, DCC antibodies inhibit the netrin-dependent outgrowth of commissural axons (Keino-Masu et al., 1996). Despite the fact that DCC has been identified as a receptor for netrins, DCC does not seem to be involved in the chemorepulsive effect of netrins on trochlear motor axons (Colamarino and Tessier-Lavigne, 1995; Keino-Masu et al., 1996; Walsh and Doherty, 1997).

The myelin-associated glycoprotein (MAG) has been shown to be an inhibitor of neurite outgrowth from adult DRG neurons and cerebellar neurons (Filbin, 1996; McKerracher et al., 1994; Mukhopadhyay et al., 1994). Interestingly, Johnson and co-workers (1989) have shown that MAG increases neurite outgrowth of young postnatal DRG neurons. Thus, the same protein either promotes or inhibits neurite outgrowth from DRG neurons, depending on the developmental stage of the cell. These observations suggest that developmental changes in the expression of yet to be identified receptor(s) or changes in signal transduction pathways determine the cellular response to a given protein. A
variety of in vitro experiments suggest that MAG may be a major inhibitor of axonal regeneration in the CNS (e.g., McKerracher et al., 1994; Mukhopadhyay et al., 1994). However, analysis of MAG-deficient mice did not reveal significantly improved regeneration of retinal ganglion cell axons and corticospinal tract axons in vivo when compared with wild-type littermates. These findings led to the conclusion that MAG may not belong to the major inhibitors of neurite outgrowth in the CNS (Bartsch et al., 1995a; Bartsch et al., 1995b). MAG is rather involved in myelination as described below in more detail. Other molecules with major inhibitory or repulsive effects on neurite/axon elongation include oligodendrocyte- and myelin-associated neurite growth inhibitors NI-35 and NI-250, the semaphorins, connectin, netrins, tenascins, proteoglycans, and the ephrine family (Goodman, 1996; Schwab, 1993).

1.3.3 Myelination of Axons

Compact myelin is only found in vertebrates, although myelin-like structures have been described in invertebrates (Waehneldt, 1990). The presence of spirally stacked glial membranes around axons functions as an insulator and increases the speed at which action potentials are propagated along axons. Myelin sheaths originate from oligodendrocytes in the CNS and from Schwann cells in the PNS. The process of myelination starts with proliferation and differentiation of glial cells from mitotically active, migratory precursor cells. Upon differentiation, these glial cells express various myelin genes, the products of which control initiation of myelination, and formation and maintenance of compact myelin (Colello and Pott, 1997; Nave, 1994).

1.3.3.1 PNS Myelination

Schwann cells derive from neural crest cells. These neural crest cells differentiate first into Schwann cell precursors, then give rise to immature Schwann cells which finally differentiate into myelinating or non-myelinating Schwann cells. The first stage of maturation, the selective differentiation of neural crest cells into Schwann cell precursors is induced by a protein family called neuregulins (NRGs). The further proliferation and differentiation of the precursor cells is under the control of growth factors such as platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF), and transforming growth factor β (TGF-β; Colello and Pott, 1997; Nave, 1994).

Cell adhesion molecules (e.g., L1 or NCAM) become expressed at the time when the cells reach the immature Schwann cell stage. For instance, L1 is
involved in the extension of Schwann cell processes over the surfaces of axons and promotes their elongation along axons (Wood et al., 1990a; Wood et al., 1990b). However, as soon as the spiraling of Schwann cell processes proceeds and the Schwann cells have enwrapped the axon, L1 and NCAM become down-regulated. However, both molecules remain present on the surface of non-myelinating Schwann cells (Martini and Schachner, 1986; Mirsky et al., 1986).

With completion of differentiation, myelinating Schwann cells start to express protein zero (P₀), myelin-associated glycoprotein (MAG), myelin basic protein (MBP), and integrins. At this time point NCAM and L1 are already down-regulated. P₀ and MBP are mainly responsible for the compaction of the myelin sheath, where in particular P₀ serves as a spacer primarily at the extracellular sides of the Schwann cell plasma membranes (Colello and Pott, 1997; Waehneldt, 1990). This notion is supported by the finding that P₀-deficient mice develop non-compacted myelin, and a severe hypomyelination resulting in impaired nerve conduction (Giese et al., 1992; Martini et al., 1995).

In the PNS, myelin-associated glycoprotein has been implicated to be critical for initiation of myelination and for formation of morphologically intact myelin sheaths. In vitro studies have demonstrated that myelination of DRG neurites is impaired when levels of MAG expression are experimentally reduced in cocultured Schwann cells (Owens and Bunge, 1991). Vice versa, myelination of DRG neurites is accelerated when MAG is over-expressed in co-cultured Schwann cells (Owens et al., 1990). Regions of myelin sheaths in the quaking mutant are morphologically abnormal in that portions of the periaxonal space may be dilated or collapsed, or that regions of the periaxonal collar may be collapsed. These regions are exactly the affected regions of myelin sheaths which lack expression of MAG, whereas morphologically intact portions of the same myelin sheath are MAG-immunoreactive. These results suggest that MAG is critical for the formation of these structures (for a review, see Trapp, 1990). However, the analysis of MAG-deficient mice did not reveal an impaired initiation of myelination in the PNS, nor formation of ultrastructurally abnormal myelin sheath by Schwann cells (Montag et al., 1994). Instead, a decreased caliber of myelinated PNS axons (Yin et al., 1998), and degeneration of myelin sheaths and axons in the PNS of aged MAG mutants (Fruttiger et al., 1995) was observed. Thus, in the PNS, MAG appears to represent a glial signal that controls radial growth of myelinated axons, and the long-term maintenance of myelin sheaths and axons. A further family of CAMs that play a role in myelination in the PNS, are the integrins which mediate the control of
myelination by the basal lamina. Antibodies to the integrin member β1 inhibit myelin formation in vitro (Colello and Pott, 1997; Lander, 1989).

1.3.3.2 CNS Myelination

Myelin in the CNS is formed by oligodendrocytes. Early in development, these oligodendrocytes arise from neural epithelial cells. Neural epithelial cells give rise to oligodendrocyte progenitor cells (O-2A progenitor cells) which migrate to developing fiber tracts and proliferate under the influence of the platelet-derived growth factor (PDGF) secreted by astrocytes and insulin-like growth factor (IGF-1). Cells that differentiate into oligodendrocytes lose their responsiveness to PDGF and myelin basic protein (MBP) becomes up-regulated (reviewed in Colello and Pott, 1997). Similar to P0 in the PNS, the membrane-spanning proteolipid protein (PLP) acts as a spacer molecule at the extracellular sides of oligodendrocyte membranes (Waehneldt, 1990). MBP appears to be important for compaction of myelin, since MBP mutant mice produce large amounts of uncompacted myelin (Matthieu et al., 1990; Nave, 1994). In the CNS, MBP and PLP are the most abundant myelin proteins (80% of the proteins by weight; Readhead et al., 1990).

Shortly before and during myelination, oligodendrocytes start to express a variety of cell adhesion molecules (e.g., N-cadherin, NCAM, and MAG; Colello and Pott, 1997). N-cadherin is expressed on oligodendrocyte cell bodies and their major processes, and NCAM is found on oligodendrocyte cell bodies and processes and in compact myelin. N-cadherin and NCAM are both found on oligodendrocytes and neurons and may, therefore, mediate initial cell contacts between myelin-forming cells and axons to initiate myelination (Bartsch et al., 1989; Bartsch et al., 1990; Colello and Pott, 1997; Lander, 1989). Analysis of MAG null mutants (Li et al., 1994; Montag et al., 1994) has revealed a role of MAG for initiation of myelination and formation of ultrastructurally intact myelin sheaths in the CNS. Formation of myelin sheaths is delayed in the mutant (Montag et al., 1994), and the adult CNS of null mutants is hypomyelinated (Bartsch et al., 1997) when compared to wild-type littermates. Moreover, the periaxonal collar of mutant myelin sheaths is only poorly developed or completely lacking, myelin sheaths often contain regions with an unfused major dense line, axons may be concentrically surrounded by two or more myelin sheaths, and some MAG-deficient oligodendrocytes may display a so-called dying-back oligodendrogliopathy (Bartsch et al., 1997; Bartsch, 1996b; Bartsch et al., 1995b; Lassmann et al., 1997; Li et al., 1994; Montag et al., 1994). Two isoforms of MAG are known which, after deglycosylation display molecular weights of 72 kDa (L-
MAG) and 67 kDa (S-MAG). It is interesting that the CNS of a mutant mouse specifically lacking the L-MAG isoform displayed all defects that were observed in the MAG null mutant. Myelin in the PNS of L-MAG mutants, in contrast, was not affected (Fujita et al., 1998). These results demonstrate that L-MAG is the functionally important MAG isoform in the CNS, whereas S-MAG is the important MAG isoform in the PNS.

1.3.4 Signal Transduction - Second Messenger Pathways

Recent findings indicate that members of the IgSF are participating in second messenger pathways. For instance, cell-cell adhesion induces neurite outgrowth and also activates second messenger pathways such as changes in the intracellular pH and Ca\(^{2+}\) concentration, in inositol diphosphate, and in inositol triphosphate (Doherty and Walsh, 1992; Doherty and Walsh, 1994; Doherty and Walsh, 1996; Schuch et al., 1989; von Bohlen und Halbach et al., 1992). Evidence has been found for NCAM (neural cell adhesion molecule), L1, and N-cadherin to activate G-proteins that increase Ca\(^{2+}\) concentration and consequently the involvement of a tyrosine kinase, phospholipase C\(\gamma\) (PLC\(\gamma\)), diacylglycerol (DAG) lipase, and arachidonic acid (reviewed in Doherty and Walsh, 1996). Williams and colleagues have proposed an involvement for L1-, NCAM-, and N-cadherin-dependent neurite outgrowth of the fibroblast growth factor receptor (FGFR) that interacts with NCAM, L1, N-cadherin in a cis conformation (Williams et al., 1994). This interaction is required to initiate neurite outgrowth since antibodies against the FGFR or expression of a dominant negative form of the FGFR inhibit L1-, NCAM-, and N-cadherin-induced neurite outgrowth (Saffell et al., 1997; Williams et al., 1994). Second messenger activation not only results in changes of neurite outgrowth, but can also lead to a reorganization of cytoskeletal components or modulation of gene expression of proteins involved in cell migration or proliferation (reviewed in Brümmendorf and Rathjen, 1995). For instance, a tyrosine residue of the cytoplasmic domain of the myelin-associated glycoprotein (L-MAG) interacts with a src-related tyrosine kinase Fyn and Fyn is involved in the process of myelination downstream of MAG. This interaction may play a crucial role in the signal transduction pathway for myelination (Umemori et al., 1994). Indeed, it has been demonstrated that the fyn tyrosine kinase site is responsible for normal myelination in the CNS (Umemori et al., 1994).
1.4 Cell Recognition Molecules in the Lesioned Nervous System

Lesioned axons in the peripheral nervous system are able to regenerate in all vertebrate species. The distal part of an injured nerve undergoes Wallerian degeneration; Schwann cells proliferate and invading macrophages remove degenerating axons and myelin debris. An up-regulation of neurotrophic factors and cell adhesion molecules follows and supports regeneration of injured axons. Regenerating axons elongate between Schwann cells and the basement membrane. As soon as they reach their targets, growth promoting molecules eventually become down-regulated and remyelination starts. Despite the ability of the PNS to regenerate after an injury, functional recovery in mammals is poor. Lesioned axons often fail to reinnervate the original target (Gordon and Fu, 1997).

In contrast to the CNS of amphibia or fish, the mammalian CNS emphasizes the repair of the lesioned tissue rather than axonal regeneration. A CNS lesion is followed by a quick wound closure, restoration of the blood-brain barrier, a controlled inflammation, and cell death. Non-neuronal cells play an important role in the processes of repair such that they secrete growth factors and neurotrophic factors. An injury induces proliferation of fibroblasts, neuroglia, and endothelial cells (Eclancher et al., 1990; Logan et al., 1994). Microglia release TGF-β and bFGF with a subsequent recruitment of astrocytes which form a glial scar believed to be inhibitory for regeneration. However, astrocytes also release neurotrophic factors, which promote regeneration (Jones and Redpath, 1998).

The regenerative capability of CNS neurons differs among vertebrate species (for a review see Chernoff and Stocum, 1995; Filogamo and Vercelli, 1995; Jones and Redpath, 1998; Larner et al., 1995; Nicholls and Saunders, 1996; Nona, 1995; Schwab and Bartholdi, 1996; von Bernhardi and Muller, 1995; Windle, 1956). In contrast to higher vertebrates, lower vertebrates exert a remarkable degree of functional recovery in the CNS after an injury. Functional recovery of the spinal cord has been found in cyclostomes, fish, urodeles (e.g., newt, axolotl, salamander), premetamorphic anurans, and to some degree in reptiles. Optic nerve regeneration has been described in fish, urodeles, and adult anurans (Larner et al., 1995; Schwab and Bartholdi, 1996). It has been suggested that the regenerative capacity of the CNS of anamniotes is related to a continuous neurogenesis in some brain regions of these animals. Newly generated nerve cells require a growth-permissive environment in order to extend their axons. This permissive environment may also be used by injured nerve cells to regrow
their axons (Holder and Clarke, 1988). In the CNS of birds and mammals, however, long-distance regeneration of lesioned axons does not occur. The response of nerve cells to an injury is limited to axonal sprouting without any functional recovery (Larner et al., 1995).

Although injured axons of the adult mammalian CNS are unable to regrow over long distances (Ramon y Cajal, 1928), these nerve cells do have the intrinsic ability to regenerate their axons if a growth-permissive environment is provided. For instance, a variety of different CNS neurons have been shown to regrow their axons over long distances through peripheral nerve grafts (e.g., Anderson et al., 1989; Benfey and Aguayo, 1982; Cho and So, 1987; David and Aguayo, 1985; Hall and Berry, 1989; Hall and Kent, 1987; Larner et al., 1995; Richardson et al., 1980; 1982; Villegas-Pérez et al., 1988; reviewed in Aguayo, 1985). Grafts from the fetal CNS were also successful in promoting regeneration of transected CNS axons (Aguayo et al., 1984; Laedtke and Turner, 1989; Lindvall et al., 1990; Sievers et al., 1988). These observations clearly demonstrate that the cellular and molecular surrounding of lesioned CNS axons critically determines the extent of axonal regrowth. Different soluble factors may be present in the PNS and CNS, and may support axonal regeneration in peripheral nerves, but not in the brain. However, when sympathetic or sensory nerve cells are cultured under identical and optimal (i.e., in the presence of growth factors) conditions, they extend neurites into peripheral nerve explants, but not into CNS (i.e., optic nerve) explants (Schwab and Thoenen, 1985). These observations indicate that the different regenerative capacity of CNS versus PNS tissue is related to differences in substrate properties, rather than to differences in soluble factors.

Axonal regrowth does occur in the immature CNS of birds and mammals (e.g., Hasan et al., 1993; Kalil, 1988; Keirstead et al., 1992; Varga et al., 1995). Interestingly, the end of this regeneration-permissive period correlates with the onset of differentiation of oligodendrocytes and the formation of myelin (e.g., Hasan et al., 1993; Keirstead et al., 1992; reviewed by Schwab and Bartholdi, 1996). Experimental delay of myelination results in a prolongation of the regeneration-permissive period (e.g., Hasan et al., 1993; Keirstead et al., 1992; Savio and Schwab, 1990; Weibel et al., 1994) indicating that differentiated oligodendrocytes and their product, CNS myelin, inhibit axonal regrowth. Cell culture experiments have in fact demonstrated that differentiated oligodendrocytes and CNS myelin inhibit neurite elongation and cause growth cone collapse (Bandtlow et al., 1990; 1993; McKerracher et al., 1994). The inhibitory effects of oligodendrocytes and CNS myelin have been related to the
expression of potent growth inhibitors (reviewed by Jones and Redpath, 1998; Schwab and Bartholdi, 1996; see below). However, robust axonal regrowth of retinal ganglion cell axons into optic nerves containing oligodendrocytes and myelin debris was observed after grafting PNS explants into the vitreous body of adult rats (Berry et al., 1996). The physiological relevance of oligodendrocyte- and myelin-associated growth inhibitors has been questioned by these results. However, soluble factors derived from the PNS grafts may induce down-regulation of neuronal receptors for neurite growth inhibitory molecules.

Altogether, nerve cells of the adult mammalian CNS do have the intrinsic capability to re-extend injured axons. Axonal regeneration in the brain of mammals may therefore be achieved by appropriate experimental manipulations. The neutralization of growth inhibitory factors has already been demonstrated to be a promising approach (see also below). Axonal regrowth in the adult CNS may also be achieved by introducing growth promoting molecules into the environment of injured axons (see below).

1.4.1 Inhibition of Axonal Regeneration

As discussed above, the regeneration-permissive period of the amniote CNS ends with the differentiation of oligodendrocytes and with the formation of myelin (Keirstead et al., 1992; Savio and Schwab, 1990; Weibel et al., 1994). In fact, CNS white matter from adult rat spinal cord, cerebellum, forebrain, or optic nerve were not permissive for adhesion or neurite outgrowth of neuroblastoma cells and sympathetic or dorsal root ganglion neurons in vitro. In contrast, gray matter of mammalian CNS, sciatic nerves, or fish CNS white and gray matter permitted cell adhesion and axon elongation (Savio and Schwab, 1989). Moreover, sciatic nerve explants, but not optic nerve explants of adult rats were permissive substrates for axon elongation of dissociated newborn rat sympathetic or sensory neurons (Schwab and Thoenen, 1985). Isolated mammalian CNS myelin inhibited neurite growth and induced growth cone collapse (Bandtlow et al., 1993; McKerracher et al., 1994). Neurite growth is also inhibited in vitro as soon as differentiated mammalian oligodendrocytes are contacted (Bandtlow et al., 1990).

In search for the molecular basis of the inhibitory properties of oligodendrocytes and CNS myelin, the neurite growth inhibitors, designated NI-35 and NI-250, have been identified which are expressed by oligodendrocytes (Caroni and Schwab, 1988b; Spillmann et al., 1997; 1998). Monoclonal antibodies (mAb IN-1) against NI-35 and NI-250 prevented $\text{Ca}^{2+}$-dependent growth cone
collapse when CNS myelin was encountered and allowed neurite elongation on oligodendrocytes and CNS myelin in vitro (Bandtlow et al., 1990; Loschingher et al., 1997). Furthermore, cerebellar Purkinje cells that exert a poor regenerative capacity, showed increased axonal growth after IN-1 treatment (Zagrebelsky et al., 1998). Application of mAb IN-1 further induced regeneration of lesioned axons in the adult spinal cord and in brains of young and adult rats over a long distance in vivo and led to a partial functional restoration (Bandtlow et al., 1993; Bregman et al., 1995; Cadelli and Schwab, 1991; Caroni and Schwab, 1988a; 1988b; Schnell and Schwab, 1990; Schwab and Bartholdi, 1996). After a lesion of the corticospinal tract and application of IN-1, corticospinal tract fibers elongated into the cervical spinal cord. However, regenerating fibers were rarely found at their original location (Raineteau et al., 1999). Despite the misplaced position of regenerating fibers, antibody-treated rats exhibited recovery in motor and sensory tests (Thallmair et al., 1998; Z'Graggen et al., 1998). Even after chronic injury, transected corticospinal fibers retained their regenerative capacity for a few weeks (von Meyenburg et al., 1998).

In addition to the potent growth inhibitors NI-35 and NI-250, several cell recognition molecules have been implicated to inhibit axonal regeneration in the injured mammalian CNS (Jones and Redpath, 1998; Schwab and Bartholdi, 1996). One of these molecules may be MAG, a member of the IgSF, which is localized on the surface of the axon-facing myelin. The substrate properties of MAG for neurite elongation may depend on the age of the nerve cells tested. Neurite outgrowth from young postnatal DRG neurites, for instance, is promoted by MAG while neurite outgrowth from adult DRG neurons is inhibited on the same substrate (Johnson et al., 1989; Mukhopadhyay et al., 1994). However, for most nerve cell types MAG represents an inhibitory substrate, and the molecule was therefore considered as an inhibitor of axonal regeneration (Filbin, 1995; 1996). For instance, Mukhopadhyay and co-workers (1994) demonstrated that neurite outgrowth from rat cerebellar neurons (postnatal day 1, 4, and 7) on MAG-transfected CHO-cells was decreased by 70% when compared to control substrates. Polyclonal antibodies against MAG reversed the inhibition by about 50%. Similar although less pronounced results were found for dorsal root ganglion (DRG) neurons from adult animals (Mukhopadhyay et al., 1994). Finally, it has been demonstrated that immunodepletion of MAG from CNS myelin removed most of its inhibitory activity. Based on this observation, it has been speculated that MAG is a significant, and possibly major inhibitor in CNS myelin (McKerracher et al., 1994). However, David and co-workers (1995) have demonstrated that laminin, a
component of the Schwann cell basal lamina, neutralized the inhibitory effects of MAG in vitro. Moreover, axonal regeneration of optic nerves or corticospinal tracts in MAG-deficient mice was not improved in comparison with wild-type littermates (Bartsch, 1996b; 1995a). In addition, application of antibodies against the neurite growth inhibitors NI-35 and NI-250 showed a similar improvement of axonal regrowth in both MAG-deficient and wild-type animals (Bartsch, 1996b; 1995a). Generation of double-mutants (C57BL/Wld^s) with a delayed lesion-induced myelin degeneration in peripheral nerves (C57BL/Wld^s) and a deficiency in MAG (MAG^s) showed an improved regrowth of femoral nerve axons when compared with C57BL/Wld^s mice that expressed MAG (Schäfer et al., 1996). Thus, in this system where myelin is only slowly removed from lesioned PNS, a role of MAG in inhibiting axonal regrowth became apparent in vivo (Schäfer et al., 1996). The different results obtained for the regenerative capacity of CNS versus PNS of MAG-deficient mice may relate to the fact that inhibitory factors predominate in the CNS, while growth-promoting factors predominate in the PNS. As a consequence, inhibitory properties of MAG may be masked in the CNS, but become apparent in the PNS. In any case, available data do not support the view that MAG is a major inhibitor of axonal regeneration in the CNS (Bartsch et al., 1995a).

Other possible inhibitors of axonal regeneration are members of the tenascin gene family (see chapter 1.2.3 or Bartsch, 1996a; Faissner, 1997; Wintergerst et al., 1997). Two of the four identified tenascins are expressed in the CNS, namely tenascin-C and -R (TN-C and TN-R, respectively). TN-C is expressed by astrocytes while TN-R is expressed by oligodendrocytes. Depending on the cell type and the way the molecules are presented to cells, TN-C and TN-R exert stimulatory or inhibitory effects which are mediated by distinct domains of the protein. Controversial results have been found regarding TN-C expression after a lesion. For instance, TN-C has been found to be up-regulated in lesioned PNS tissue at the neuromuscular junction, in lesioned peripheral nerves of mice, chicken, and frogs (Bartsch, 1996a; Faissner, 1997; Martini et al., 1990), and in the CNS after performing stab wounds in the cerebral or cerebellar cortex of mice (Laywell et al., 1992) or after transecting the postcommissural fornix of adult rats (Lips et al., 1995). In contrast, no up-regulation of TN-C was found in the CNS after an optic nerve crush or after 3-acetylpyridine-induced degeneration of nerve cells in the inferior olivary nucleus (Bartsch, 1996a; Wintergerst et al., 1997). These diverse results found in the CNS led to the hypothesis that the differential expression of TN-C in the injured CNS may depend on the type of
lesion (invasive, mechanically-induced versus non-invasive, chemically-induced lesion; Wintergerst et al., 1997).

Additional molecules involved in neurite outgrowth inhibition include collapsins/semaphorins, ephrin receptor tyrosine kinases, proteoglycans, netrins, Thy-1, thrombin, and lysophosphatidic acid (reviewed in Flanagan and Vanderhaeghen, 1998; Mark et al., 1997; Mukhopadhyay et al., 1994; Schwab and Bartholdi, 1996; Sefton and Nieto, 1997; Winberg et al., 1998; Zisch and Pasquale, 1997).

1.4.2 Promotion of Axonal Regeneration

Neurite outgrowth stimulating molecules include the cell adhesion molecules NCAM (Drosophila fasciclin II), L1 (and its relatives chick Ng-CAM, CHL1, Nr-CAM/Bravo, neurofasern, and Drosophila neuroglian), F11, axonin-1 (TAG-1), and integrins (for an overview see Goodman, 1996; Walsh and Doherty, 1996).

NCAM is a well studied molecule which mediates cell recognition and supports neurite outgrowth. A special feature of NCAM is that it may carry polysialic acid (PSA). The binding site of PSA has been attributed to the fifth Ig domain of NCAM with an overlap to the adjacent domain (Fig. 1; Nelson et al., 1995). NCAM-PSA is described as the embryonic form of NCAM, since it is widely expressed during development of the nervous system. In the adult brain, NCAM-PSA remains expressed only in regions with a high degree of synaptic plasticity. During regeneration in the visual system of fish and amphibia and in the sciatic nerve, PSA becomes re-expressed. A re-expression of PSA has also been found in muscle after denervation (Rutishauser, 1998; Rutishauser and Landmesser, 1996). Doherty and co-workers have proposed that the removal of PSA leads to an increase in the NCAM homophilic binding and thus a decrease in the interaction with other molecules that may be needed for signal transduction pathways ultimately resulting in axon growth (Walsh and Doherty, 1996).

L1 and its possible roles during neurite outgrowth and regeneration are described in more detail in chapter 1.5. Most studies involving L1 have been conducted under in vitro conditions. These experiments revealed that L1 is a good promoter for neurite outgrowth when binding to itself, chick axonin-1 (and its rat homolog TAG-1), F11, or integrins (Brümmendorf and Rathjen, 1995). In fact, L1-transfected fibroblasts (L-cells) grafted into a lesioned rat spinal cord successfully promoted regrowth of lesioned axons through the glial scar into
and along the graft (Kobayashi et al., 1995). Antibodies against Ng-CAM inhibited neurite outgrowth on axonin-1 (Kuhn et al., 1991). However, it is proposed that axonin-1 requires a second binding partner like itself or β1 integrins (Felsenfeld et al., 1994; Montgomery et al., 1996; Rader et al., 1993). Interaction of Nr-CAM (an L1-relative) with F11 or bound neurofascin also promotes neurite outgrowth (Morales et al., 1993; Volkmer et al., 1996). CHL1, another member of the L1-family, is also involved in neurite outgrowth. CHL1- and L1-transfected cells enhanced neurite outgrowth of embryonic hippocampal neurons and small cerebellar neurons in vitro (more details about CHL1 in chapters 1.5.6-9; Hillenbrand et al., 1999).

Members of the tenascin gene family, specifically TN-C and TN-R, have been described as neurite inhibitors in the previous chapter. However, their functional roles are more complex. Indeed, TN-C and TN-R have also been implicated in adhesion activities in the developing and lesioned nervous tissue (Faissner, 1997). Certain domains of TN-C promote neurite outgrowth, specifically the FNIII repeat eleven. Homogeneous TN-C substrates enhanced neurite outgrowth of embryonic mesencephalic and hippocampal neurons (Lochter et al., 1991) and of small cerebellar neurons of 6-8 day-old mice (Husmann et al., 1992). Interestingly, Zhang and co-workers have shown that regenerating CNS axons grow through TN-C positive peripheral nerve grafts and that peripheral axons grow inside crushed dorsal roots which express high levels of TN-C (Zhang et al., 1995a; 1995b).
1.5 The L1 Family

The L1 family is a subgroup of the immunoglobulin superfamily. Members of the L1 family include L1, CHL1, Nr-CAM, and neurofascin (Fig. 1 and Table 1). These family members share a basic structural organization that includes six Ig domains, four to five FNIII domains, a hydrophobic membrane-spanning region, and a short and highly conserved cytoplasmic domain (about 110 amino acid residues; Brümmendorf and Rathjen, 1995). The molecules are predominantly expressed in the developing PNS and CNS, but also in the adult nervous system mainly during regenerative processes or concomitant with synaptic plasticity. L1 and L1-related molecules are involved in various processes including cell migration, neurite outgrowth, axon pathfinding and fasciculation, growth cone morphology, myelination, and synaptic plasticity (see chapters 1.3 and 1.4). Members of the L1 family are expressed by neurons and by glial cells, and are mainly found on the surface of axons or at sites of cell-cell contact (Brümmendorf and Rathjen, 1995; Hortsch, 1996).

1.5.1 Neural Cell Adhesion Molecule L1

One of the most widely studied cell recognition molecules is L1. L1 and its species homologs are expressed in both, the vertebrate and invertebrate nervous system (for reviews see Brümmendorf et al., 1998; Hortsch, 1996) and also in non-neural tissue (e.g., Kowitz et al., 1992; Kowitz et al., 1993; Probstmeier et al., 1990). The various species homologs of L1 are listed in Table 2. Alternative splicing of the pre-mRNA generates diverse isoforms of different lengths and expression patterns. All members of the L1 family mediate Ca\(^{2+}\)-independent cell-cell adhesion (Hortsch, 1996).

The sequence similarity among the species homologs of L1 ranges between 30% to 60% (Table 3, Holm et al., 1996). Additionally, the analysis of homology indicates that the intracellular part of L1-related molecules is highly conserved among species whereas the extracellular domain is more variable (Table 3).

Mammalian L1 including human, rat, and mouse homologs, consists of six Ig-like domains of the C2-type, five FNIII repeats, a transmembrane stretch, and a short cytoplasmic domain (Fig. 1 and 3; Brümmendorf and Rathjen, 1995; Moos et al., 1988). The sequence similarity is 92% between human and mouse L1 and 97% between mouse and rat L1, with the intracellular part being completely identical (Hlavin and Lemmon, 1991; Miura et al., 1991). The length of the L1 protein ranges between 1256 and 1260 amino acids (Table 2). Various molecular
mass forms have been described (see Table 2), as a result of proteolytic cleavage or differential glycosylation patterns, and serve different functions (Faissner et al., 1985; Sadoul et al., 1988; Salton et al., 1983; Thor et al., 1987). Two major integrin-binding sites (RGDs: arginine-glycine-aspartate) are present in the sixth Ig domain of mammalian L1 (Kadmon and Altevogt, 1997). The L1 gene is composed of 28 exons. Two alternatively spliced exons have been described for mammalian L1 corresponding to exon 2 and 27. The alternatively spliced exon 2 codes for the amino acids YEGHH in human and YKGGH in mouse (Jouet et al., 1995). The second alternatively spliced exon 27 codes for the amino acids RSLE (arginine-serine-leucine-glutamine) in the intracellular part of L1. Both exons are present in neuronal L1, but are spliced out of L1 mRNAs from non-neuronal cells (Hlavin and Lemmon, 1991; Miura et al., 1991; Reid and Hemperly, 1992; Takeda et al., 1996).

Table 2: Summary of the species homologs of L1

<table>
<thead>
<tr>
<th>Phyla</th>
<th>Species</th>
<th>Name</th>
<th>Protein (aa)</th>
<th>Isoforms</th>
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<tbody>
<tr>
<td>Higher Vertebrates</td>
<td>Human¹</td>
<td>L1CAM, L1, 5G3</td>
<td>1256</td>
<td>190, 180, 130, 105, 80, 62</td>
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<td></td>
<td>Rat²</td>
<td>NILE</td>
<td>1259</td>
<td>230, 200, 160, 80, 30</td>
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<td></td>
<td>Mouse³</td>
<td>L1</td>
<td>1260</td>
<td>200, 180, 140, 80, 50, 30</td>
</tr>
<tr>
<td></td>
<td>Chick⁴</td>
<td>Ng-CAM, G4, 8D9</td>
<td>1265, 1280</td>
<td>200, 180, 190, 135, 80, 65</td>
</tr>
<tr>
<td>Lower Vertebrates</td>
<td>Goldfish⁵</td>
<td>E587</td>
<td>1232</td>
<td>200, 170, 110, 57, 40</td>
</tr>
<tr>
<td></td>
<td>Zebrafish⁶</td>
<td>L1.1</td>
<td>1197*</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>L1.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Invertebrates</td>
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<td>Neuroglian</td>
<td>1239</td>
<td>180, 167, 155</td>
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<td></td>
<td>Manduca⁸</td>
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<td>156</td>
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<td>Leech⁹</td>
<td>Tractin</td>
<td>1880</td>
<td>200, 130, 100</td>
</tr>
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</table>

¹ Hlavin and Lemmon, 1991; Wolff et al., 1988
² Miura et al., 1991; Prince et al., 1989; Salton et al., 1983; Stallcup and Beasley, 1985
³ Moos et al., 1988; Sadoul et al., 1988
⁴ Buchstaller et al., 1996; Burgoon et al., 1991; Grumet et al., 1984
⁵ Giordano et al., 1997; Vielmetter et al., 1991
⁶ Tongiorgi et al., 1995
⁷ Bieber et al., 1989
⁸ Chen et al., 1997
⁹ Huang et al., 1997
* almost complete length of L1.1 (including 6 Ig domains, 5 FNIII repeats, transmembrane and cytoplasmic domain)
Table 3: Sequence comparison of the extra- and intracellular domains of species homologs of L1

<table>
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<tr>
<th>extraacellular domain</th>
<th>L1 (m, r, h)</th>
<th>Ng-CAM (c)</th>
<th>L1.1 (zf)</th>
<th>Neuroglian (d)</th>
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<td>Ng-CAM (c)</td>
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<td>28</td>
<td>30</td>
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<tr>
<td>L1.1 (zf)</td>
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<td>Neuroglian (d)</td>
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<td></td>
<td></td>
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<tr>
<td>L1.1 (zf)</td>
<td>61</td>
<td></td>
<td>50</td>
<td></td>
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<tr>
<td>Neuroglian (d)</td>
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<td>36</td>
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<td>L1.2 (zf)</td>
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<td>44</td>
<td>22</td>
</tr>
</tbody>
</table>

The values indicate the degree of homology of the extra- and intracellular parts of presumed L1 species homologs. They represent the percentage of amino acid identity after alignment. The animal species in brackets are the following: c: chicken, d: Drosophila, h: human, m: mouse, r: rat, zf: zebrafish (Holm et al., 1996).

Figure 3: Structure of L1. The L1 molecule consists of six immunoglobulin domains (numbered I - VI) and five complete fibronectin type III repeats (FN1-5) next to the N-terminal. The transmembrane region is indicated by the green box and is followed by a short intracellular domain.

The sequence identity of Ng-CAM in chick with mouse L1 is in the range of 40-50% (Buchstaller et al., 1996; Burgoon et al., 1991), with the intracellular domain being more conserved (61% identity) than the extracellular part (Table 3; Reid and Hemperly, 1992). Ng-CAM comprises six Ig-like domains, five FNIII repeats, a transmembrane region, and a cytoplasmic domain (Fig. 1). Ng-CAM contains an RGD motif in the third FNIII repeat and an RSLE stretch in the cytoplasmic domain (Burgoon et al., 1991).

Another L1-like molecule, E587, has been found in goldfish. It shares a homology of 40% with mouse L1 and chick Ng-CAM and of 65% with zebrafish L1.1. Several isoforms have been identified with molecular weights of 200, 170, 110, 57, and 40 kDa (Table 2). Like in mouse L1, the sixth Ig domain of E587
contains an RGD motif, which may function as an integrin binding site (Giordano et al., 1997; Vielmetter et al., 1991).

*L1.1* and *L1.2* are L1-related proteins in zebrafish with a 40% sequence identity of *L1.1* and with a 50% identity of the partial sequence of *L1.2* with mouse, rat, and human L1 (Tongiorgi et al., 1995). *L1.1* consists of six Ig-like domains, five FNIII repeats, a transmembrane region, and an intracellular domain.

*Neuroglian* in *Drosophila* has first been described by Bieber and co-workers (Bieber et al., 1989). The protein comprises six Ig-like domains (type C2), five FNIII repeats, a transmembrane stretch, and a short intracellular domain. Neuroglian and mouse L1 are 28% identical at the amino acid level. Although most of the L1 homologs include an RGD motif in an Ig-domain, neuroglian does not. Neuroglian protein isolated from nerve cord membrane extracts yielded bands with molecular masses of 180, 167, and 155 kDa, with the 167 kDa band being the major form (Table 2). These different molecular weights are due to differential glycosylation of a core protein of 155 kDa (Bieber et al., 1989). A selective intracellular binding to ankyrin at cell contact sites has been demonstrated by Hortsch and co-workers (Hortsch et al., 1998).

*3B11* in the moth Manduca sexta shares a homology of 58% and 31% with the *Drosophila* neuroglian and the mouse L1 protein, respectively (Chen et al., 1997). The Manduca L1 comprises six Ig domains of the C2-type, five FNIII repeats, a transmembrane region, and a cytoplasmic domain. So far only one isoform has been described (Chen et al., 1997).

*Tractin* in leeches comprises six Ig domains, four FNIII repeats, an acidic domain, 12 repeats of a novel proline- and glycine-rich motif, a membrane-spanning region, and a short cytoplasmic domain. A putative cleavage site in the third FNIII repeat infers a secreted half of the protein. The presence of three isoforms with molecular masses of 200, 130, and 100 kDa is proposed (Table 2). Tractin also contains an RGD integrin binding site which is located in the second FNIII repeat and an ankyrin binding site in the intracellular part. These binding sites suggest an interaction with RGD-specific integrin heterodimers or an interaction with the cytoskeleton, respectively. Sequence comparison revealed a 33% homology of tractin to *Drosophila* neuroglian (Huang et al., 1997).
1.5.2 Expression of L1

L1 in human, rat, and mice is expressed both in the CNS and the PNS. In the CNS, L1 is only expressed by post-mitotic neurons and not by glial cells (Mohajeri et al., 1996; Persohn and Schachner, 1987). During the development of the CNS, L1 is predominantly expressed in early-forming axon tracts, but down-regulated on surfaces of myelinated processes (Bartsch et al., 1989; Faissner et al., 1984; Lagenaur and Lemmon, 1987; Rathjen and Schachner, 1984). In the cerebellum, L1 does not become expressed until precursor cells become postmitotic, i.e. precursors of granule, stellate, and basket cells are devoid of L1 (Persohn and Schachner, 1987). As soon as the granule cells are postmitotic, L1 is expressed by granule cells which migrate from the external granular layer (EGL) to the internal granular layer (IGL) of the cerebellum of mice (Lindner et al., 1983).

After embryonic day 9 in the neocortex of mice, L1 becomes detectable on young neurons in the marginal zone only. Progressive expression of L1 is found on afferent fibers in the intermediate zone of subcortical areas and also on migrating neurons, in close analogy to migrating granule cells in the cerebellum. Expression of L1 in the cortical plate and the subplate is detectable between embryonic days 13 to 16 (Fushiki and Schachner, 1986). Moreover, during the development of the mouse cortex and olfactory bulb, L1 is detected on various unmyelinated fiber tracts including thalamocortical fibers, olfactory nerve, and inner plexiform layer of the olfactory bulb (Chung et al., 1991).

In the developing hippocampus, L1 is expressed only on fasciculating axons and not on dendrites or cell bodies of pyramidal cells, granule cells or interneurons. Synapses (pre- and postsynaptic membranes) are devoid of L1-immunoreactivity. Also, no expression of L1 is detected at contact sites between astrocytes and axons (Persohn and Schachner, 1990). All these observations suggest that expression of L1 is one of the first signs of neuronal differentiation and may be crucial during the dynamic phase of neural development. L1 may not initiate neuronal migration, but may play an important role during migration and aggregation of nerve cells at their final location.

The expression pattern of L1 in the developing primary visual system of mice is similar to that observed in the brain. A predominant expression of L1 is detectable on fasciculating, non-myelinated retinal ganglion cell axons with or without contact of glial cells. However, expression of L1 on retinal ganglion cell axons disappears completely as soon as axons become myelinated. Furthermore,
growth cones of retinal ganglion cells are L1-positive at contact sites with axons, but not at contact sites with glial cells. In contrast to observations in the hippocampus (Persohn and Schachner, 1990), L1 is not detectable at glia-glia contact sites (Bartsch et al., 1989).

In contrast to the central nervous system, L1 in the PNS is not only expressed by neurons, but additionally by glial cells including non-myelinating Schwann cells and satellite cells surrounding neurons (Martini and Schachner, 1986; Mirsky et al., 1986). L1 has further been shown to be present on small, non-myelinated, and fasciculated axons ensheathed by non-myelinating Schwann cells (Martini and Schachner, 1986). L1 was also detected on myelinating Schwann cells during the onset of myelination until the first 1.5 loops of the myelinating Schwann cell around the axon have been completed. Afterwards L1 was down-regulated in both axons and myelinating Schwann cells. Accordingly, L1 has not been found in compact myelin, Schmidt-Lanterman incisures, paranodal loops, and finger-like processes of Schwann cells at nodes of Ranvier (Martini and Schachner, 1986). However, Mirsky and co-workers (1986) have reported low expression of L1 at nodes of Ranvier. In contrast to the long form of L1, a short form of L1, lacking the exons 2 and 27 (see also chapter 1.5.1), has been described to be solely expressed in non-neuronal cells including Schwann cells, astrocytes, and oligodendrocytes (Takeda et al., 1996).

The expression of Ng-CAM, the chick homolog of L1, is restricted to the nervous system. The spatio-temporal pattern of expression of Ng-CAM is very similar to that of L1 in mammals. In the developing cerebellar cortex, for instance, Ng-CAM is expressed by migratory granule cells as soon as they have become postmitotic (Chuong et al., 1987; Daniolff et al., 1986; Thiery et al., 1985). Antibodies against Ng-CAM interfere with neuronal migration from the subependymal zone to the neostriatum in song birds and cause process retraction and cell rounding (Barami et al., 1994). Ng-CAM is expressed on fasciculated axons in the visual system and antibodies against Ng-CAM cause defasciculation of commissural axons, demonstrating that Ng-CAM plays a role in axon fasciculation (Lemmon and McLoon, 1986; Stoeckli and Landmesser, 1995). More details are given in chapter 1.5.3.

L1-related proteins in lower vertebrates are also expressed predominantly in nervous tissue. E587, a presumed goldfish homolog of L1, is expressed in almost all neuronal structures of the developing nervous system and expression of E587 declines in the adult tissue. E587 is re-expressed during axonal regeneration. Expression of E587 is found on regenerating retinal ganglion cell axons and
oligodendrocyte-like cells in the regenerating fish optic nerve (Bastmeyer et al., 1993; Giordano et al., 1997; Vielmetter et al., 1991). During embryogenesis in zebrafish, L1 and L1.2 mRNA is restricted to postmitotic neurons and becomes detectable at the onset of axogenesis (Tongiorgi et al., 1995). Both, L1.1 and L1.2 mRNA are upregulated in the lesioned spinal cord of adult zebrafish by regenerating nerve cells, but not by nerve cells which show no robust axonal regeneration (Becker et al., 1998). Interestingly, L1.1 and L1.2 mRNAs are also expressed by glial cells in the lesioned optic nerve of zebrafish, but not in unlesioned control nerves (Bernhardt et al., 1996). These observations imply a role of E587, L1.1, and L1.2 during regenerative processes in the injured CNS of fish.

Species homologs of L1 have also been found in invertebrates. For instance, neuroglian in the fruitfly Drosophila is widely expressed in the developing nervous system. High levels of expression were found on surfaces of neurons and glia of the peripheral nerve roots and on fasciculating axons in the developing CNS (Bieber et al., 1989). Embryonic lethal neuroglian mutants showed errors in motor axon pathfinding (Hall and Bieber, 1997). 3B11 of the moth Manduca is expressed at sites of cell-cell contact in various tissues including the nervous system and may be involved in cell aggregation and cell migration. First expression is found on primordial cells (Chen et al., 1997). Tractin in leeches is expressed in the peripheral and central nervous system. Differential glycosylation regulates neurite outgrowth and fasciculation of distinct neuronal subpopulations as has been demonstrated in antibody perturbation experiments (Huang et al., 1997).

1.5.3 Functional Characterization of L1

The cell recognition molecule L1 is a multifunctional molecule in that it promotes adhesion via homophilic or heterophilic binding, mediates cell migration, axonal outgrowth, axonal pathfinding, axonal fasciculation, and myelination, and is involved in synaptic plasticity.

1.5.3.1 Homophilic and Heterophilic Adhesion

Cell recognition molecules are cell-surface molecules that mediate adhesion either in a homophilic or heterophilic manner. Homophilic interaction is mediated by binding only to itself, that means that the same protein serves as ligand and receptor, whereas heterophilic binding always involves two different proteins. Proteins can further interact either in cis conformation (on the same cell, in the plain of the same plasma-membrane) or in trans configuration (on
opposing membranes of different cells). Homophilic adhesion is mostly mediated by the immunoglobulin domains due to the better accessibility, whereas heterophilic adhesion is proposed to be mediated by both the Ig domains and the FNIII repeats (Brümmendorf and Rathjen, 1995; Walsh and Doherty, 1997).

L1 mediates cell recognition via both, homophilic and heterophilic binding (for review, see Brümmendorf and Rathjen, 1995). Antibodies against Ng-CAM (the presumed L1 homolog in chicken) or mouse L1 inhibited neurite outgrowth on L1 substrates and blocked aggregation of L1-expressing cells (Grumet and Edelman, 1988; Kadmon et al., 1990b; Keilhauer et al., 1985; Lagenaur and Lemmon, 1987; Lemmon et al., 1989; Miura et al., 1991; Rathjen and Schachner, 1984; Seilheimer and Schachner, 1988). Grumet and Edelman (1988) demonstrated that Ng-CAM mediates cell adhesion via homophilic binding. Covaspheres coated with Ng-CAM bind to neurons and glia, and binding of these Ng-CAM-coated beads was blocked when neurons were pretreated with antibodies against Ng-CAM (Grumet and Edelman, 1988). In addition, when purified L1 from chick or mouse brain was plated on nitrocellulose-coated dishes, neurite growth of chick and mouse neurons was detectable on both, chick and mouse L1. Neurite outgrowth of chick neurons, but not mouse neurons, was inhibited when the dishes were pretreated with chick L1-antibodies which were pre-adsorbed against mouse L1 (Lemmon et al., 1989).

Homophilic adhesion is mediated by specific domains of the extracellular part of L1, namely the Ig domains 1 and 2 and the FNIII repeats 3 to 5 as demonstrated in aggregation assays using microspheres coated with different L1 fragments (Holm et al., 1995). Strong binding of microspheres coated with the Ig domains 1 and 2 or with the FNIII repeats 3 to 5 to small cerebellar neurons confirmed this notion (Holm et al., 1995). Zhao and co-workers (1995) further defined more precisely the adhesive region of L1 to the second Ig domain with a promoting effect of the first Ig domain. Solely the second Ig domain bound to itself on opposing membranes, demonstrating its homophilic binding property (Zhao and Siu, 1995). Interestingly, the second Ig domain represents the most highly conserved region in the extracellular domain of L1 among various species (Hlavin and Lemmon, 1991; Lemmon et al., 1989).

Heterophilic binding of L1 to various ligands is mediated either in cis or trans configuration (Brümmendorf and Rathjen, 1995). Several members of the IgSF are involved in heterophilic interactions with L1. NCAM, for example, has been shown to enhance homophilic binding of L1 by interacting with L1 in cis, a
process called "assisted homophilic" binding (Kadmon et al., 1990b; Simon et al., 1991). The L1-NCAM interaction in cis is dependent on carbohydrates, and a carbohydrate recognition domain has been identified in the extracellular domain of NCAM (Horstkorte et al., 1993; Kadmon et al., 1990a). The interaction of Ng-CAM with NCAM is also dependent on N-glycosylation (Grumet et al., 1984). Other members of the IgSF such as axonin-1 (Kuhn et al., 1991), F11 (Brümmendorf et al., 1993), and DM-GRASP (DeBernardo and Chang, 1996) also bind to Ng-CAM, and TAG-1 (rat homolog of axonin-1) to L1 (Felsenfeld et al., 1994; Rader et al., 1996). Ng-CAM antibodies inhibit neurite outgrowth of neurons that are cultured on axonin-1 or DM-GRASP, but not on F11 (Brümmendorf et al., 1993). The interaction of axonin-1 and Ng-CAM takes place in cis configuration (Buchstaller et al., 1996).

Other binding partners of L1 are found in the family of integrins. For instance, \( \alpha_5\beta_1 \) (VLA-5) and \( \alpha v \beta_3 \) bind to the RGD sequence of L1, which is located in the sixth Ig domain (Kadmon and Altevogt, 1997). Both integrins are mainly expressed outside the nervous system. During the formation of the nervous system, the interaction of L1 with \( \alpha_5\beta_1 \) plays a role in the migration of neural crest cells (Ruppert et al., 1995). Laminin, an extracellular matrix molecule, facilitates migration of L1-transfected L-cells (Malinda and Kleinman, 1996; Timpl and Brown, 1994). L-cells transfected with L1 lacking the RSLE sequence adhered to, but did not migrate on laminin, suggesting a role of the RSLE stretch for cell migration (Takeda et al., 1996). Grumet and co-workers have further shown an interaction of laminin with Ng-CAM (Grumet et al., 1993b). Phosphacan and neurocan, two chondroitin sulfate proteoglycans, act as ligands for Ng-CAM and inhibit or modulate cell adhesion and neurite outgrowth (Friedlander et al., 1994; Grumet et al., 1993a; Grumet et al., 1996; Margolis et al., 1996; Milev et al., 1994).

1.5.3.2 Nerve Cell Migration

In the early eighties L1 has been described to be involved in the migration of postmitotic granule cells in mice. L1 antibodies added to cerebellar explant cultures interfered with granule cell migration from the external granular layer (EGL) through the molecular layer (ML) into the internal granular layer (IGL; Lindner et al., 1983). Similar results were found in the chick cerebellum, where antibodies against Ng-CAM (chick L1) arrested granule cell migration (Chuong, 1990; Chuong et al., 1987; Grumet et al., 1984; Hoffman et al., 1986).
Migrating granule cells are closely associated with specific cerebellar astrocytes known as Bergman glia (Rakic, 1971). A hypothesized L1-mediated interaction with these glia (proposed by Grumet et al., 1984; Lindner et al., 1983) was, however, never proven (Stitt and Hatten, 1990). At the electron-microscopic level Persohn and co-workers (1987) did not detect L1 on glia or at contact sites between neurons and glia in the cerebellum. The inhibitory effect of L1 antibodies on migration of granule cells is thus unlikely to result from a disturbed granule cell - Bergmann glial interaction. Rather, L1 antibodies may interfere with the elongation and fasciculation of parallel fibers, the axons of granule cells, and may thereby disturb cell migration. Alternatively, L1 antibodies may disrupt interactions between migrating granule cells which often migrate in close association with each other (Altman, 1972). On the other hand, L1 is expressed at contact sites between retinal ganglion cell axons and glia in the mouse optic nerve (Bartsch et al., 1989). Similar results have been obtained in the developing olfactory bulb of mice (Miragall et al., 1988; 1989).

1.5.3.3 Outgrowth, Guidance, and Fasciculation of Axons

L1 plays also an important role in neurite outgrowth and axon fasciculation during the development of the nervous system (for review, see Walsh and Doherty, 1997). This notion has been confirmed recently by the finding that mutations in the L1 gene of humans or mice lead to hypoplasia and/or pathfinding errors of axon fiber tracts such as the corpus callosum or the corticospinal tract (Cohen et al., 1997; Dahme et al., 1997; Fransen et al., 1995; Wong et al., 1995b). Before these findings, several in vitro studies have demonstrated the importance of L1 for outgrowth and fasciculation of neurites. Fischer and colleagues (1986) reported reduced neurite outgrowth from cerebellar explant cultures when L1 antibodies were applied to the culture. In addition, when neurons from various brain regions or from dorsal root ganglia were cultured on cells expressing L1 such as Schwann cells, L1 antibodies reduced neurite extension (Bixby et al., 1988; Kleitman et al., 1988a; Kleitman et al., 1988b; Seilheimer and Schachner, 1988). These neurite outgrowth properties of L1 are mediated by homophilic binding (see chapter 1.5.3.1; Dahme et al., 1997; Lemmon et al., 1989). Nerve cells cultivated on L1 transfected astrocytes extended longer neurites and showed increased migratory activity when compared to nerve cells maintained on non-transfected astrocytes (Yazaki et al., 1996). CHL1, a member of the L1 family (Holm et al., 1996), is also involved in neurite outgrowth. CHL1- and L1-transfected cells enhanced neurite outgrowth of embryonic hippocampal neurons and small cerebellar neurons in vitro (for more details about CHL1 see chapters 1.5.6-9; Hillenbrand et al., 1999).
Similar results have been described in chick and lower vertebrates. When Ng-CAM antibodies were applied to chick retinal explants or to embryonic cortical neurons cultivated on Müller cells or immature astrocytes, neurite extension was significantly diminished (Drazba and Lemmon, 1990; Smith et al., 1990). Furthermore, antibodies against Ng-CAM inhibited neurite outgrowth on axonin-1 (Kuhn et al., 1991). However, it is proposed that axonin-1 requires a second binding partner like itself or β1 integrins to promote neurite elongation (Felsenfeld et al., 1994; Montgomery et al., 1996; Rader et al., 1993). Buchstaller and co-workers (Buchstaller et al., 1996) proposed a trans homophilic binding of Ng-CAM concomitant with a cis interaction with axonin-1 to promote neurite outgrowth. In addition, G4 (Ng-CAM) promoted neurite outgrowth of sympathetic neurons when used as a substrate and antibodies against G4 reduced neurite outgrowth (Chang et al., 1990; Chang et al., 1987). In lower vertebrates such as goldfish or zebrafish, E587 mediates outgrowth and fasciculation of axons extending from retinal ganglion cells or other types of nerve cells (Bastmeyer et al., 1995; Weiland et al., 1997).

Besides the neurite outgrowth properties, L1 is further implicated in axon fasciculation and guidance. When L1 antibodies are added to cerebellar explant cultures, neurites extend from these explants in a highly defasciculated manner (Fischer et al., 1986). Moreover, antibodies against Ng-CAM induced defasciculation of commissural neurites, whereas axonal pathfinding was not disrupted. A correct axonal crossing of the floor plate required an interaction between axonin-1 and Nr-CAM, a close relative of L1 (Stoeckli and Landmesser, 1995). Furthermore, antibodies against G4 (Ng-CAM) induced defasciculation of retinal axons and diminished fasciculation of DRG neurites (Chang et al., 1990; Chang et al., 1987; Rathjen et al., 1987b).

In the chick the neurite outgrowth property of L1 has also been assigned to the second Ig domain (same domain that is responsible for adhesion; Zhao and Siu, 1995). In mice, however, a specific site of L1 mediating neurite extension was not found. All Ig domains and FNIII repeats 1-2 increased outgrowth of cerebellar neurons, and outgrowth was best when the entire L1 molecule was used as a substrate (Appel et al., 1993). The alternatively spliced exon 27 (RSLE) in the cytoplasmic domain appears not to be involved in mediating neurite outgrowth (Takeda et al., 1996).
1.5.3.4 Myelination of Axons

Non-myelinating Schwann cells express L1 (Martini and Schachner, 1986; Mirsky et al., 1986; Nieke and Schachner, 1985). Myelinating Schwann cells also express L1, but down-regulate L1 after they have formed 1.5 loops around the axon (Martini and Schachner, 1986). This temporal pattern of L1 expression by Schwann cells, together with the fact that L1 mediates adhesion between nerve cells and Schwann cells, suggests a role of L1 for the initiation of myelination. This hypothesis was tested in a culture system in which Schwann cells normally myelinate the neurites of co-cultured DRG neurons. L1 antibodies interfered with the formation of contacts between Schwann cells and neurites and with the ensheathment of neurites (Seilheimer et al., 1989; Wood et al., 1990a; 1990b). As a consequence, Schwann cells failed to express myelin-specific proteins and to myelinate co-cultured nerve cells, strongly suggesting that L1 is critical for the initiation of myelination in the PNS.

1.5.3.5 Axonal Regeneration

This functional property of the protein suggests that L1 supports the elongation of axons during normal development. It also raises the possibility that L1 supports the regrowth of axons after injury of adult nervous tissue. The latter hypothesis is supported by several observations.

Axons in the PNS are able to regenerate after a lesion in all vertebrates. Successful regeneration depends on the presence of Schwann cells (Berry et al., 1988; Hall, 1986). A variety of trophic factors and growth promoting cell surface and extracellular matrix molecules are expressed by Schwann cells, which are considered to be essential for axonal regrowth. Among them is L1 which has been demonstrated to potently promote the growth of neurites over Schwann cell surfaces in vitro (Bixby et al., 1988; Kleitman et al., 1988b; Seilheimer and Schachner, 1988). It is of particular interest in this context that expression of L1 is up-regulated in the distal stump of lesioned peripheral nerves (Danillof et al., 1986; Martini and Schachner, 1988; Nieke and Schachner, 1985; Tacke and Martini, 1990), and that high levels of L1 are present at times of axonal regrowth. Regenerating axons are L1-positive and in contact with L1-positive Schwann cells (Martini and Schachner, 1988). L1 on the cell surface of Schwann cells may thus support axonal regrowth in the injured PNS.

The inability of the amniote CNS to regenerate injured axons may, in part, be related to the fact that CNS glial cells do not express L1. To test this hypothesis, transgenic mice were generated in which L1 is ectopically expressed by
astrocytes under regulatory sequences of the GFAP gene (Mohajeri et al., 1996). The transgene improved substrate properties of cultivated astrocytes and differentiated CNS tissue cryosections for growing neurites in vitro. Regrowth of injured retinal ganglion cell axons in vivo, however, was not improved in transgenic mice when compared to wild-type controls (Mohajeri et al., 1996). The GFAP-L1 transgenic mouse lines are described in more detail in chapter 1.5.4.

In contrast to the low regenerative capacity in the CNS of higher vertebrates, lower vertebrates such as fish or amphibians are able to regenerate injured axons in the CNS. Axonal regeneration correlates with an up-regulation of L1-related proteins by the injured nerve cells. Retinal ganglion cells, for instance, up-regulated expression of E587 or L1.1/L1.2 after an optic nerve crush in goldfish or zebrafish, respectively (Bernhardt et al., 1996; Vielmetter et al., 1991). Similarly, expression of L1.1 and L1.2 is up-regulated by regenerating nerve cells in the lesioned spinal cord of zebrafish (Becker et al., 1998). More interesting in the present context is the fact that axonal regeneration in the CNS of fish is accompanied by an expression of L1-like molecules by glial cells. Oligodendrocyte-like cells, for instance, have been isolated from lesioned optic nerve of goldfish (Vielmetter et al., 1991). These oligodendrocyte-like cells are E587-positive and support neurite elongation from rat or fish retinal ganglion cells, whereas neurite outgrowth from the same nerve cell types is inhibited by rat oligodendrocytes (Bastmeyer et al., 1993; Bastmeyer et al., 1991). Expression of L1-related proteins by glial cells has also been observed in the lesioned CNS of zebrafish. Both L1.1 and L1.2 mRNA are detectable in glial cells of the lesioned optic nerve, but not in the unlesioned control nerve of zebrafish (Bernhardt et al., 1996).

In summary, observations suggest a correlation between the ability of nervous tissue to regenerate axons and the expression of L1 and L1-related proteins by glial cells. L1 or L1-related proteins are expressed by glial cells in the regeneration-permissive PNS and CNS of fish. Glial cells in the mammalian CNS, in contrast, do not express L1 and axonal regeneration does not occur in this tissue.

1.5.3.6 Learning and Memory

Learning and memory are believed to require structural changes in the brain such as alteration in synaptic morphology or formation of new synaptic connections (reviewed in Wheal et al., 1998). Synaptic plasticity can take place
after a short high-frequency train of stimuli applied to the afferent fiber tracts (e.g., the perforant path) of the hippocampal formation (Bliss and Lomo, 1973). Such stimuli produce an increased excitatory synaptic potential which has been termed long-term potentiation (LTP; Bliss and Lomo, 1973). In vitro and in vivo studies suggest a role of cell adhesion molecules in synaptic plasticity (Edelman, 1986; Schachner, 1997; Wheal et al., 1998). Altered expression of cell recognition molecules can induce changes in synaptic efficacy and these alterations in turn may affect again expression of adhesion molecules (Wheal et al., 1998).

Evidence for an involvement of CAMs in synaptic plasticity has been presented for NCAM and L1 (Lüthi et al., 1994). Application of antibodies against L1 or application of L1 fragments impaired the initiation of LTP in the CA1 region of the hippocampus. Already established LTP, however, was not changed (Lüthi et al., 1994). L1 ectopically expressed in astrocytes interfered with the establishment of LTP after theta-burst stimulation (Lüthi et al., 1996). On the other hand, Wolter and colleagues (Wolter et al., 1998) reported an improved learning ability of transgenic mice expressing L1 ectopically in astrocytes (see also chapter 1.5.4). In agreement with this finding is the observation that a continuous intraventricular infusion of polyclonal antibodies against L1 in rats reduced spatial learning in the Morris water maze (MWM). The ability to use spatial cues required to find the platform in the MWM was clearly decreased in these animals (Arami et al., 1996). In addition, mice deficient in L1 showed learning deficits and an impaired exploratory behavior, but normal LTP (Fransen et al., 1998a). A more detailed description of mutant L1 mice is given in chapter 1.5.5.

1.5.3.7 L1 and the Human Disease CRASH

The functional importance of L1 for normal brain development is demonstrated by the fact that mutations in the L1 gene result in severe defects of the nervous system. This disease is known as CRASH syndrome (corpus callosum hypoplasia, mental retardation, adducted thumbs, spastic paraplegia, and hydrocephalus; Fransen et al., 1995). CRASH syndrome includes five diseases which were originally described as distinct clinical entities based on examination of patients and on results of necropsy: HSAS (hydrocephalus due to stenosis of aqueduct of Sylvius; Bickers and Adams, 1949; Willems et al., 1987), MASA (mental retardation, adducted thumbs, shuffling gait, aphasia; Bianchini and Lewis, 1974; Schrander-Stumpel et al., 1990; Winter et al., 1989), SPG1 (spastic paraplegia type 1; Kenrick et al., 1986), ACC (agenesis of the corpus callosum; Fransen et al., 1995; Kaplan, 1983) or DCC (dysgenesis of the corpus callosum; Fransen et al., 1995; Kaplan, 1983) or DCC (dysgenesis of the corpus callosum; Fransen et al., 1995; Kaplan, 1983)
callosum; Fransen et al., 1995; Kaplan, 1983), and MR-CT (mental retardation with clasped thumbs; Straussberg et al., 1991). So far, over 90 pathogenic mutations have been described in the human L1 gene (see Appendix), and most mutations were found in only one family.

X-linked hydrocephalus is a common inherited disease with a 2-7% occurrence rate in males (Halliday et al., 1986). The severity of this disease ranges from embryonic lethality or early postnatal death to long-term survival with moderate to mild mental retardation. Hydrocephalus is characterized in a variable degree of dilatation of the lateral ventricles, often resulting in increased intracranial pressure due to an excessive accumulation of the cerebrospinal fluid. Some patients with a progressive hydrocephalus show severe macrocephaly which require ventriculo-peritoneal (VP) shunting (Jouet et al., 1993; Schrander-Stumpel et al., 1995; Serville et al., 1992; Takechi et al., 1996; Willems et al., 1987; Yamasaki et al., 1997). Hydrocephalus is also often associated with the stenosis of the aqueduct of Sylvius, as indicated by the acronym HSAS (Bickers and Adams, 1949). However, a complete occlusion of the aqueduct of CRASH patients is extremely rare (Renier et al., 1982; Yamasaki et al., 1995) and usually results in embryonic death.

The most common symptom of CRASH syndrome includes mental retardation with variable severity and an IQ often below 50. In cases with severe hydrocephalus shunting does not improve mental function. Therefore, hydrocephalus may not be the cause for mental retardation in CRASH patients (Kamiguchi et al., 1998a). In addition to hydrocephalus or mental retardation, spastic paraplegia, hyper-reflexia, or shuffling gait has been noted in many patients suffering from CRASH. These symptoms are likely related to the abnormal development of the corticospinal tract (Yamasaki et al., 1995). Other malformations frequently associated with L1 mutations include adducted thumbs, agenesis or dysgenesis of the corpus callosum, fusion of thalami, and hypoplasia of the cerebellar vermis, all of which may result either from impaired outgrowth or fasciculation of axons or from a defective midline development and abnormal cell migration (Kamiguchi et al., 1998a).

In both mice and humans, the L1 gene has been mapped to the X-chromosome, more specifically to the Xq28 locus (Chapman et al., 1990; Djabali et al., 1990). Wong and co-workers (1995b) classified L1 mutations into three categories. Class 1 is ascribed to mutations in the intracellular domain of L1, class 2 is assigned to point mutations in the extracellular domain of L1, and class 3 mutations result in truncations of the extracellular domain of the L1 protein.
These three different classes of mutations correlate with the severity of the disease, with the most severe phenotypes falling into class 3 (Fig. 4; Yamasaki et al., 1997).

**Figure 4:** Correlation of the different classes of L1 mutations with the severity of the disease (clinical signs). The percentage of patients with varying degrees of survival, hydrocephalus, mental retardation, and presence of adducted thumbs in each class of L1 mutations is indicated (Yamasaki et al., 1997).

Mutations in the cytoplasmic domain (class 1) include missense mutations, nonsense mutations, frameshifts, duplications, and deletions. These mutations may result in a failure or alteration of signal transduction and/or binding of L1 to the cytoskeleton. However, adhesion may not be affected, since deletion of most of the intracellular part of L1 does not change the adhesive properties of L1 (Wong et al., 1995a). Class 1 patients show relatively mild symptoms including modest mental retardation and only a slightly enlarged or normal ventricular system. All patients survive the first year of life, and over 90% of the patients develop adducted thumbs (Fig. 4; Yamasaki et al., 1997).
Class 2 mutations, representing missense mutations in the extracellular part of L1, may affect homophilic or heterophilic binding depending on the location of the mutation (for a description of various binding sites of L1 see chapter 1.5.3.1). A detailed analysis of 22 missense mutations that cause neurological diseases has in fact revealed that these mutations are not randomly distributed. Instead, they cluster in regions in which they cause conformational changes or alterations of the surface properties of the L1 protein (Bateman et al., 1996). These mutations, therefore, likely affect homophilic or heterophilic interactions of the L1 protein. Such mutations may also disrupt an interaction in the cis-conformation which is needed for neurite outgrowth or may prevent conformational changes in the L1 protein and therefore, abolish intracellular signal transduction (Kamiguchi et al., 1998a; Yamasaki et al., 1997). Class 2 mutations cause an intermediate severity of CRASH syndrome, and often result in spasticity associated with abnormal corticospinal tract development. Patients develop severe hydrocephalus with a 50% possibility and grave mental retardation with a 30% possibility. About 30% of class 2 patients do not survive the first year of life and about 85% are born with adducted thumbs (Fig. 4; Yamasaki et al., 1997).

Class 3 mutations result in premature stop codons in the extracellular domain and lead to a complete loss of L1 functions such as L1-mediated adhesion, as well as loss of the intracellular signaling pathways and cytoskeletal binding, thus resulting in the most severe phenotypes (Yamasaki et al., 1997). Moreover, class 3 mutations may result in the production of a secreted form of L1 with negative consequences for the developing nervous tissue. The phenotype of class 3 mutations is characterized by severe hydrocephalus and serious mental retardation. Only about 50% of such patients survive the first year of life and all are described with adducted thumbs (Fig. 4; Yamasaki et al., 1997).

Recently, a fourth class of mutations has been added (Fransen et al., 1998b). This fourth class includes mutations in highly conserved sequences of the extracellular part including intron-exon boundary sequences, branch point signals, or XGGG sequences in the 5' end of an intron. These mutations may result in aberrant splicing of pre-mRNA. However, their effect on the protein and thus on the phenotype is not predictable (Fransen et al., 1998b).

1.5.4 The GFAP-L1 Transgenic Mouse

Regeneration of injured axons does not occur in the central nervous system of higher vertebrates. Lack of axonal regeneration in the CNS of amniotes has been
attributed to the presence of inhibitory molecules which prevent axonal regrowth (reviewed by Schwab and Bartholdi, 1996), but may also be related to low levels of molecules which promote axon growth. To test this hypothesis, transgenic mice were generated in which L1 is ectopically expressed in astrocytes (Mohajeri et al., 1996). A 4.05 kb mouse L1 cDNA containing the entire coding sequence of L1, was inserted into the exon 1 of a modified GFAP gene (total length: 14.5 kb). The L1 transgene is thus expressed under the control of the regulatory sequences of the glial fibrillary acidic protein (GFAP) gene. Three mouse lines with different levels of transgene expression were generated, namely GFAP-L1 3418, 3426, and 3427. The strongest expression of the L1 transgene was found in line 3426, intermediate levels in line 3427 and lowest levels in line 3418. Analysis of brains from transgenic mice of the line 3426 by Northern and Western blot showed a 34% increase in L1 mRNA and a 40% increase in L1 protein when compared to wild-type animals (Mohajeri et al., 1996).

In the primary visual system of wild-type mice, only retinal ganglion cells, horizontal cells and amacrine cells express L1 (Bartsch et al., 1989; Wintergerst et al., 1993). The expression of transgenic L1 has been shown to be localized in astrocytes of optic nerves at the mRNA and protein level (Mohajeri et al., 1996) similar to the expression of GFAP (Eng, 1985). In contrast, wild-type mice do not express L1 in glial cells. The intensity of the L1 mRNA staining was slightly increased in the non-myelinated, proximal part (i.e., lamina cribrosa) when compared with the myelinated, distal part of the optic nerve. Similar regional differences in the intensity of expression levels have been found for the GFAP mRNA (Mohajeri et al., 1996). The weak expression level of transgenic L1 was suggested to be due to the short 5' flanking sequence (2 kb) of the GFAP gene (Mohajeri et al., 1996). It was demonstrated by in vitro studies that the region 2-6 kb upstream to the transcriptional start site of the GFAP gene is involved in elevating the expression of GFAP-driven fusion genes (Sarid, 1991). Cultures of astrocytes prepared from 6-day-old transgenic mice revealed an expression of L1 on the cell surface. Cultivated astrocytes of wild-type animals, in contrast, did not express L1 (Mohajeri et al., 1996).

1.5.4.1 Learning and Memory

Different antibodies against L1 and recombinant L1 fragments reduced LTP and it was, therefore, suggested that L1 is involved in the modulation of the development or stabilization of LTP (Lüthi et al., 1994). Lüthi and co-workers (1996) have further found that the basal synaptic transmission was not changed
in GFAP-L1 transgenic mice when compared to wild-type mice. Furthermore, no differences were described in the resting membrane potential, the input resistance, or the stimulation current required to evoke baseline EPSPs nor in paired-pulse facilitation of LTP between GFAP-L1 transgenic and wild-type animals (Lüthi et al., 1996). However, LTP induced by theta-burst stimulation was decreased in transgenic mice. Similar results were also found with pairing-induced LTP (Lüthi et al., 1996). It has been suggested that ectopic expression of L1 in astrocytes close to synapses may affect L1-mediated signal transduction and thus may lead to reduced LTP. Ectopically expressed L1 in astrocytes may further contribute to immobilise L1 on neighboring synapses and may thus prevent homophilic binding between neurons. Such a reduced homophilic binding capability may, therefore, prevent LTP-induced morphological changes of the synapse (Lüthi et al., 1996).

Although LTP has been shown to be reduced in GFAP-L1 mice, spatial learning in the Morris water maze (Morris, 1984) appears to be improved in L1 transgenic animals (Wolfer et al., 1998). Transgenic mice are more flexible and more selective in searching a hidden platform in the water maze than wild-type animals. When the mutant mice were placed close to the platform, they tended to stay in this region. When they were placed more distantly to the platform, mutant mice changed their swimming behavior to a straighter, more directed style. These results imply that GFAP-L1 mice have an increased behavioral flexibility which enables them to adapt faster to a new situation. However, L1-overexpressing mice showed a reduced spatial retention, when the platform was moved to another quadrant (Wolfer et al., 1998). Compared to controls, they spent less time in the quadrant, where the platform was previously located. The latter finding may be in line with reduced LTP in transgenic mice (Lüthi et al., 1996).

1.5.4.2 Neurite Outgrowth and Axonal Regeneration

In vitro and in vivo experiments were performed to analyze whether transgenic L1 improves the substrate properties of astrocytes and CNS cryosections in vitro and allows axonal regeneration in the CNS in vivo.

Mouse small cerebellar neurons extended significantly longer neurites on astrocyte monolayers from transgenic mice than on astrocyte monolayers from wild-type mice. Neurite elongation on transgenic astrocytes was significantly reduced when astrocytes were pre-incubated with anti-L1 antibodies. Similarly, neurite outgrowth from chick DRG neurons was significantly increased on
transgenic astrocytes when compared to wild-type astrocytes. Neurite outgrowth was again reduced by antibodies to mouse L1. The latter experiments unequivocally demonstrates that transgenic L1 improves the substrate properties of astrocytes, since the antibodies were raised against mouse L1 and thus recognize L1 on astrocytic cell surfaces, but not L1 expressed by chick DRG neurons (Mohajeri et al., 1996).

Neurite outgrowth from small cerebellar neurons was also analyzed on cryosections prepared from unlesioned optic nerves of wild-type and transgenic mice. Neurite outgrowth was significantly increased on cryosections of transgenic nerves when compared to wild-type nerves. Interestingly, increase in neurite outgrowth was proportional to the levels of transgene expression in the different mouse lines (3418 < 3427 < 3426). Neurite length on unlesioned nerves from line 3426 was about 3-fold increased when compared to neurite length on unlesioned nerves of wild-type mice.

An optic nerve crush induces reactive astrogliosis which is characterized, for instance, by increased expression of GFAP. Lesioning the optic nerve of transgenic mice, therefore, increases expression of the L1 transgene by astrocytes, since it is expressed under the regulatory sequence of the GFAP gene. Levels of L1 protein in lesioned wild-type nerves, in contrast, are reduced due to degeneration of unmyelinated L1 positive ganglion cell axons.

Neurite outgrowth on cryosections from lesioned wild-type mice was similar to that observed on unlesioned wild-type nerves. Neurite outgrowth on cryosections from lesioned transgenic nerves was, however, significantly increased when compared to neurite outgrowth on unlesioned transgenic nerves. Pre-incubation of cryosections from unlesioned or lesioned nerves of the transgenic mouse line 3426 with L1 antibodies reduced neurite outgrowth by about 50%, indicating that the improved substrate properties are related to transgenic L1 (Mohajeri et al., 1996).

Based on these positive in vitro observations, the optic nerve of wild-type and transgenic mice was crushed, and regrowth of ganglion cell axons was studied in vivo. Regrowth of injured retinal ganglion cell axons was not significantly improved in transgenic mice when compared to wild-type mice. The length of ganglion cell axons distal to the lesioned site two weeks after a lesion was $348 \pm 53 \mu m$ for wild-type and $443 \pm 44 \mu m$ for transgenic mice (Mohajeri et al., 1996).
1.5.5 The L1 Knock-Out Mouse

To establish animal models for the human disease CRASH (chapter 1.5.3.8), mouse lines were generated that lack expression of L1. Up to date two different research groups reported mouse mutants with a targeted null mutation in the L1 gene (Cohen et al., 1997; Dahme et al., 1997). For an easier discrimination of the two L1 mutant mouse lines, mice generated by Dahme et al. (1997) are denoted here as mutants, since L1 expression is not completely abolished, whereas mice generated by Cohen et al. (1997) are denoted as KO (for knock out) mice, because no L1 expression was detectable in these animals.

In the mouse model generated by Dahme et al. (1997) exon 8 of the L1 gene was interrupted by inserting a neomycin/thymidine kinase cassette. The founder animals belonged to the 129/SvEv strain, but were later backcrossed with C57BL/6J. Western blot analysis of whole brain proteins with poly- and monoclonal antibodies gave a single, weak band of 190 kDa in the mutant as compared to wild-type mice with bands of 200, 180, 140, and 80 kDa (Dahme et al., 1997). Northern blot analysis of brains from mutant mice revealed a band with a slightly increased mobility and with a 90% reduced intensity when compared to wild-type mice (Dahme et al., 1997).

The KO mouse line of Cohen and co-workers (1997) was generated by inserting a neomycin/thymidine kinase cassette into exons 13 and 14 which encode the sixth Ig domain of the L1 gene. These mice were bred in a 129/SvEv background. Protein analysis of whole brain lysates revealed a complete loss of L1 demonstrating that the mutation resulted in a null mutation (Cohen et al., 1997).

1.5.5.1 In Vitro Studies

L1 is a neurite outgrowth promoting molecule as has been demonstrated for different types of neurons on L1 substrates (Appel et al., 1993; Fischer et al., 1986; Lemmon et al., 1989; Williams et al., 1994). Evidence has been presented that L1 performs this function by homophilic interactions (Lemmon et al., 1989). Therefore, small cerebellar neurons of the mutant generated by Dahme et al. (1997) and of wild-type mice were cultured on poly-γ-lysine (PLL), PLL and laminin, or PLL and L1. PLL alone did not induce neurite extension from neurons of either genotype. When cultured on laminin, neurite length was similar for neurons isolated from mutants and wild-type mice. However, when neurons were grown on L1, neurite length of wild-type cells was significantly
increased when compared with control substrates, whereas neurons of mutants did not extend neurites (Dahme et al., 1997).

Similar results were reported for dorsal root ganglion neurons isolated from KO or wild-type mice (Fransen et al., 1998a). L1-Fc substrates induced neurite outgrowth from wild-type neurons, but did not induce neurite elongation from KO neurons. Laminin or control substrates (PLL and anti-Fc antibodies) showed no differences in neurite elongation between nerve cells from both genotypes.

The results of both groups demonstrate that DRG KO neurons or small cerebellar mutant neurons maintain the ability to extend neurites in a L1 independent fashion. However, results clearly demonstrate that L1-mediated neurite outgrowth requires homophilic binding of L1 on the neurite and L1 in the substrate, which is consistent with results previously presented by Lemmon and co-workers (1989) using functionally perturbing antibodies.

Antibody perturbation experiments have demonstrated that L1 mediates fasciculation of neurites (e.g., Fischer et al., 1986). Cerebellar microexplants from wild-type mice and L1 mutants were, therefore, cultivated on a PLL substrate, and fasciculation of neurons was assayed. Neurites extending from cerebellar explants of wild-type mice formed thick bundles of processes. Neurites extending from cerebellar explants of mutant mice, in contrast, were highly defasciculated, and many processes appeared to grow as individual fibers (Dahme et al., 1997).

1.5.5.2 Behavior of the Mutant

Analysis of both mouse lines revealed a very typical phenotype. They were smaller in size, less viable, and showed a weakness of their hindlimbs. Their eyes appeared smaller and lacrimous and were sunken into the eye sockets (Cohen et al., 1997; Dahme et al., 1997). Mutants showed a decreased sensitivity to touch and pain and a delayed motor response in the hot plate paradigm (Dahme et al., 1997; Mah et al., 1980). Since mice homozygous for the mutation were usually sterile, heterozygous females were bred with wild-type males to obtain mutant males (Cohen et al., 1997; Dahme et al., 1997). In contrast to the mutants, KO mice had no difficulties in a wire suspension test in a time frame of 60 s (Dahme et al., 1997; Fransen et al., 1998a). However, in the rotarod test KO mice failed at least once during the first five attempts to stay on a rotating rod, whereas wild-type mice stayed on the rod tube during all five trials. KO mice displayed hypoactivity in the cage and the open field, and social exploration
tests revealed a reduced exploratory activity when compared to wild-type littermates (Fransen et al., 1998a).

Additional tests for memory and cognitive functions were performed. KO mice were able to learn avoidance tasks similarly well as wild-type mice. However, KO mice showed deficits in the Morris water maze (MWM) test. These mice were able to find the platform, but needed more time than wild-type mice. KO mice further demonstrated reduced spatial retention in that they spent less time in the quadrant where the platform was initially located. In addition, these mice spent more time along the wall of the pool than wild-type littermates after habituation (Fransen et al., 1998a).

1.5.5.3 Morphology of the Nervous System

Morphological analysis of both L1 mutants revealed significantly enlarged ventricles which were lined by morphologically normal appearing ependymal cells (Dahme, 1998; Dahme et al., 1997; Fransen et al., 1998a). Furthermore, corticospinal tracts were reduced in size by approximately 40% in both mutants. Corticospinal tract fibers projected normally to the caudal end of the medulla, but only rarely crossed to the contralateral side at the pyramidal decussation (Cohen et al., 1997), demonstrating a role of L1 in axonal pathfinding.

Analysis of cerebella of adult mutant mice revealed no evidence for an impaired migration of granule cells (i.e., presence of ectopic granule cells; Dahme et al., 1997), although L1 has been demonstrated to mediate granule cell migration in vitro (Lindner et al., 1983). Also, no change was found in the location and number of basket and stellate cells in the molecular layer and of Golgi cells in the internal granular layer (Dahme et al., 1997). At the ultrastructural level, fasciculation of parallel fibers in the molecular layer appeared normal in the mutant when compared to wild-type animals. Similar to the cerebellum, preliminary inspection of the hippocampus revealed no histological abnormalities. In the PNS, myelination of axons was not affected in the mutant. However, non-myelinating Schwann cells developed abnormal processes that did not ensheath small caliber axons, but instead protruded into the endoneurium, resulting in incomplete separation of axons (Dahme et al., 1997).

Analysis of fiber tracts in adult KO mice demonstrated normal appearance of the corpus callosum, of the descending corticofugal projection to the thalamus and internal capsule, and of the retinal ganglion cell axons at the level of the optic chiasm (Cohen et al., 1997). High resolution magnetic resonance imaging
Introduction

(MRI) of KO mouse brains demonstrated a reduction in total brain volume and in cerebellar vermis size, an abnormal shape and increased volume of the fourth ventricle, dilatation of the lateral ventricles (but not of the third ventricles), and a different shape, but no changes in volume and no stenosis of the Aqueduct of Sylvius (Fransen et al., 1998a).

1.5.6 Close Homolog of L1 (CHL1)

Cell recognition molecules mediate cell-matrix and cell-cell interactions in the developing, adult, and lesioned nervous system. CHL1 (a close homolog of L1) is a recently identified member of the L1 family of cell recognition molecules. The molecule comprises an N-terminal signal sequence, six immunoglobulin (Ig)-like domains, 4.5 fibronectin type III (FNIII)-like domains, a transmembrane domain, and a short C-terminal intracellular domain (Fig. 5; Holm et al., 1996).

Figure 5: Structure of CHL1. The CHL1 molecule consists of six immunoglobulin domains (numbered I - VI) and four complete fibronectin type III repeats (FN1-4) next to the N-terminal. FN5 comprises a half FNIII repeat. The transmembrane region is indicated by the green box and is followed by a short intracellular domain.

Immunoblot analysis using polyclonal CHL1 antibodies revealed three different bands of 185, 165, and 125 kDa in insoluble and soluble fractions of brain membranes. The 185 kDa form represents the membrane-bound form of CHL1, whereas the 165 and 125 kDa forms represent proteolytically cleaved, soluble fragments of CHL1 (Holm et al., 1996; Hillenbrand et al., 1999).

The second Ig-like domain of CHL1 contains an RGD sequence (Holm et al., 1996) that contributes to the binding of integrins (Ruoslahti and Pierschbacher, 1987). This RGD sequence is also found in other members of the L1 family. A DGEA sequence is found in the sixth Ig-like domain of CHL1 (Holm et al., 1996).
This sequence is involved in αβ, integrin recognition of type I collagen (Staatz et al., 1991).

Glycosylation analysis revealed that CHL1 contains N-glycosidally linked carbohydrates which account for about 20% of the entire molecular weight of CHL1 (Hillenbrand et al., 1999; Holm et al., 1996). Several neural cell recognition molecules carry an HNK-1 carbohydrate which is involved in cell adhesion and in binding to laminin (Hall et al., 1997; Künemund et al., 1988; Martini, 1994; Martini and Schachner, 1986; Martini et al., 1992; Nieke and Schachner, 1985; Nolte and Martini, 1992; Poltorak et al., 1989; Rathjen et al., 1987b; Sanes et al., 1986). Western blot analysis showed that CHL1 also carries the HNK-1 epitope.

Structural analysis revealed that the extracellular part of CHL1 is most closely related to chicken Ng-CAM (see Table 4). The intracellular part of CHL1 is most similar to L1, Nr-CAM, and neurofascin (see Table 5). Tables 4 and 5 summarize a sequence comparison of CHL1 with the different cell adhesion molecules (Holm et al., 1996).

**Table 4: Sequence comparison of the extracellular regions of L1 related molecules**

<table>
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<tr>
<th></th>
<th>CHL1</th>
<th>L1 (m)</th>
<th>Ng-CAM (c)</th>
<th>Nr-CAM (c)</th>
<th>Neurofascin (c)</th>
<th>L1.1 (zf)</th>
<th>Neurogian (d)</th>
<th>F3 (m)</th>
<th>TAG-1 (r)</th>
<th>BIG-1 (r)</th>
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The values indicate the percentage of amino acid identity after alignment of the extracellular parts of members of the L1 family and other cell recognition molecules of the Ig superfamily. The animal species in brackets are the following: c: chicken, d: Drosophila, h: human, m: mouse, r: rat, zf: zebrafish (Table from Holm et al., 1996).

The most conserved regions of members of the L1 family (L1, CHL1, neurofascin, Nr-CAM) are present in the intracellular domain of these molecules. This aspect is interesting in that the cytoplasmic domain of L1 members can interact with cytoskeletal proteins via ankyrin (Davis and Bennett, 1994; Davis et al., 1993). High conservation of the cytoplasmic domain further
suggests that members of the L1 family may use similar signal transduction pathways to exert their biological function.

Table 5: Sequence comparison of the intracellular regions of L1 related molecules

<table>
<thead>
<tr>
<th></th>
<th>CHL1 (m)</th>
<th>L1 (m,r,h)</th>
<th>Nr-CAM (m)</th>
<th>Nr-CAM (c)</th>
<th>Ng-CAM (c)</th>
<th>ABGP (r)</th>
<th>Neurofascin (m)</th>
<th>Neurofascin (c)</th>
<th>Neuroglial (d)</th>
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<td>7</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>13</td>
</tr>
<tr>
<td>PO (m)</td>
<td>11</td>
<td>6</td>
<td>12</td>
<td>13</td>
<td>6</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>28</td>
</tr>
</tbody>
</table>

The values indicate the percentage of amino acid identity after alignment of the intracellular parts of members of the L1 family and other cell recognition molecules of the Ig superfamily. The animal species in brackets are the following: c: chicken, d: Drosophila, h: human, m: mouse, r: rat, zf: zebrafish. Mouse Nr-CAM and neurofascin are partial sequences of 91 and 86 amino acid residues, respectively (Table from Holm et al., 1996).

1.5.7 Expression of CHL1

CHL1 is expressed early during development of the nervous system. In lysates of mouse forebrain, CHL1 protein becomes detectable at embryonic day (ED) 13, is strongest between ED 18 and postnatal day (PD) 7, and declines afterwards with increasing age of the animal (Hillenbrand et al., 1999). This finding is in contrast to the developmental regulation of L1 expression which does not decrease as dramatically with increasing age. Besides the expression of CHL1 in the CNS, all three isoforms of CHL1 are found in the PNS in lysates of the femoral and sciatic nerves of adult mice. Further, CHL1 has been detected on cell surfaces of cultivated neurons isolated from various regions of the nervous system such as hippocampus, cortex, mesencephalon, spinal cord, and dorsal root ganglia. However, CHL1 could not be found in primary cultures of small cerebellar neurons prepared from early postnatal (6-7 day old) mouse brains. A characteristic feature of CHL1 in the CNS is its expression by glial cells. This is in marked contrast to the expression of L1 which is restricted to postmitotic
nerve cells. Table 6 displays a summary of CHL1 expression in different primary cultures and cell lines (Hillenbrand et al., 1999).

**Table 6: Expression of CHL1 in primary cultures and cell lines of neural origin**

<table>
<thead>
<tr>
<th>CELL TYPE</th>
<th>IMMUNOREACTIVITY FOR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CHL1</td>
</tr>
<tr>
<td><strong>primary culture</strong></td>
<td></td>
</tr>
<tr>
<td>hippocampal neurons*</td>
<td>++</td>
</tr>
<tr>
<td>cortical neurons</td>
<td>++</td>
</tr>
<tr>
<td>mesencephalic neurons</td>
<td>++</td>
</tr>
<tr>
<td>spinal cord neurons*</td>
<td>+</td>
</tr>
<tr>
<td>small cerebellar neurons</td>
<td>-</td>
</tr>
<tr>
<td>dorsal root ganglion neurons</td>
<td>++</td>
</tr>
<tr>
<td>oligodendrocyte progenitors (A2B5*)</td>
<td>+</td>
</tr>
<tr>
<td>oligodendrocyte precursors (O4+/O1*)</td>
<td>+</td>
</tr>
<tr>
<td>oligodendrocytes (MAG+)</td>
<td>-</td>
</tr>
<tr>
<td>astrocytes (GFAP+, A2B5+/GFAP+)</td>
<td>+</td>
</tr>
<tr>
<td>Schwann cells (S-100-)</td>
<td>++</td>
</tr>
<tr>
<td><strong>cell lines</strong></td>
<td></td>
</tr>
<tr>
<td>PC12 pheochromocytoma</td>
<td>-</td>
</tr>
<tr>
<td>PC12 pheochromocytoma (NGF-treated)</td>
<td>-</td>
</tr>
<tr>
<td>N2A neuroblastoma</td>
<td>-</td>
</tr>
<tr>
<td>AtT-20 pituitary tumor</td>
<td>+</td>
</tr>
<tr>
<td>C6 glioma</td>
<td>+</td>
</tr>
<tr>
<td>S-16 Schwann cells</td>
<td>+</td>
</tr>
</tbody>
</table>

Polyclonal antibodies against recombinant CHL1 were used to detect the molecule on live cells by indirect immunofluorescence, either in combination with monoclonal antibodies to cell-type specific marker molecules or in combination with monoclonal antibodies against L1. The level of expression is given by an arbitrary scale: +++ strong, ++ intermediate, + low, - no expression. *: only subpopulations of cells were CHL1-immunoreactive (Table from Hillenbrand et al., 1999).

Expression of CHL1 by cells of the oligodendrocyte cell lineage in vitro decreases with increasing maturity of the cells. About 50% of oligodendrocyte progenitor cells (A2B5+) express CHL1, whereas only about 5% of all oligodendrocyte precursor cells (O4+/O1+) with unbranched processes and none of the mature oligodendrocytes (O4+/O1+ or MAG+) express CHL1. In the PNS, only immature or non-myelinating Schwann cells express CHL1. Further, it has been found that in vivo CHL1 is often expressed only by subpopulations of specific cell types, as has been observed, for instance, for GFAP-positive astrocytes or Schwann cells. Astrocytes located in the most proximal unmyelinated part of the optic nerve, for instance, are CHL1-positive, whereas astrocytes located in the myelinated distal part of the nerve lack detectable levels of CHL1 expression (Hillenbrand et al., 1999; Holm et al., 1996).
The expression pattern of CHL1 mRNA in vivo differs significantly between 7-day-old and 3-week-old mice. Frontal sections of 7-day-old mouse forebrain show an intense staining of pyramidal cells of the hippocampus, but not of granule cells of the dentate gyrus. Different to the CA1 and CA3 regions, RNA expression is less prominent in the CA2 and CA4 regions. Interneurons of the hippocampus proper and of the hilus reveal also CHL1 RNA staining. In the cerebral cortex, expression of CHL1 mRNA is most evident in layer V. The thalamus shows strong expression of CHL1 in all nuclei except in the medial nucleus. No staining of CHL1 was found in the white matter tracts as for example the hippocampal fimbria and the internal capsule (Hillenbrand et al., 1999). Expression of CHL1 undergoes an overall down-regulation with maturation of the animals. After three weeks of age, expression of CHL1 is much less prominent in the cerebral cortex than in 7-day-old mice. In the hippocampus, expression in the CA1 region remains high, but decreases in CA3 and remains low in CA2 and CA4. In contrast, CHL1 staining of granule cells of the dentate gyrus increases with increasing age. In general, CHL1 is expressed by less neuronal cell types than L1, but many nerve cell types co-express both proteins (e.g., Holm et al., 1996).

These findings show that expression of CHL1 is highest during development of the nervous system and decreases with differentiation in most brain areas. Granule cells in the dentate gyrus are the only exception, showing an increase of CHL1 expression with increasing age. Table 7 summarizes CHL1 expression in 7-day-old and 3-week-old mice.

In situ hybridization analysis of PNS tissue revealed that CHL1 is expressed in femoral nerves of 13-day-old mice. The number of cells expressing CHL1 is similar to the number of cells expressing L1 (Hillenbrand et al., 1999). This finding indicates that CHL1 is expressed by non-myelinating Schwann cells, similar as has been shown for L1 (Martini and Schachner, 1986; 1988).
Table 7: Comparison of CHL1 expression between 7-day-old and 3-week-old mice

<table>
<thead>
<tr>
<th>Tissue</th>
<th>7-DAY-OLD MICE</th>
<th>3-WEEK-OLD MICE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebral cortex</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Hippocampus:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CA₁</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>CA₂</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CA₃</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>CA₄</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Dentate gyrus</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Thalamus:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole thalamus</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Medial nucleus</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>White matter tracts:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fimbria</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Internal capsule</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Subthalamic nuclei</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Nucleus lateralis habenulae</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

CHL1 expression was analyzed in vivo using in situ hybridization. The intensity of CHL1 in situ hybridization signals was arbitrarily categorized: ++ strong, + weak, - no CHL1 expression (after Hillenbrand et al., 1999).

1.5.8 Functional Characterization of CHL1

1.5.8.1 Heterophilic Adhesion

L1 mediates adhesion or promotes neurite outgrowth by either homophilic or heterophilic interactions with other adhesion molecules, such as TAG-1/axonin-1 (Dahme et al., 1997; Kuhn et al., 1991; Lemmon et al., 1989). Since CHL1 is structurally related to L1 and other members of the L1 family, it was reasonable to assume that CHL1-dependent adhesion is also mediated by homophilic interactions. In aggregation tests with CHL1 transfected mouse L929 fibroblast cells (L cells) or Drosophila S2 cells, CHL1 was expressed as a membrane-bound molecule and was present as a soluble, smaller molecule in the culture medium (Hillenbrand et al., 1999). In contrast to L1, CHL1-transfected S2 cells did not aggregate. Also, the presence or absence of Ca²⁺ did not change the aggregation behavior of these cells. These observations indicate that CHL1 does not mediate cell-cell interactions via homophilic binding. Moreover, L1- and CHL1-transfected cells did not co-aggregate, indicating that CHL1 does not undergo heterophilic binding with L1 (Hillenbrand et al., 1999). With regard to the heterophilic binding, CHL1 seems to be similar to GPI-linked members of the immunoglobulin superfamily such as F3/F11/contactin, BIG-1/PANG, or BIG-2 (reviewed in Brümmendorf and Rathjen, 1994). Potential binding partners other than L1 have, however, not yet been tested.
1.5.8.2 Neurite Outgrowth

L1 and other members of the L1 family are strong promoters of neurite outgrowth (reviewed in Brümmendorf and Rathjen, 1994). CHL1 has also been identified as a potential promoter of neurite outgrowth in vitro. Neurite outgrowth assays were performed with hippocampal neurons of embryonic rats and small cerebellar neurons of early postnatal mice that were grown on a monolayer of CHL1- or L1-transfected L cells. Remarkably, neurite outgrowth from hippocampal neurons on CHL1-transfected cells was strikingly stronger than on L1-transfected cells. In the case of CHL1-transfected cells, neurite length of hippocampal neurons increased by approximately 60% compared to parental L-cells. On L1-transfected L-cells, neurite outgrowth was increased by 20% compared to non-transfected L-cells. Polyclonal antibodies against CHL1 completely blocked increased neurite outgrowth on transfected cells (Hillenbrand et al., 1999). Similar results were found for small cerebellar neurons grown on CHL1- and L1-transfected cells. The fact that small cerebellar neurons do not express CHL1 in vitro supports the view that CHL1 mediates neurite outgrowth by heterophilic rather than by homophilic interactions. In addition, CHL1 becomes easily proteolytically cleaved. Neurite outgrowth assays have shown that not only the membrane-bound form, but also the soluble form of CHL1 is a strong promoter for neurite outgrowth. Thus, CHL1 mediates neurite outgrowth as a substrate-bound and as a soluble molecule (Hillenbrand et al., 1999).

1.5.9 The CHL1 Knock-out Mouse

A mutant mouse deficient in CHL1 has been generated to study the functional role(s) of CHL1 in vivo. Since CHL1 is strongly expressed in the developing nervous system, and since the molecule has a potent neurite outgrowth promoting activity, a severe phenotype of mutant mice was expected. However, preliminary morphological analysis of the central nervous system of CHL1 mutants did not reveal any major defects (Dirk Montag, Monique Sallaz, and Melitta Schachner, unpublished observations). A more profound analysis of the CHL1 knock-out mouse is needed to eventually reveal some subtle morphological changes in the nervous system.

It is also tempting to speculate that the presence of other molecules which perform similar functions as CHL1 compensate, at least partially, for the lack of CHL1 in the mutant. Members of the L1 family are of particular interest in this context, and a mutant deficient in L1 and CHL1 has been generated to test this
hypothesis. Finally, biological functions of CHL1 \textit{in vivo} may become apparent in experimentally manipulated neural tissue, for instance after lesioning the CNS (see chapter 3.2).
2. **Aim of the Project**

The aim of this study was to obtain insights into the functions of two members of the L1 family, L1 and CHL1, in the developing, adult, diseased, and lesioned CNS. The neural adhesion molecule L1 is a potent promoter of neurite outgrowth and mediates cell adhesion, cell migration, axon fasciculation, and axonal pathfinding. Injured axons in the PNS of all vertebrates and in most regions of the CNS of fish are able to regenerate. Glial cells in these tissues express L1 or L1-related proteins, and up-regulate these proteins in response to a lesion. Axonal regeneration does not occur in the CNS of mammals, and glial cells in the mammalian CNS do not express L1. A transgenic mouse was generated in which L1 is ectopically expressed by differentiated astrocytes. Regrowth of lesioned retinal ganglion cell axons was analyzed in this mutant after increasing levels of transgene expression by (1) cross-breeding separate transgenic mouse lines or (2) intravitreal application of bFGF.

CHL1 is a recently identified new member of the L1 family. Expression of CHL1 was analyzed in the developing CNS, in the lesioned optic nerve, and in the primary visual pathway after application of growth factors to obtain first insights into possible functions of this protein.

Mutations in the L1 gene which is located on the X-chromosome cause a severe disease in humans, termed CRASH. The clinical picture of affected males is complex and highly variable between families, but also within the same family. Surprisingly, heterozygous women are considered to be healthy. L1-deficient male mice were analyzed to investigate the variability of the phenotype. Heterozygous female mice were analyzed to explain the apparent lack of a phenotype of carrier females.
3. PROJECTS

Axonal regeneration was studied in the optic nerve of transgenic mice ectopically expressing L1 in astrocytes. Different lines of transgenic mice were cross-bred, and bFGF was applied to the retina to increase levels of transgene expression.

To obtain first insights into possible functions of CHL1, a recently identified new member of the L1 family, expression of CHL1 was studied in the developing brain, lesioned optic nerve, and in the retina after application of bFGF.

The variability of defects associated with L1 mutations was studied in L1-deficient male mice. Heterozygous L1 female mice were analyzed to explain the apparent lack of a phenotype of carrier females.
3.1 Analysis of Axonal Regeneration in Single and Double GFAP-L1 Transgenic Mice after Application of bFGF

3.1.1 Introduction

Regeneration of injured axons in adult mammals occurs in the PNS, but not in the CNS (Chemoff and Stocum, 1995; Filogamo and Vercelli, 1995; Jones and Redpath, 1998; Larner et al., 1995; Nicholls and Saunders, 1996; Schwab and Bartholdi, 1996; Windle, 1956). In contrast, lower vertebrates, e.g. fish or amphibia, are able to regenerate injured axons in many regions of the CNS (Chemoff and Stocum, 1995; Larner et al., 1995; Nona, 1995; von Bernhardi and Muller, 1995; Windle, 1956).

It is well established that injured nerve cells of the adult mammalian CNS are able to regrow their axons if a regeneration-permissive environment is provided. For instance, differentiated neurons re-extend lesioned axons over long distances through transplants of peripheral nerves (Aguayo et al., 1984; Cheng et al., 1996; David and Aguayo, 1985; Davies et al., 1997; Kobayashi et al., 1995; Lindvall et al., 1990; Richardson et al., 1982; Sievers et al., 1988; Villegas-Pérez et al., 1988). These results strongly suggest that the cellular and molecular environment of lesioned axons determines the extent of axonal regrowth. Lack of axonal regeneration in the adult mammalian CNS may be related, in part, to physical barriers for regrowing axons, such as the glial scar (discussed in detail by Schwab and Bartholdi, 1996), or to a low abundance of neurotrophic factors (e.g., Berry et al., 1996; Schnell et al., 1994). However, a large body of evidence indicates that the inability of axonal regeneration is mainly related to molecules which actively prevent axonal regrowth. This inhibitory activity may be associated with astrocytes or the extracellular matrix (e.g., Bähr et al., 1995; Laywell et al., 1992; McKeon et al., 1991; Shewan et al., 1995; Wintergerst et al., 1997). Clearly, potent inhibitory activity is associated with oligodendrocytes and CNS myelin. For instance, growing neurites strongly avoid differentiated oligodendrocytes and CNS myelin in vitro and react with growth cone collapse (e.g., Bandtlow et al., 1990; Vanselow et al., 1990). More importantly, delayed myelination has been demonstrated to result in significantly improved axonal regeneration and eventually functional recovery in vivo (e.g., Keirstead et al., 1992; Savio and Schwab, 1990). Part of the inhibitory activity of oligodendrocytes and myelin could be attributed to two antigenically related proteins with an apparent molecular weight of 35 kDa and 250 kDa, designated NI35/250. In fact, application of antibodies to these proteins resulted in improved axonal
regeneration in the adult mammalian CNS (for reviews, see Schwab, 1993; Schwab and Bartholdi, 1996).

The inability of the adult mammalian CNS to regenerate axons may also be related to the low abundance of molecules which support axonal regrowth. L1 is a potent promoter of axonal growth (see chapter 1.5; Bixby et al., 1988; Fischer et al., 1986; Kleitman et al., 1988a; Lemmon et al., 1989; Schachner, 1990; Seilheimer and Schachner, 1988). Interestingly, L1 is expressed by glial cells of the regeneration-competent PNS of mammals (Daniloff et al., 1986; Martini and Schachner, 1988; Nieke and Schachner, 1985). Moreover, L1-like molecules are also expressed by glial cells of the regeneration-competent CNS of fish (Bastmeyer et al., 1993; Bernhardt et al., 1996; Vielmetter et al., 1991). In contrast, L1 is not expressed by glial cells of the developing, adult, or lesioned CNS of mammals (Bartsch et al., 1989; Jucker et al., 1996; Mohajeri et al., 1996; Gschwend and Bartsch, unpublished observations).

Transgenic mice ectopically expressing L1 in astrocytes under regulatory sequences of the GFAP gene were generated to test whether growth promoting molecules on the cell surface of differentiated glial cells improve axonal regeneration in the mature CNS (Mohajeri et al., 1996). Expression of the L1 transgene by astrocytes was demonstrated both in vitro and in vivo. As expected for a transgene expressed under regulatory sequences of the GFAP gene, expression of transgenic L1 by astrocytes was significantly increased in the lesioned optic nerve. Importantly, in vitro experiments demonstrated increased neurite outgrowth from nerve cells cultivated on astrocyte monolayers or optic nerve cryosections prepared from transgenic mice in comparison to similar substrates prepared from wild-type littermates (Mohajeri et al., 1996). Regeneration of injured retinal ganglion cell axons in vivo, however, was not increased in transgenic mice when compared to age-matched wild-type mice (Mohajeri et al., 1996; see also chapter 1.5.4). A possible explanation for the latter observation is an expression level of the transgene which may be too low to support regrowth of injured axons in mutant mice.

Basic fibroblast growth factor (bFGF) is a growth factor with a variety of biological functions (for reviews, see Hicks, 1998; Logan and Berry, 1993). For instance, bFGF has been demonstrated to enhance survival of peripheral and central nervous system neurons in culture (e.g., Petroski et al., 1991; Unsicker et al., 1987) and to induce retinal regeneration in chick embryos (Park and Hollenberg, 1989). Two functional properties of bFGF are of particular interest in the context of the present study. Application of acidic or basic FGF to lesioned
optic nerves significantly reduced lesion-induced death of retinal ganglion cells in adult rats (Sievers et al., 1987). Moreover, application of exogeneous bFGF to CNS tissue induced a significant astrogliosis, accompanied by a massive up-regulation of GFAP expression (Eclancher et al., 1990; Lewis et al., 1992).

Application of bFGF to the eye of transgenic GFAP-L1 mice should enhance the survival of axotomized retinal ganglion cells and increase expression of GFAP, and thus of transgenic L1 in astrocytes. To analyze whether both effects of bFGF induce increased regrowth of axotomized retinal ganglion cell axons in transgenic mice, bFGF was injected into the eye and regrowth of ganglion cell axons was studied following an optic nerve crush. To further increase levels of transgene expression, experiments were additionally performed on double-mutants obtained by crossing GFAP-L1 transgenic mice from line 3426 and 3427.

3.1.2 Material and Methods

3.1.2.1 Breeding and Genotyping of GFAP-L1 Mice

Three lines of transgenic mice expressing L1 under regulatory sequences of the GFAP gene have been generated, with high (line 3426), intermediate (line 3427), and low (3418) levels of transgene expression (Mohajeri et al., 1996). Lines 3426 and 3427 were used in this study. Heterozygous transgenic mice were always back-crossed with C57BL/6J mice to obtain a homogenous genetic background. Further, to increase expression levels of transgenic L1, homozygous GFAP-L1 mice of line 3426 were mated with homozygous mice of line 3427. The resulting animals are termed double-mutants in this study.

Genotyping of GFAP-L1 transgenic and wild-type mice was performed by means of PCR analysis. Short tail pieces were incubated overnight in 400 µl tailcut buffer (0.5% SDS, 100mM NaCl, 50mM Tris-HCl (pH 7.5), 1mM EDTA) and 120 µg proteinase K (Boehringer Mannheim) at 56°C. The DNA was extracted with 1.5 M KAc and chloroform and precipitated with ethanol. The DNA was resolved in 350 µl H2O, of which 1 µl was used for PCR amplification. To identify transgenic mice, the following primers were used (Mohajeri et al., 1996): 1) 5' GCACCC TAT TCT GGC TCC TT 3' and 2) 5' ATG CTG TTC GTG GGC TTG AC 3'. PCR was performed with 35 cycles for 45 sec at 94°C, 60 sec at 53°C, and 30 sec at 72°C in PCR buffer with 2mM MgCl2, 200mM dNTPs, and 2.5 pM/µl of each primer. The presence of the wild-type L1 gene was monitored in the same reaction as a positive control using the following primers (Dahme, 1998): 1) 5' CAG TCA TTG ATC CTG GAG TGC 3' and 2) 5' GGT AGG CAG GAG ATA AGG TCA 3' at a concentration of 8 pM/µl.
3.1.2.2 Intravitreal Application of bFGF

Adult GFAP-L1 double-mutants (derived from lines 3426 and 3427) and GFAP-L1 transgenic mice of line 3426 with strongest expression of the transgene were used (Mohajeri et al., 1996). Wild-type littermates served as controls. Mice with an age of at least 2 months were deeply anesthetized by an i.p. injection of a 10% Nembutal solution (100 µl/10g body weight). About 1 µl of vitreous fluid was removed from the eye, and a similar volume of recombinant human bFGF (0.1 µg/µl; Pepro Tech EC Ltd., London) was injected into the vitreous cavity using a micropipette. The left, uninjected eye and eyes injected with a similar volume of a 0.9% solution of NaCl served as controls. The injection site was close to the edge of the retina. After 1, 3, and 7 days, animals were killed with a lethal dose of Nembutal. The eyes were excised, immediately embedded in OCT-medium, and frozen in 2-methylbutane cooled in liquid nitrogen. Tissue was used to analyze bFGF-induced changes in GFAP and L1 transgene expression at the mRNA and protein level.

3.1.2.3 In Situ Hybridization

Digoxigenin-labeled antisense cRNA probes of equal size corresponding to the extracellular parts of L1 and CHL1 and a GFAP probe were generated by in vitro transcription of plasmids (Bartsch et al., 1994; Holm et al., 1996). Sense probes were transcribed from similar constructs with the inserts in opposite direction. Probes were hydrolyzed to an average fragment length of 250 nucleotides and in situ hybridization was performed as described (Bartsch et al., 1992; Dörries and Schachner, 1994; Molthagen et al., 1996). Fresh-frozen cryosections of 14 µm thickness from mouse optic nerves with attached retinas were mounted onto 3-aminopropyltriethoxysilane- (Sigma) coated coverslips. Sections were immediately fixed in 4% paraformaldehyde (in PBS pH 7.3) for at least 30 min. or overnight. Then, sections were immersed for 5 min. each in 3x PBS, 1x EtOH (70%), 2x H₂O, 1x HCl (0.1 M), 2x PBS, 20 min. triethanolamine (pH 8.0; 0.1 M) containing 0.25% acetic anhydride, followed by 2x 5 min. in PBS. The tissue was then dehydrated in an ascending series of ethanol (70%, 80%, and 100%). Prehybridization was performed at 37°C for at least 4 hrs and hybridization followed overnight at 55°C. The prehybridization buffer contained 50% formamide, 2.5x Denhardt’s solution, 25mM EDTA, 50mM Tris-HCl (pH 7.6), 20mM NaCl, and 0.25 mg/ml tRNA (Boehringer-Mannheim). The hybridization buffer consisted of 50% formamide, 0.33M NaCl, 0.1M dithiothreitol, 10% dextran sulfate, 20mM Tris-HCl pH 7.5, 1mM EDTA, 1x Denhardt’s solution, 0.5 mg/ml tRNA (Boehringer-Mannheim), 0.1 mg/ml
polyA RNA (Sigma), and the corresponding antisense or sense cRNA probes. The sections were washed three times in 0.2x SSC (30mM NaCl, 3mM Na-citrate pH 7.4) at 55°C for 15 min., followed by three washes with 0.2x SSC and 50% formamide at 55°C for at least 60 min. each. The washes were continued at room temperature once with 0.2x SSC (10 min.), twice for 15 min. with P1-Dig (100mM Tris-HCl and 150mM NaCl pH 7.5), followed by incubation in modified blocking solution P2-Dig (1% Boehringer blocking reagent, 0.5% BSA fraction V [Sigma], in P1-Dig) for 30 min. The sections were incubated with alkaline phosphatase-conjugated antibodies to digoxigenin at a dilution of 1:350 in P2-Dig at 4°C overnight. The sections were then washed twice with P1-Dig for 15 min., followed by a two-minute pH-equilibration with P3-Dig (100mM Tris-HCl, 100mM NaCl, 50mM MgCl₂ pH 9.5), and developed in P3-Dig containing 0.35 mg/ml 4-nitroblue tetrazolium chloride (Sigma), 0.175 mg/ml 5-bromo-4-chloro-3-indolyolphosphate (Sigma), and 0.25 mg/ml levamisol (Sigma). The development of the color reaction was stopped by washing sections in P4-Dig (= TE; 10mM Tris-HCl and 1mM EDTA pH 8.0) and rinsing in H₂O. Finally, sections were air-dried and analyzed using an Axiophot microscope (Zeiss).

3.1.2.4 Immunocytochemistry

Cryosections of 14 µm thickness prepared from fresh-frozen mouse optic nerves with attached retinai were mounted onto poly-L-lysine coated coverslips and air-dried overnight. Sections were then immersed in PBS and 0.1% BSA fraction V (Sigma) for 30 min. Incubation of the sections with polyclonal L1 and GFAP antibodies (1:200) for 1 hr followed. The tissue was washed three times in PBS, incubated with a fluorescein isothiocyanate-conjugated secondary antibody (FITC, Dianova, 1:50) for 45 min., washed in PBS, and mounted.

3.1.2.5 Indirect Immunoelectron Microscopy

Eyes of adult GFAP-L1 double mutants, and heterozygous transgenic mice and wild-type littermates (at least 4 months old) were injected with bFGF or NaCl as described above. The animals were killed with a lethal dose of Nembutal and eyes with attached optic nerves were removed. The retinai and optic nerves were immersed in 4% paraformaldehyde and 0.25% glutaraldehyde in PBS overnight, and were then embedded in 6% agar (in PBS). Longitudinal sections of optic nerves with attached retinai, 200 µm in thickness, were washed in PBS for 2 hrs at room temperature on a shaker, followed by another 2 hrs in PBS containing 1% BSA fraction V (Sigma). The sections were incubated with polyclonal L1 antibodies (1:50) overnight at 4°C on a shaker. The incubation was followed by washing the sections four times for 15 min. each in PBS. Sections
were then incubated with the AB complex (Immuno Diffusion) for 90 min., washed four times for 15 min. each in PBS, preincubated with 0.03\% 3,3'-diaminobenzidine-4HCl (DAB, Sigma) in 0.04M Tris-HCl (pH 7.4) for 30 min., and incubated for 30-60 min. in the same DAB solution containing additionally 0.01\% H_2O_2 (all steps at room temperature on a shaker). The sections were washed in PBS overnight on a shaker. After postfixation with 1\% OsO_4 for 30 min., sections were dehydrated in 30\%, 50\%, 70\%, 90\%, and 2x 100\% aceton for 15 min. each, and embedded in Spurr. Ultrathin sections were analyzed with a Zeiss EM10.

3.1.2.6 Anterograde Tracing of Axons

Adult GFAP-L1 double-mutants, heterozygous GFAP-L1 mutants, and wild-type littermates were injected with bFGF into the right eye (described in chapter 3.1.2.2). Three days later, the right optic nerve was crushed for 15 sec using watchmaker's forceps about one millimeter behind the eye. After additional 13 days, N-hydroxysuccinimidobiotin (an anterograde axonal tracer) dissolved in DMSO and 100\% ethanol was injected into the right eye. The next day, animals were killed with a lethal dose of Nembutal. The eyes were fixed in 4\% paraformaldehyde in PBS (pH 7.4) for one hour and immersed in sucrose solutions with increasing concentrations (10\%, 20\%, and 30\%). After sucrose treatment, eyes were embedded in OCT-medium, and frozen. Cryosections of 14 \mu m thickness were mounted onto poly-L-lysine-coated coverslips and air-dried overnight. Sections were immersed in PBS containing 0.1\% BSA for 30 min., incubated with fluorescein-isothiocyanate-conjugated Avidin-D (1:300) for one hour, followed by three washes with PBS and mounting.

3.1.3 Results

3.1.3.1 GFAP and L1 mRNA and Protein Expression after bFGF Injection in vivo

Expression of detectable levels of GFAP mRNA is restricted to astrocytes located at the vitread margin of the retina of wild-type and transgenic mice (Fig. 6 a,d). Intravitreal application of NaCl did not change the expression pattern of GFAP transcripts in both genotypes (Fig. 6 b,e). The distribution of GFAP mRNA, however, changed after intravitreal application of bFGF in both transgenic and wild-type mice (compare Fig. 6 b,e with Fig. 6 c,f). Most remarkably, cells expressing GFAP transcripts became detectable in the inner nuclear layer after bFGF application, both in wild-type (Fig. 6c) and mutant (Fig. 6f) mice. Moreover, in these retinae, a weak and diffuse in situ hybridization
signal was apparent throughout the inner plexiform layer (Fig. 6 c,f). Finally, the intensity of GFAP transcript expression increased significantly at the vitread margin of bFGF-treated retinae of wild-type (Fig. 6c) and transgenic (Fig. 6f) mice when compared to control (Fig. 6 a,d) or NaCl-injected (Fig. 6 b,e) retinae of both genotypes. Thus application of bFGF resulted in increased expression of GFAP by retinal astrocytes and induced expression of GFAP transcripts in cells located in the inner nuclear layer, most probably corresponding to Müller cells. No differences in the distribution and intensity of GFAP mRNA expression could be detected between wild-type and transgenic mice after bFGF application (compare Fig. 6 c and f).

L1 transcripts in the primary visual system of adult mice are detectable in retinal ganglion cells and weakly in amacrine cells (Fig. 6g). Intravitreal application of NaCl did not change the cellular distribution or intensity of expression of L1 mRNA in wild-type retinae (Fig. 6h). Application of bFGF also did not change the distribution or intensity of expression of L1 transcripts in wild-type retinae (Fig. 6j) when compared to untreated control retinae (Fig. 6g) or NaCl-treated (Fig. 6h) retinae. The distribution and levels of L1 transcripts expressed in retinae of untreated control (Fig. 6k) or NaCl-treated (Fig. 6l) GFAP-L1 transgenic mice were not detectably different from those observed in retinae of wild-type mice (Fig. 6 g,h). However, in close analogy to what we observed for the expression of GFAP transcripts in bFGF-treated retinae, L1 transcripts became detectable in cells located in the inner nuclear layer of bFGF-treated transgenic retinae (Fig. 6m). Moreover, some intensively stained cells became apparent in the nerve fiber layer (Fig. 6m). These data suggest that expression of the L1 transgene which is expressed under the control of regulatory sequences of the GFAP gene is induced or up-regulated in Müller cells and retinal astrocytes in response to application of exogeneous bFGF. The cellular distribution of L1 transcripts and the intensity of L1 expression were not significantly different between bFGF-treated retinae of heterozygous transgenic mice and double mutants (not shown). Finally, intravitreal application of bFGF had no detectable effect on the expression of the L1 transgene by optic nerve glial cells (not shown). The different cellular response of retinal and optic nerve astrocytes to intravitreal application of bFGF is possibly related to an inability of the growth factor to penetrate into the nerve.
Figure 6: Expression of GFAP and L1 mRNA in normal control retinae and in retinae after application of NaCl or bFGF. Expression of GFAP mRNA was analyzed in retinae of wild-type (a-c) and L1-transgenic (d-f) mice without experimental treatment (a,d) or after intravitreal application of NaCl (b,e) or bFGF (c,f). A similar expression pattern of GFAP mRNA was detectable in control retinae (a,d) and NaCl-injected retinae (b,e) of wild-type (a,b) and transgenic (d,e) mice. Application of bFGF strongly up-regulated expression of GFAP mRNA and induced expression in the inner plexiform and nuclear layers of wild-type (c) and transgenic (f) animals when compared to the respective control (a,d) or NaCl-treated (b,e) retinae. Expression of L1 transcripts was analyzed in retinae of wild-type (g-j) and transgenic (k-m) animals without experimental treatment (g,k) or after intravitreal application of NaCl (h,l) or bFGF (j,m). L1 transcripts are expressed in a similar distribution and with a similar intensity in wild-type control retinae (g) and retinae exposed to NaCl (h) or bFGF (j), and in transgenic control retinae (k) and retinae exposed to NaCl (l). In contrast, significant up-regulation of L1 expression is visible in the inner nuclear layer of transgenic mice after intravitreal application of bFGF (m). Scale bars 250 μm.
Since exogeneous bFGF affected expression of GFAP transcripts in wild-type retinae, and expression of GFAP and L1 transcripts in transgenic retinae, expression of both molecules was additionally analyzed at the protein level (Fig. 7).

GFAP protein was detectable in retinal astrocytes located predominantly at the vitread margin of wild-type (Fig. 7a) and transgenic (Fig. 7g) retinae. A similar distribution of GFAP expression with a similar intensity of immunoreactivity was observed in retinae of both genotypes after application of NaCl (see Fig. 7 b and h). Thus, intravitreal application of a substance per se did not induce detectable astrogliosis. After application of bFGF, GFAP-immunoreactive radial processes became detectable spanning the entire width of wild-type (Fig. 6c) and transgenic (Fig. 6j) retinae.

There were no obvious differences in the distribution and intensity of L1-immunoreactivity between normal control and NaCl-injected wild-type (Fig. 7d and e) and transgenic (Fig. 7k and l) retinae. Similarly, expression of L1 protein in wild-type retinae was not detectably changed by intravitreal application of bFGF (Fig. 7f) when compared to control (Fig. 7d) or NaCl-treated (Fig. 7e) retinae. In all cases, prominent L1-immunoreactivity was observed in the nerve fiber layer and inner and outer plexiform layer (Fig 7 d-f, k, l). However, in bFGF-treated retinae of transgenic mice, L1-immunoreactivity was additionally associated with radial processes spanning the entire width of the retina, and was also detectable above the outer limiting membrane (Fig. 6m). Thus, immunohistochemical data confirm observations at the mRNA level: application of bFGF induced a more widespread and elevated expression of GFAP in wild-type retinae, and of GFAP and L1 in transgenic retinae, when compared to untreated retinae or NaCl-injected retinae.
Figure 7: GFAP and L1 protein in normal control retinae and in retinae after intravitreal NaCl and bFGF application. Expression of GFAP (a-c, g-j) and L1 (d-f, k-m) protein was analyzed in normal control (a,d,g,k) and NaCl- (b,e,h,l) and bFGF- (c,f,j,m) retinae of wild-type (a-f) and L1-transgenic (g-m) mice. A similar expression pattern of GFAP protein was found in control retinae (a,g) and NaCl-injected retinae (b,h) of wild-type (a,b) and transgenic mice (g,h). Intravitreal application of bFGF up-regulated GFAP protein at the vitread margin of the retina and induced expression in Müller cells of wild-type and transgenic mice (c,j). Expression of L1 protein was detectable in the nerve fiber layer and inner and outer plexiform layer of control retinae (d,k) and NaCl-injected retinae (e,l) of both wild-type (d,e) and transgenic mice (k,l), and in bFGF-injected retinae (f) of wild-type mice. In transgenic mice, intravitreal application of bFGF induced additional expression of L1 by Müller cells. 1: ganglion cell and nerve fiber layer; 2: inner plexiform layer; 3: inner nuclear layer; 4: outer plexiform layer; 5: outer nuclear layer. Scale bars 250 μm.
Immuno-electron microscopic analysis confirmed that application of bFGF induced expression of L1 protein in retinal glial cells of GFAP-L1 transgenic mice. Specifically, L1 protein was expressed on cell surfaces of Müller cell endfeet contacting the basal lamina (Fig. 8b), and on microvilli of Müller cells extending above the outer limiting membrane (Fig. 8d). A similar distribution of L1-immunoreactivity was detectable on Müller cells in retinae of double-mutant mice (data not shown). In wild-type littermates, no L1-immunoreactivity was detectable on cell surfaces of Müller cells, neither in untreated control retinae or NaCl-injected retinae (not shown), nor in bFGF-injected retinae (Fig. 8a,c). L1-immunoreactivity on the cell surface of retinal ganglion cell axons (Fig. 8a) located in direct vicinity to Müller cell endfeet served as a control for a successful immuno-staining of transgenic and wild-type tissues. These data unequivocally demonstrate expression of the L1 transgene by CNS glial cells, and provide evidence for the insertion of transgenic L1 protein into the cell membrane.
Figure 8: Ultrastructural location of L1 protein in wild-type and transgenic retinae after bFGF application. Ultrastructural location of L1 was studied at the vitread margin (a,b) and above the outer limiting membrane (c,d) of wild-type (a,c) and transgenic (b,d) retinae after intravitreal application of bFGF. Müller cell end feet at the vitread margin of the retina are L1-negative in wild-type animals (a), but strongly L1-positive in transgenic mice (some labeled with arrowheads in b). Similarly microvilli of Müller cells above the outer limiting membrane are L1-negative in wild-type mice (c), but strongly L1-immunoreactive in mutant mice (some labeled with arrowheads in d). L1-immunoreactivity associated with retinal ganglion cell axons (asterisks in a) served as an internal control for successful immunostaining. Scale bars 2 μm.
3.1.3.2 Analysis of Axonal Regeneration in the Optic Nerve of GFAP-L1 Mice

A transgenic mouse with an ectopic expression of L1 under regulatory sequences of the GFAP gene was generated to analyze whether growth-promoting molecules on the cell surface of glial cells support axonal regrowth in the adult mammalian CNS (Mohajeri et al., 1996). Here we have increased levels of transgene expression by (a) generating a double-mutant by cross-breeding transgenic lines 3426 and 3427 and (b) intravitreal application of bFGF which up-regulates GFAP and thus transgene expression.

The effect of increased levels of transgene expression on axonal regrowth was studied in the lesioned optic nerve. To this aim, basic FGF was injected into the vitreous of wild-type mice, heterozygous mutants, and double-mutant mice, the optic nerve was crushed intraorbitally three days later and retinal ganglion cell axons were anterogradely labeled 13 days after the lesion.

In some animals of each genotype, a few axons grew across the lesion site and extended into the distal stump of the optic nerve (for a wild-type, see Fig. 9a; for a heterozygous L1 transgenic mouse, see Fig. 9b). Retinal ganglion cell axons with a beaded and curved appearance (Fig. 9) were considered as axons that have succeeded to regrow into the distal stump. Optic nerves which contained straightly running axons in their distal stumps were classified as incompletely lesioned nerves, and the corresponding animals were excluded from the analysis.

The length of the longest regrown axon was measured distal to the lesion site in each animal to compare the efficacy of axonal regrowth between experimental groups (Fig. 10a). No obvious differences in the lengths of regrown axons were apparent between wild-type mice, heterozygous mutants, and double mutants (Fig. 10a). To further compare axonal regrowth between different genotypes, only those animals were considered that contained axons which had regrown for more than 0.5 mm into the distal nerve stump. Comparison of average values of different genotypes revealed that axonal regrowth was not significantly improved in heterozygous mutants or double mutants, when compared with wild-type mice (Fig. 10b).
Figure 9: Regrowth of retinal ganglion cell axons after bFGF application and injury. Regrowth of retinal ganglion cell axons was analyzed after bFGF injection into the eye and a subsequent optic nerve crush in wild-type (a) and GFAP-L1 transgenic (b) mice. Regrown axons were visualized with an anterograde tracer. Regrown axons appeared beaded and curved which is in contrast to the straight course taken by axons in unlesioned or incompletely lesioned optic nerves. Scale bars 200 μm.
Figure 10: Length of regrown axons after injury. The length of regrown retinal ganglion cell axons was determined in wild-type (wt), GFAP-L1 transgenic (het), and GFAP-L1 double-mutant (dm) mice after bFGF application and optic nerve crush. (a) In all genotypes, regrown axons extended for up to 1.6 mm across the lesion site into the distal part of the optic nerve. Regrown axons shorter than 0.2 mm were not measured. (b) For all three genotypes, only those axons were considered that extended at least 0.5 mm into the distal nerve stump. No significant differences are apparent between different experimental groups. Bars indicate mean values (± standard deviation; P>0.1). The number of animals analyzed for each experimental group is indicated on top of each bar. wt = wild-types; het = heterozygous L1 transgenic mice; dm = double mutants.

3.1.4 Discussion

Axons in the CNS of birds and mammals are unable to regenerate over long distances after an injury (Ramon y Cajal, 1928). However, these nerve cells do have the intrinsic capacity for axonal regrowth when a growth-permissive environment is provided, such as a PNS explant (see Aguayo, 1985 for a review). Lack of axonal regrowth in the CNS of adult amniotes has been attributed to inhibitory molecules expressed by oligodendrocytes and associated with myelin (see Schwab, 1993; Schwab and Bartholdi, 1996 for reviews), and to non-permissive or inhibitory substrate properties of differentiated and reactive...
aimed to experimentally induce axonal regrowth in the adult mammalian CNS thus include the neutralization of inhibitory molecules and the introduction of growth promoting molecules into CNS glial cells.

The neural adhesion molecule L1 is a potent promoter of axonal elongation (see chapters 1.5.2 and 1.5.3). L1 is expressed on the surface of neurons and glial cells in the regeneration-permissive PNS of mammals. Interestingly, expression of L1 by Schwann cells is up-regulated in lesioned peripheral nerves, and the protein has thus been hypothesized to support axonal regrowth (Daniloff et al., 1986; Martini and Schachner, 1988; Nieke and Schachner, 1985). CNS glial cells in the regeneration-permissive CNS of lower vertebrates have also been reported to express L1-like proteins (Bastmeyer et al., 1993; Becker et al., 1998; Bernhardt et al., 1996; Vielmetter et al., 1991). Glial cells in the non-regenerating CNS of mammals, in contrast, do not express L1 (e.g., Jucker et al., 1996; Mohajeri et al., 1996). While it is generally accepted that the cellular and molecular environment critically determines the regenerative capability, the nerve cell response may also play an important role. Regenerating nerve cells in the CNS of fish up-regulate or re-express the growth promoting proteins GAP-43, the E587 antigen, NCAM, neurolin, L1.1, and L1.2 (Bastmeyer et al., 1990; Bernhardt et al., 1996; Paschke et al., 1992; Skene, 1989; Vielmetter et al., 1991).

Developing retinal ganglion cells of rodents express L1, DM-GRASP/SC-1, TAG-1/axonin-1, and GAP-43. After axotomy, retinal ganglion cells continue to express L1, up-regulate expression of GAP-43, but stop to express DM-GRASP/SC-1 and TAG-1/axonin-1. When axonal regeneration of retinal ganglion cells is experimentally induced by grafting a PNS transplant to the proximal optic nerve stump, regenerating cells also express L1 and GAP-43, but not DM-GRASP/SC-1 and TAG-1/axonin-1 (Jung et al., 1997; Schaden et al., 1994). Since L1 supports axonal elongation by homophilic interactions (Dahme et al., 1997; Lemmon et al., 1989), successful axonal regeneration may relate, at least partially, on L1-mediated interactions between retinal ganglion cell axons and L1-positive Schwann cells within the PNS graft.

All these data suggest that axonal regeneration may be achieved in the adult mammalian CNS when L1 is expressed by CNS glial cells. To test this hypothesis experimentally, transgenic mice were generated which express L1 ectopically in astrocytes under regulatory sequences of the GFAP gene (Mohajeri et al., 1996). Immunohistochemical analysis and in situ hybridization experiments revealed expression of L1 by astrocytes of transgenic mice, but not of wild-type controls. Expression of L1 by astrocytes of GFAP-L1 mutants was increased after a lesion,
as expected for a transgene expressed under the GFAP promoter. Interestingly, neurite outgrowth from young postnatal cerebellar neurons or chick dorsal root ganglion neurons was increased on transgenic astrocytes when compared to wild-type astrocytes, and this outgrowth-promoting effect could be partly reversed with anti-L1 antibodies (Mohajeri et al., 1996). Neurite outgrowth from cerebellar neurons was also increased on cryosections of heavily myelinated optic nerve of mutant mice when compared to wild-type nerves, and neurite outgrowth increased with increasing levels of transgene expression (three different transgenic lines with different levels of transgenic L1 expression were tested). When cryosections were prepared from lesioned transgenic nerve, GFAP and thus transgenic L1 were elevated and neurite outgrowth was further improved up to 400% of that observed on lesioned wild-type nerves. Incubation of cryosections with L1 antibodies partly reversed this growth promoting effect (Mohajeri et al., 1996).

In an independent study, full length human or rat L1 was introduced into primary rat cortical astrocytes using a defective herpes simplex virus (HSV) vector (Yazaki et al., 1996). Together, observations demonstrate that an ectopic expression of L1 by CNS glial cells makes these cells more permissive for growing neurites.

The optic nerve of transgenic mice was lesioned to test whether transgenic L1 on astrocytic cell surfaces promotes axonal regrowth in vivo. These experiments revealed no significant differences between the lengths of the longest regrown axons of GFAP-L1 mutants and wild-type controls (Mohajeri et al., 1996). Since low levels of transgene expression may explain these negative results, we attempted to increase expression of L1 on astrocytes by two approaches. Since application of exogenous bFGF induces a significant up-regulation of GFAP (Eclancher et al., 1990; Lewis et al., 1992), such a treatment should also elevate levels of the L1 transgene. In addition to up-regulate GFAP and thus transgene expression, bFGF also protects axotomized nerve cells from lesion-induced cell death and supports axonal sprouting (e.g., Anderson et al., 1988; Fagan et al., 1997; Peterson et al., 1996; Ramirez et al., 1999; Sievers et al., 1987; Weibel et al., 1994). The other approach to elevate levels of the L1 transgene was to generate "double" mutants by cross-breeding two independent GFAP-L1 transgenic mouse lines.

Application of bFGF to the vitreous induced significant up-regulation of GFAP in retinal astrocytes, and induced expression of GFAP in Müller cells, both at the mRNA and protein levels. As expected for a protein expressed under the
GFAP promoter, similar results were obtained for transgenic L1. In fact, induction of transgene expression by Müller cells was strong enough to enable detection of the L1 transgene on Müller cell surfaces at the electron microscopic level. However, despite these elevated levels of L1 on CNS glial cells, axonal regeneration was not significantly improved in bFGF-treated GFAP-L1 mutants or in "double" mutants.

In a recent study, L1-transfected mouse fibroblast L cells were grafted into the lesioned spinal cord of young adult rats (Kobayashi et al., 1995). Neurofilament immunohistochemistry revealed that numerous regenerating axons had penetrated the graft over long distances two weeks after the lesion. In contrast, only a few axons penetrated control grafts consisting of non-transfected L cells (Kobayashi et al., 1995). These observations contrast with our negative in vivo results. Although we were successful to increase levels of transgenic L1 on astrocytes by application of exogeneous bFGF, these levels may still be too low to promote axonal regrowth in vivo. In fact, application of exogeneous bFGF only increased levels of transgenic L1 on retinal glial cells. A significant increase of transgenic L1 expression by optic nerve glial cells, in contrast, was not detectable. The latter observation is most likely due to a limited diffusion of bFGF into the heavily myelinated optic nerve. However, improved axonal regrowth was also not detectable in double mutant mice obtained from cross-breeding transgenic lines 3426 and 3427. Apparently, non-permissive or inhibitory molecules associated with reactive astrocytes, oligodendrocytes, and CNS myelin dominate the growth-promoting effects of L1 on transgenic astrocytes and prevent axonal regeneration. These inhibitory components are absent from the grafts of L1-positive fibroblasts which support axonal growth (Kobayashi et al., 1995). In an attempt to experimentally address this possibility, transgenic mice were generated which express L1 ectopically in oligodendrocytes under regulatory sequences of the myelin basic protein gene. However, transgenic L1 was only weakly detectable in cell bodies of oligodendrocytes, and did not associate or integrate into CNS myelin (Mohajeri, Bartsch, and Schachner, unpublished observations).

It will be interesting to study axonal regeneration in GFAP-L1 transgenic mice or double mutants with an experimentally delayed myelination of spinal cords (Savio and Schwab, 1990) or optic nerves (Weibel et al., 1994) and simultaneous exposure to bFGF.

Axonal sprouts distal to the lesion site appeared more numerous in transgenic mice when compared to wild-type controls. However, this effect could not be
quantified since the experiments were designed to determine the length of the longest regrown axon. A detailed quantitative analysis of axonal sprouting in transgenic mice, either in the lesioned optic nerve or in oligodendrocyte- and myelin-poor CNS regions may uncover the growth promoting properties of glial-derived L1 which have been demonstrated in in vitro experiments (Mohajeri et al., 1996; Yazaki et al., 1996).
3.2 Regulation of Expression of CHL1: Effect of Intravitreal Application of bFGF and Optic Nerve Lesions

3.2.1 Introduction

CHL1 (close homolog of L1) is a recently identified member of the L1 family of cell recognition molecules (Holm et al., 1996). Originally, a partial cDNA clone of CHL1 has been isolated from an expression library with a cross-reactive polyclonal anti-L1 antibody (Tacke et al., 1987). A CHL1 full length cDNA clone has been isolated recently, and encodes a novel protein of 1209 amino acids with a calculated molecular weight of 134.9 kDa (Holm et al., 1996). As all members of the L1 family, CHL1 is a transmembrane molecule. The extracellular region comprises six Ig-like domains and five FNIII repeats, with the fifth repeat being only rudimentary. Homology analysis revealed that the intracellular domain of CHL1 (57% of similarity) is more similar to L1 than the extracellular domain (37% of similarity). Two major isoforms of 185 kDa and 165 kDa and a minor form of 125 kDa have been found in the nervous system of mice (Holm et al., 1996). The 185 kDa form is possibly the membrane-bound form, whereas the 165 and 125 kDa forms are possibly proteolytically cleaved fragments of CHL1. Proteolytically cleaved fragments have also been reported for L1 (Faissner et al., 1985; Sadoul et al., 1988). For both CHL1 and L1, the functional role of these fragments has yet to be determined.

In the CNS, CHL1 and L1 are both expressed by a variety of neuronal cell types. CHL1 (but not L1) is additionally expressed by some glial cells (Holm et al., 1996). Expression of CHL1 becomes detectable at embryonic day 13, and is strongest between embryonic day 18 and postnatal day 7. With increasing age of the animal, expression of CHL1 decreases again in many regions of the nervous system (Hillenbrand et al., 1999). In contrast to L1, CHL1 is often found to be expressed by a subset of certain cell types. For example, whereas in vitro CHL1 is expressed by most astrocytes, the molecule is expressed by only a few astrocytes restricted to the lamina cribrosa of the adult optic nerve in vivo (Holm et al., 1996). There are also striking differences between developmental stages at which certain cell types start to express L1 or CHL1. For instance, granule cells in the developing cerebellar cortex start to express L1 as soon as they become postmitotic and initiate their migration from the external granular layer to the internal granular layer (Lindner et al., 1983). In contrast, the same cell types start to express CHL1 only after they have reached their final positions in the internal granular layer (Holm et al., 1996; our unpublished observations). Due to the lack of anti-CHL1 antibodies which can be used for immunohistochemical analysis,
the expression pattern of CHL1 has so far been studied at the mRNA level using \textit{in situ} hybridization analysis. Knowledge about the subcellular distribution of CHL1 protein will eventually give more insights into possible functions of this protein during the development of the nervous system. For a detailed description of CHL1 see chapters 1.5.6 through 1.5.9.

CHL1 strongly promotes neurite elongation from cerebellar and hippocampal neurons when offered as a substrate-bound or soluble molecule (Hillenbrand et al., 1999). Since cerebellar neurons did not express detectable levels of CHL1 when they were used for neurite outgrowth assays, a yet to be identified receptor for CHL1 on neuronal surfaces has to be postulated. Expression of L1, but not of CHL1 in heterologous surfaces promotes formation of cell aggregates, again indicating that CHL1 acts as a heterophilic cell recognition molecule (Hillenbrand et al., 1999).

Altogether, CHL1 is closely related to L1, has been identified as a cell recognition molecule, and promotes neurite outgrowth \textit{in vitro}. To obtain further insights into possible functions of CHL1 \textit{in vivo}, we have studied the molecule's expression in the lesioned optic nerve of adult mice. Possible functional roles of CHL1 in lesioned CNS tissue have recently been suggested, based on the observations that (i) motoneurons in the spinal cord up-regulate CHL1 expression after lesioning peripheral nerves (Roslan et al., 1998); (ii) certain subsets of CNS neurons up-regulated CHL1 expression after insertion of PNS grafts into the adult rat brain (Chaisuksunt et al., 1998). Here, we focused on the question whether a CNS lesion modifies expression of CHL1. We additionally analyzed the effect of exogeneous bFGF on the glial expression of CHL1, since applications of bFGF to the developing and adult brain have been demonstrated to induce astrogliosis (Eclancher et al., 1990; Finklestein et al., 1988; Hicks, 1998; Lewis et al., 1992; Logan and Berry, 1993; Petroski et al., 1991; Sievers et al., 1987).

Hence, we have studied the expression of CHL1 mRNA and protein in the retina and optic nerve of adult mice after lesioning the optic nerve and after intravitreal application of bFGF. We chose this system because it is well accessible for experimental manipulations, such as mechanical lesions and application of substances. We further investigated in more detail the expression of CHL1 mRNA during development of the nervous system of young postnatal mice, specifically in the cerebellum and the primary visual system.
3.2.2 Material and Methods

3.2.2.1 Intravitreal Application of bFGF, Optic Nerve Lesions, and in situ Hybridization

Adult C57BL/6J mice (at least 2 months old) were subjected to intravitreal application of 1 μl bFGF (0.1 μg/μl; see chapter 3.1.2.2). Control animals received a similar volume of a 0.9% solution of NaCl. Three days after injection, animals were killed and sections of their optic nerves and retinas were prepared as described in chapter 3.1.2.2. Lesions of optic nerves of adult C57BL/6J mice were performed as described in chapter 3.1.2.6. Animals were killed 2, 7, 14, 28, and 63 days after the optic nerve crush and the tissue was prepared as described in chapter 3.1.2.2. All experimental tissues and appropriate control tissues were subjected to in situ hybridization analysis (chapter 3.1.2.3). Expression of CHL1 transcripts in the developing central nervous system was analyzed in neonatal, 3-, 7-, and 14-day-old C57BL/6J mice. The cerebellum, retina, and optic nerve were chosen for this analysis. In situ hybridization was performed as described in detail in chapter 3.1.2.3.

3.2.2.2 Immunoblot Analysis

Retinal tissue from C57BL/6J mice 7 days after intravitreal application of bFGF and optic nerves 7 and 14 days after an intraorbital crush were prepared for immunoblot analysis as described (Rathjen and Schachner, 1984; Sadoul et al., 1989) with minor modifications. Retinal tissue 7 days after intravitreal application of NaCl, unlesioned optic nerves, purified L1, and cerebellar tissue served as controls. The tissue was homogenized in 40mM Tris-HCl, pH 7.4, containing 150mM NaCl, 5mM EDTA, 5mM EGTA, 1% (vol/vol) Triton X-100, 1mM phenylmethylsulfonylfluoride (PMSF), 0.5mM iodoacetamide (solubilization buffer), 1mM dithiothreitol (DTT) and agitated for 15 min to 12 h at 4°C on an end-over-end shaker. The non-soluble material was pelleted by centrifugation at 100,000 g for 45 min at 4°C. Protein concentrations of supernatants were determined by the bicinchoninic acid-method (BCA) according to the manufacturer’s instructions (Pierce). Proteins were resolved by 8-10% SDS-PAGE (Lämmli, 1970) and transferred (Towbin et al., 1979) to nitrocellulose filters (0.45 μm, BA 85; Schleicher & Schuell). Immunodetection was performed as described (Faissner et al., 1985) using a CHL1 antiserum (1:10,000 diluted; Hillenbrand et al., 1999), L1 polyclonal antibodies (1:5,000 diluted; Bartsch et al., 1989), monoclonal antibodies against rabbit GAPDH (0.25 μg/ml; Chemicon) or polyclonal GFAP antibodies (1:10,000 diluted; Bartsch et al., 1990). Bound antibodies were detected with HRP-conjugated secondary
antibodies and visualized by enhanced chemoluminescence according to the manufacturer's instruction (ECL, Amersham). For comparison of expression levels and sizes of the different proteins, bound antibodies were removed as described by the manufacturer (Amersham) and reprobed with another primary antibody.

3.2.3 Results

3.2.3.1 Expression of CHL1 Transcripts in Selected Brain Regions during Development

According to Hillenbrand and co-workers (1999), expression of CHL1 mRNA is not detectable in the brain before embryonic day (ED) 13, is highest between ED 18 and postnatal day (PD) 7, and declines thereafter until adulthood. Young, untreated C57BL/6J mice were analyzed in more detail for their CHL1 mRNA expression by in situ hybridization in the primary visual system and the cerebellum.

Expression of CHL1 and L1 transcripts was examined at postnatal days 0, 3, 7, and 14 in retinae and optic nerves, and in the cerebellar cortex (Fig. 11; PD 14 not shown). At postnatal day 0 and 3, CHL1 transcripts were strongly expressed at the retinal end of the optic nerve, and weakly by cells located throughout the entire distal length of the nerve (Fig. 11 a and b). In the retina, labeled cells were located directly above the developing inner plexiform layer, suggesting that they correspond to amacrine cells (Fig. 11 a and b). CHL1-positive cells were also found at the vitread margin of the retina, and these cells may either represent retinal astrocytes, ganglion cells, and/or displaced amacrine cells (Fig. 11 a and b). In comparison, L1 mRNA at these developmental stages was absent from the optic nerve, and present in developing horizontal and amacrine cells, in ganglion cells, and presumably in displaced amacrine cells (not shown). At postnatal day 7, CHL1 message in the optic nerve is still strongly expressed in its retinal end, but almost completely absent from the distal part of the nerve (Fig. 11c). In the retina, CHL1 expression is drastically down-regulated, and only detectable in a few cells located at the inner margin of the inner nuclear layer (Fig. 11c). This principal pattern of CHL1 expression was also observed in older animals (i.e., 14-day-old mice; not shown) and did not change until adulthood.

In the developing cerebellar cortex, intense CHL1 expression was detectable in the deep cerebellar nuclei at all developmental ages analyzed (for neonatal and 3-day-old animals, see Fig. 11 d and e). In the cortex, CHL1 transcripts were present in granule and Golgi cells, Purkinje cells, stellate cells, and basket cells
did not express detectable levels of CHL1 message until the end of the first postnatal week (Fig. 11 d-f). At later developmental stages, weakly CHL1-positive cells became apparent, possibly corresponding to stellate cells (not shown; see Holm et al., 1996). A striking difference between expression patterns of CHL1 and L1 was that granule cells started to express CHL1 not before they entered the internal granular layer (Fig. 11 d-f), whereas the same cell types started to express L1 as soon as they became postmitotic and started their migration (not shown, see Lindner et al., 1983).

Figure 11: Expression of CHL1 transcripts in the developing retina, optic nerve, and cerebellar cortex. CHL1 mRNA is strongly expressed in the retinal end of the optic nerve at all developmental ages studied (a: PD 0; b: PD 3; c: PD 7). In the distal part of the nerve, expression decreases with increasing age (a-c). In the retina, transcripts are present in cells located at the inner margin of the inner nuclear layer, and at the vitread margin of the retina. Again, expression decreases with increasing age (a-c). In the developing cerebellar cortex (d: PD 0; e: PD 3; f: PD 7), CHL1 is expressed in the cerebellar nuclei and by granule and Golgi cells in the internal granular layer (d-f). Note that proliferating, postmitotic and premigratory, and migratory granule cells are CHL1-negative (d-f). Scale bars 500 μm.
3.2.3.2 Expression of CHL1 Transcripts after Intravitreal Application of bFGF and Optic Nerve Lesions

In the retina of adult mice, GFAP transcripts are restricted to the inner margin (i.e., nerve fiber layer and ganglion cell layer) of the retina (Fig. 12f). In the optic nerve, GFAP transcripts are detectable in numerous astrocytes present throughout the entire length of the nerve. Highest levels of GFAP mRNA are detectable in astrocytes located in the unmyelinated retina-near end of the optic nerve (Fig. 13f), known as the lamina cribrosa. Three and 7 days after a single intravitreal injection of 1 μl bFGF (0.1μg/μl), in situ hybridization analysis revealed an up-regulation of the expression of GFAP transcripts in the retina (Fig. 12b) when compared with untreated (Fig. 12f) or NaCl-injected (Fig. 12d) retinae. Application of bFGF resulted in elevated levels of GFAP mRNA in astrocytes located in the nerve fiber layer (Fig. 12b). Moreover, bFGF induced expression of GFAP in cells located in the inner nuclear layer. These cells correspond to Müller cells, the only cell type in the inner nuclear layer which is able to express GFAP. While GFAP transcripts in control and NaCl-treated retinae were restricted to cell bodies (see Fig. 12 d and f), they were diffusely distributed between the outer plexiform layer and vitread margin of bFGF-treated retinae (Fig. 12 b). This observation suggests the presence of GFAP transcripts in basal processes of Müller cells.

CHL1 transcripts were hardly detectable in untreated control retinae or NaCl-injected retinae, and were only present in a few cells located in the inner nuclear layer (Fig. 12 c and e, not shown). After application of bFGF, CHL1-positive cells became apparent at the vitread margin of the retina (Fig. 12 a). In addition, numerous CHL1 expressing cells became detectable in the inner nuclear layer (Fig. 12a). The location and density of CHL1-positive cells in bFGF-treated retinae suggests that these cells correspond to retinal astrocytes and Müller cells, respectively (compare Fig. 12 a and b).

Since up-regulation of CHL1 (Fig. 12a) and GFAP (Fig. 12b) expression was only observed after intravitreal application of bFGF, but not of NaCl (Fig. 12 c and d), it represents a specific cellular response to bFGF and not a response to the experimental manipulation (i.e., intravitreal injection).

In the optic nerve, there were no detectable changes in the expression of CHL1 and GFAP after bFGF or NaCl application, when compared to untreated control tissue (data not shown). In untreated control nerves and after application of NaCl or bFGF, GFAP mRNA was present in astrocytes distributed throughout
the entire length of the nerve, and highest levels were found in astrocytes located in the lamina cribrosa (for an untreated control nerve, see Fig. 13f). CHL1 transcripts were not detectable in the myelinated part of the nerve, and were restricted to cells of the lamina cribrosa under all experimental conditions (for a control nerve, see Fig. 13b and d).

Figure 12: Expression of GFAP and CHL1 mRNA after bFGF injection. In situ hybridization of CHL1 is shown in (a,c,e), and of GFAP in (b,d,f). Basic FGF injection in adult C57BL/6J mice resulted in an increase of CHL1 and GFAP mRNA, (a) and (b) respectively. Control injections of NaCl did not change CHL1 or GFAP expression (c,d) when compared to untreated control retinae (e,f). Scale bars 300 µm.

The expression pattern of CHL1 in bFGF-treated retinae is similar to that of GFAP, suggesting that CHL1 is expressed by reactive astrocytes and Müller cells. Optic nerves of adult C57BL/6J mice were lesioned intraorbitally to further investigate whether CHL1 is expressed by reactive astrocytes.
Figure 13: Expression of CHL1 and GFAP transcripts in lesioned nerves. In unlesioned control nerves, CHL1 transcripts are restricted to astrocytes located in the unmyelinated retinal end of the nerve (b). One week after an intraorbital crush of the nerve, CHL1-positive cells become detectable throughout the entire length of the nerve (a). Elevated levels of CHL1 mRNA are still detectable in the distal stump of the nerve 63 days after the lesion (c; d shows the unlesioned contralateral nerve). GFAP transcripts are expressed by astrocytes present throughout the entire length of the nerve, and are detectable at elevated levels in astrocytes located in the lamina cribrosa (f). After a lesion, expression of GFAP is increased in all astrocytes (for a 7 day post-lesion interval, see e). Scale bar 500 μm.

In untreated control nerves, expression of CHL1 transcripts was restricted to astrocytes located in the unmyelinated, retina-near end of the nerve (Fig. 13b). However, in lesioned nerves CHL1 mRNA became detectable in cells located throughout the entire length of the nerve (Fig. 13a and c and Fig. 14). Lesion-induced expression of CHL1 in the entire distal myelinated part was already apparent 2 days after the lesion (not shown), the earliest post-lesion interval investigated. Elevated levels of CHL1 transcripts persisted for at least 63 days post-lesioning, the latest post-lesion time point studied (Fig. 13c).

Figure 14: Expression of CHL1 mRNA in the lesioned optic nerve. In situ hybridization revealed an increase of CHL1 in cells located along the entire length of the optic nerve 7 days after an intraorbital crush (a). CHL1 expression in the untreated control nerve is restricted to cells located in the unmyelinated retinal end of the nerve (b). Scale bar 500 μm.

For each experimental animal, expression of GFAP was studied in parallel to control the lesioning of the nerve. In unlesioned control nerves, GFAP transcripts were present in astrocytes located throughout the entire length of the nerve (Fig. 12f). Highest levels of GFAP expression were observed in the
unmyelinated retinal end of the nerve (Fig. 12f). Optic nerve lesions induced a significant increase of GFAP expression throughout the nerve (Fig. 12e).

3.2.3.3 Immunoblot Analysis of CHL1 Expression in Different Experimental Tissues

We have demonstrated that expression of CHL1 mRNA is induced in Müller cells and is increased in retinal astrocytes in response to intravitreal application of bFGF. We have also demonstrated that expression of CHL1 transcripts is induced in glial cells of the optic nerve in response to an intraorbital nerve crush. Immunoblot analysis of the different experimental tissues was performed to obtain information about the expression of CHL1 at the protein level. As internal controls, we also analyzed expression of L1, GFAP, and GAPDH (Fig. 15a and b).

CHL1 protein was detectable in unlesioned control nerves (Fig. 15a). This observation suggests that optic nerve preparations contain unmyelinated retinal ends of nerves (with CHL1 mRNA-positive cells), or that cells in the myelinated distal nerves express low levels of CHL1 not detectable by in situ hybridization analysis. One and two weeks post-lesion, CHL1 levels were significantly increased in lesioned nerves when compared to control nerves (Fig. 15a), in agreement with the in situ hybridization data. L1 protein was detectable in unlesioned nerves (Fig. 15a). Different to CHL1, L1 protein was slightly down-regulated 7 days after the lesion, and was drastically down-regulated 14 days after the lesion (Fig. 15a). Levels of GFAP protein were not significantly changed 7 days after the lesion, but were significantly up-regulated 14 days after the lesion, when compared to untreated control nerves (Fig. 15a). Levels of GAPDH were studied in the same tissues to control equal loading of proteins (Fig. 15a).

Intravitreal application of bFGF induced CHL1 expression by retinal astrocytes and Müller cells, and up-regulated GFAP expression in retinal astrocytes and induced expression of GFAP transcripts in Müller cells. NaCl- or bFGF-treated retinal tissue was therefore probed with CHL1 and GFAP antibodies to study the expression of these molecules at the protein level (Fig. 15b). Whereas only low levels of CHL1 protein were detected in NaCl-treated retinas, the protein was significantly up-regulated after bFGF application (Fig. 15b). In contrast to CHL1, levels of L1 were not changed by bFGF treatment when compared to NaCl application (Fig. 15b). Similar to CHL1, we observed elevated levels of GFAP protein in bFGF-injected retinas when compared to control tissue (Fig. 15b). Purified L1 and cerebellar tissue was probed with all four antibodies (to CHL1, L1, GFAP, and GAPDH) as an internal control. To
control equal loading of proteins, all tissues were additionally analyzed with GAPDH antibody (Fig. 15b).

![Diagram](image.png)

**Figure 15:** Expression of CHL1, L1, and GFAP protein after optic nerve crush and intravitreal bFGF application. Immunoblot analysis of different proteins after optic nerve crush is shown in (a) and after intravitreal application of bFGF in (b). (a) Optic nerve lesions induced an up-regulation of CHL1 protein 7 and 14 days after the crush, when compared to unlesioned nerves. Levels of L1 protein, in contrast, were progressively down-regulated 7 and 14 days after a crush. (b) Basic FGF application increased CHL1 protein expression in the retinae 7 days after injection when compared to retinae injected with NaCl. Levels of L1 protein did not change after bFGF treatment when compared to NaCl application (b). Levels of GFAP protein are up-regulated in lesioned optic nerves (a) and bFGF-treated retinae (b) when compared to corresponding control tissues. All tissues were probed with GAPDH antibodies to control equal loading of proteins. 20 µg of protein was loaded per lane. Molecular weights (in kDa) are indicated at the right margin.

3.2.4 Discussion

CHL1 belongs to the family of cell recognition molecules with structural similarities to L1. Most abundant expression of CHL1 has been found during development of the nervous system, in close analogy to L1. CHL1 becomes detectable at day 13 of embryonic development and decreases with aging of the animal (Hillenbrand et al., 1999). The expression pattern of CHL1 and L1 is partly overlapping in that both molecules are simultaneously expressed by a
variety of nerve cell types (Hillenbrand et al., 1999; Holm et al., 1996). However, there are also striking differences between the expression patterns of both proteins. While CNS glial cells do not express detectable levels of L1, subpopulations of them express high levels of CHL1 during early postnatal ages. In young postnatal mice, for instance, CHL1 transcripts are present in glial cells along the entire length of the optic nerve (see also Holm et al., 1996), whereas in adults, CHL1 expression is restricted to the most proximal part of the optic nerve. There are also differences in expression patterns with regard to the nerve cells. In general, more nerve cell types express L1 than CHL1 (Hillenbrand et al., 1999). Nerve cells which express both proteins start to express CHL1 at later developmental stages than L1. Cerebellar granule cells, for instance, start to express L1 before they migrate from the external granular layer to the internal granular layer, while CHL1 is not expressed before migration of these nerve cells is completed.

The spatio-temporal pattern of expression of both molecules, the high degree of homology between both proteins (Holm et al., 1996), and the fact that both members of the L1 family support neurite elongation (Hillenbrand et al., 1999), suggest that both proteins may perform similar functions in vivo. Similar to L1 mutations, mutations in the recently discovered human ortholog of the mouse CHL1 (named CALL with a 90% homology to mouse CHL1) may also result in mental retardation. CALL is highly expressed during development of the brain and it has been mapped to chromosome 3 at the locus 3p26. This locus has been described to contribute to the 3p- and ring (chromosome 3) syndromes which manifest in mental retardation (Wei et al., 1998). However, while mice deficient for L1 show a severe phenotype (for a recent review, see Bartsch and Schachner, 1999), CHL1-deficient mice have been reported to develop normally (Montaget et al., 1997). Recent studies suggest that CHL1 may play a role in the lesioned PNS and CNS. For instance, motoneurons in the spinal cord of adult rats up-regulate CHL1 expression after crushing the sciatic nerve, and down-regulate expression six weeks after the lesion. A similar time course of CHL1 expression by motoneurons was observed after cutting or ligating the sciatic nerve (Roslan et al., 1998). Thus, up-regulation of CHL1 by motoneurons appears to be related to axotomy, while down-regulation of the molecule appears to occur independent from axonal regeneration and re-innervation of targets. In close analogy, it has been demonstrated that nerve cells in the deep nuclei of the cerebellum and in the brainstem up-regulate CHL1 (and L1 and GAP-43) after grafting of a PNS explant. Evidence has been presented that these proteins are up-regulated in those nerve cells which extend axons into the PNS grafts. Interestingly, nerve
cells which do not grow axons into the PNS grafts, such as Purkinje cells, do not
up-regulate CHL1 (or L1 or GAP-43; Chaisuksunt et al., 1999). CHL1 supports
neurite elongation by heterophilic interactions (Hillenbrand et al., 1999). The
first regenerating CHL1-positive nerve cells may thus facilitate regrowth of later
growing axons while this hypothesis implicates CHL1 in axonal regeneration, it
also suggests that CHL1 is not essential for axonal regrowth. In fact, many DRG
neurons up-regulate CHL1 after lesioning peripheral nerves with the exception
of the large-sized nerve cells (Roslan et al., 1998). Since soluble CHL1 also
supports neurite elongation (Hillenbrand et al., 1999), CHL1 may also support
axon regrowth as an autocrine or paracrine factor.

To obtain further insights into possible functional roles of CHL1 in the
lesioned CNS, we applied bFGF, a growth factor known to induce glia
proliferation, astrocyte hyperplasia, and increased expression of GFAP, to the
vitreous of adult mice. In addition, optic nerves of adult mice were crushed
intraorbitally. In both experimental tissues, we observed a significant induction
of CHL1 expression by astrocytes and Müller cells. In all experiments, there was
a striking co-localization of CHL1 and GFAP, suggesting that CHL1 is expressed
by reactive astroglial cells. Up-regulation of CHL1 expression by retinal nerve
cells in response to an optic nerve crush was not observed, supporting the view
that induction/up-regulation of CHL1 by adult nerve cells is correlated with
axon regrowth (Chaisuksunt et al., 1998; Roslan et al., 1998).

Interestingly, bFGF has been reported to influence expression of another cell
recognition molecule in vitro. When this growth factor is added to cultured rat
cerebral cortex astrocytes, levels of the extracellular matrix molecule tenascin-C
were increased approximately 9-fold (Meiners et al., 1993). Since expression of
other extracellular matrix components such as fibronectin or laminin was not
affected by this treatment, up-regulation of tenascin-C was considered as a
specific cellular response to bFGF exposure. Remarkably, exposure of cultured
astrocytes to bFGF reduced their ability to support neuronal adhesion. In
addition, there is evidence that such astrocytes are less permissive substrates for
growing neurites (Grierson et al., 1990; Meiners et al., 1993; Petroski et al., 1991).
Both tenascin-C and bFGF are up-regulated around a stab wound of the cerebral
cortex (Finklestein et al., 1988; Laywell et al., 1992) and it has been hypothesized
that bFGF induces tenascin-C expression in the lesioned CNS, thereby reducing
axon regrowth (Meiners et al., 1993).

There are several analogies between these data and our findings. We provide
evidence that bFGF up-regulates expression of another cell recognition molecule
on astrocytes, CHL1. We also demonstrate that CNS glial cells up-regulate CHL1 after an optic nerve crush. Interestingly, bFGF is also up-regulated in lesioned optic nerves of adult rats (Eckenstein, 1994). Thus, the rapid up-regulation of CHL1 along the entire length of the mechanically injured optic nerve may, at least in part, be induced by the elevated levels of endogenous bFGF. The functional significance of an elevated level of CHL1 expression by reactive astrocytes remains to be elucidated. Substrate-bound and soluble CHL1 has been shown to be a potent promoter of neurite elongation (Hillenbrand et al., 1999). However, reactive astrocytes are considered as poor or non-permissive substrates for growing neurites (e.g., McKeon et al., 1991; Snow et al., 1990; Snow and Robson, 1995). Laminin is, like CHL1, a potent promoter of neurite elongation (Rogers et al., 1983; Tomaselli et al., 1990) and is also expressed on reactive astrocytes (McKeon et al., 1991). It is reasonable to assume that the effect of growth promoting molecules on astrocytes may be masked by inhibitory components, such as chondroitin sulfate, heparan sulfate, dermatan sulfate, or keratan sulfate containing proteoglycans (McKeon et al., 1995). It is interesting in this context that proteoglycans have been demonstrated to bind to permissive molecules and thereby reduce or neutralize their effects (Dou and Levine, 1994; Friedlander et al., 1994; Smith-Thomas et al., 1995). In contrast to differentiated and reactive astrocytes, immature astrocytes support neurite elongation and axonal regrowth (e.g, Smith et al., 1986; Smith et al., 1990; Wunderlich et al., 1994). On these cells, growth promoting molecules (Smith et al., 1990) apparently dominate inhibitory components and support axonal regrowth, and CHL1 may contribute to the permissive substrate properties of immature CNS glial cells.

One may also speculate about other possible roles of CHL1 in the lesioned CNS. Since we found a strong correlation between reactive astrogliosis and CHL1 expression, the molecule may mediate astrocyte-astrocyte interactions and may thus contribute to the formation of a glial scar. Furthermore, bFGF supports the survival of a variety of nerve cell types in vitro (e.g., Ferrari et al., 1989; Grothe et al., 1989; Morrison et al., 1986; Unsicker et al., 1987) and of axotomized nerve cells in vivo (e.g., Anderson et al., 1988; Sievers et al., 1987). CHL1 has recently been shown to also support neuronal survival in vitro (Chen et al., 1999). Basic FGF may thus exert part of its neurotrophic effect by increasing expression of other molecules which also show neurotrophic properties.
Finally, it is tempting to speculate that CHL1 improves the substrate properties of astrocytes. Exogeneous and endogeneous bFGF have been demonstrated to support axonal sprouting in the denervated hippocampus (Fagan et al., 1997; Ramirez et al., 1999). Assuming that bFGF induces up-regulation of CHL1 not only on retinal astrocytes or Müller cells, but also on hippocampal astrocytes, the molecule may support axonal sprouting in the hippocampus. This hypothesis predicts reduced axonal sprouting in the hippocampus of CHL1-deficient mice, either without or after application of exogeneous bFGF.

Altogether, up-regulation of CHL1 expression by CNS nerve cells which regenerate their axons (Chaisuksunt et al., 1999; Chaisuksunt et al., 1998; Roslan et al., 1998) and by glial cells in response to a CNS injury or application of bFGF (the present study) suggests a functional role of this protein in the lesioned nervous tissue. A detailed study of lesioned nervous tissue of CHL1-deficient mice (Montag et al., 1997) may help to elucidate this yet unknown function(s).
3.3 Genotype-Phenotype Relation in L1 Mutant Mice

3.3.1 Introduction

Four X-linked severe diseases in humans, HSAS (hydrocephalus as a result of stenosis of the aqueduct of Sylvius), MASA (mental retardation, aphasia, shuffling gait, and adducted thumbs), SP1 (spastic paraplegia type 1), and ACC (agenesis of corpus callosum) have previously been considered as distinct clinical entities (Fransen et al., 1995). The clinical picture of patients affected by either of these four syndromes is complex and highly variable. Importantly, clinical signs of HSAS-, MASA-, SP1-, and ACC-families overlap considerably. Mental retardation and adducted thumbs, for instance, were considered to represent constant features of all four syndromes. Moreover, an SP1 family (Kenwrick et al., 1986) was redefined as a family affected by MASA syndrome, since members of this family displayed mental retardation, aphasia, and adducted thumbs in addition to spastic paraplegia (Winter et al., 1989). Furthermore, members of HSAS families were reported to have no hydrocephalus (Fried, 1972; Willems et al., 1987), and MASA families were reported to contain HSAS patients displaying severe hydrocephalus (Schrander-Stumpfel et al., 1990). Finally, a family has been reported containing HSAS-, MASA-, and SP1 patients and all these syndromes were linked to Xq28 of the X-chromosome (Fryns et al., 1991).

Since the L1 gene has been mapped to Xq28 (Chapman et al., 1990; Djabali et al., 1990), L1 mutations have first been proposed and later been demonstrated to be the cause of all syndromes (Rosenthal et al., 1992; Van Camp et al., 1993; Willems et al., 1992). The four syndromes were therefore lumped together and termed CRASH (for corpus callosum hypoplasia, retardation, adducted thumbs, spastic paraplegia, and hydrocephalus; Fransen et al., 1995). The severe phenotypes associated with L1 mutations confirm an important role of L1 for normal brain development. However, the high variability of phenotypes even within the same family (see above) is puzzling, and suggest that modifier genes and/or environmental factors together with L1 mutations determine the severity of the disease.

To establish an animal model for CRASH syndrome, an L1 mutant mouse was generated by inserting a neomycin/thymidine kinase cassette into exon 8 of the L1 gene (Dahme et al., 1997). Northern blot analysis of mutant brains revealed significantly reduced levels of L1 transcripts (~ 10% of wild-type levels) with a slightly reduced size. Immunoblot analysis of mutant brains revealed residual
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(less than 0.5% of wild-type level) expression of an aberrant L1 protein of 190 kDa.

Heterozygous L1 females of the F1 generation were 129/SvEv-C57BL/6J hybrids, and these mice were either cross-bred with 129/SvEv or C57BL/6J inbred wild-type males for up to eight generations to establish two L1 mutant lines with a distinct genetic background. Mutant males of both lines were analyzed for the variability of the phenotype. Results are compared with observations obtained for an independently generated L1 mutant mouse with a pure 129/SvEv genetic background (Cohen et al., 1997; Fransen et al., 1998a).

3.3.2 Material and Methods

Wild-type and mutant male L1 mice (Dahme et al., 1997), 5-12 months old, were genotyped (chapter 3.4.2.1) and fixed by perfusion, and nervous tissue was prepared as described in chapter 3.4.2.2. Semithin sections, 5 μm in thickness, were prepared from forebrain and medulla of wild-type and mutant mice, mounted on glass slides, and stained with toluidin-blue. Animals with either a predominant 129/SvEv or a predominant C57BL/6J genetic background were analyzed.

3.3.3 Results

Analysis of the brain of L1 mutant mice with a predominant 129/SvEv genetic background revealed an apparently normal development of the ventricular system. The size of the lateral ventricles, third ventricle, and fourth ventricle was not significantly enlarged when compared to wild-type littermates (not shown; see Dahme et al., 1997). Some L1 mutants with a C57BL/6J genetic background also developed an apparently normal ventricular system (not shown). The majority of mutant males (~ 80% of all mutants analyzed) of the C57BL/6J line, however, developed significantly enlarged lateral ventricles when compared to wild-type littermates (Fig. 16).

The severity of the defect was highly variable between individual animals. In some mutants, lateral ventricles were moderately increased when compared to wild-type mice. In other mutants, lateral ventricles were severely enlarged, resulting in significant thinning of the cerebral cortex (Fig. 16). Four L1 mutants of the C57BL/6J line developed macrocephalus (Fig. 17). The first of these animals was obtained in the fourth generation of back-crosses with C57BL/6J wild-type mice.
Figure 16: *Enlarged lateral ventricles in L1 mutant mice.* Male mice of the C57BL/6J L1 mutant mouse line display severely enlarged lateral ventricles (a) when compared to wild-type (b) littermates. Sections from both genotypes were taken from corresponding levels of the brain. Scale bars 1 mm.

Figure 17: *A mutant mouse of the C57BL/6J line with macrocephalus.* Comparison of a mutant male mouse on the left with a normal wild-type littermate on the right. A few L1 mutants develop macrocephalus characterized by a significant dorsal enlargement of the skull. Note the significantly reduced body size of the mutant when compared to the wild-type littermate.

Macrocephaly correlated with a dramatic enlargement of the skull (Fig. 18) and ventricular system and with massive thinning of the cerebral cortex, or with complete atrophy of large portions of the cerebral cortex (see Fig. 18b).
Figure 18: Macrocephalus of a mutant male mouse. A few L1 mutants developed macrocephalus, characterized by massive enlargement of the skull (a), and eventually by a complete atrophy of large portions of the cerebral cortex (b).

Analysis of the corticospinal tract at the caudal end of the medulla oblongata revealed a significant hypoplasia of the tract in L1 mutants when compared to wild-type mice (Dahme et al., 1997). A reduction of the size of the tract by more than 30% was observed in mutants with a 129/SvEv and a C57BL/6J genetic background. Importantly, there was no clear correlation between the presence of massively enlarged ventricles and the degree of hypoplasia of the corticospinal tract, except for the few mutants which developed macrocephalus and showed almost complete atrophy of the cerebral cortex.

Hypoplasia of the corticospinal tract was observed in all L1 mutants analyzed, and was not dependent on the genetic background. Hypoplasia of the corticospinal tract is thus a constant defect in the brain of L1 mutants. However, in one L1 mutant mouse of the C57BL/6J line a corticospinal tract was completely missing at the caudal end of the medulla oblongata (Fig. 19). Lateral ventricles of this animal were significantly enlarged with only a moderate atrophy of the cerebral cortex.
Figure 19: Absence of the corticospinal tract in an L1 mutant mouse. The corticospinal tract with its densely packed myelinated axons is a well delineated structure at the ventral margin of the medulla of wild-type mice (a; outlined with arrow heads). This tract is not detectable in the brain of a mutant mouse (b). Scale bars 1 mm.

3.3.4 Discussion

The variability of the phenotype of CRASH patients is striking, both between families but also within the same family. The presence of families with L1 mutations containing members with a normally developed ventricular system and members with severe hydrocephalus is particularly striking in this context (Ruiz et al., 1995; Van Camp et al., 1993). A recent review of clinical descriptions of 132 CRASH patients from 35 families has, however, claimed that the intrafamilial variability has been overestimated (Yamasaki et al., 1997). Actually, only three families with substantial phenotypic variability were reported, and it has been suggested that this variability depends on genetic background or other non-L1 associated factors. We studied the variability of the phenotype of a large number of mice, all carrying the same L1 mutation. Importantly, the genetic background of these mice was relatively homogeneous, due to the fact that they have been cross-bred either into the 129/SvEv or C57BL/6J inbred mouse strain for up to eight generations.

Most defects typically associated with the L1 null mutations were observed to a similar extent in L1 mutants of either a 129/SvEv or C57BL/6J genetic background. These include decreased body weight, reduced life span, lacrimal eyes, deficits in locomotion, an impaired association of non-myelinating Schwann cells with axons, or a reduced size of the corticospinal tract. All these abnormalities have also been described for an independently generated L1-deficient mouse with a pure 129/SvEv genetic background (Cohen et al., 1997; Fransen et al., 1998a).
These results indicate that the phenotype of a given L1 mutation is predictable, independent of genetic background and with only moderate variability between individuals. Results thus support the view that the intrafamilial variability of CRASH syndrome has been overestimated (Yamasaki et al., 1997).

The situation is, however, strikingly different for the development of the ventricular system. Conventional histological analysis of mutants from a mouse line continuously cross-bred into a 129/SvEv genetic background revealed an apparently normal development of ventricles (Dahme et al., 1997). Similar results were reported for an independently generated L1-deficient mouse with a pure 129/SvEv background (Cohen et al., 1997). A more detailed analysis of the brain of the latter mutant line by high resolution magnetic resonance imaging, however, revealed a slight but significant enlargement of the ventricular system in all mutants analyzed (Fransen et al., 1998a). These results are in agreement with findings in humans, demonstrating that L1 mutations leading to truncations of the extracellular domain of L1 usually lead to the development of enlarged ventricles as macrocephalus (Fransen et al., 1995; Kamiguchi et al., 1998a; Wong et al., 1995b; Yamasaki et al., 1997).

While there was essentially no variability in the size of the ventricular system between individual mutants with a predominant or pure 129/SvEv genetic background, a dramatic variability was observed for L1 mutants with a C57BL/6J background. Analysis of these animals revealed the presence of normal, moderately enlarged, or massively enlarged ventricles. In analogy to observations in humans (e.g., Wong et al., 1995b; Yamasaki et al., 1995), significantly enlarged ventricles correlated with a thinning of the cerebral cortex.

Observations suggest that the L1 mutation together with C57-specific modifier genes determine the final size of the ventricular system. It is interesting in the present context that C57BL/6J wild-type mice have significantly larger lateral ventricles than 129/SvEv wild-type mice. Two other observations also indicate a critical role of C57-specific modifier genes. (1) Only L1 mutants with a predominant C57BL/6J genetic background develop macrocephalus. The first animal with such a phenotype was found in the fourth generation of C57BL/6J back-crosses. (2) The number of L1 mutants obtained from the C57BL/6J breeding colony was significantly lower than expected according to Mendelian frequencies, and decreased dramatically with an increasing C57BL/6J background. These observations may relate to an increased mortality of L1
mutants during embryonic development as a result of dramatically enlarged ventricles or macrocephalus.

Hypoplasia of the corticospinal tract is much less variable than development of hydrocephalus, and independent of genetic background. Some individual variability is indicated by the lack of a corticospinal tract in one C57BL/6J L1 mutant (this study), and the failure of essentially all corticospinal axons to cross contralaterally at the pyramidal decussation in one 129/SvEv L1 mutant (Cohen et al., 1997).

The apparent lack of a corticospinal tract in one L1 mutant did not correlate with severe hydrocephalus and massive thinning of the cerebral cortex, excluding that this defect is the result of increased cell death of cortical motoneurons. Observations rather support the view that hypoplasia of the corticospinal tract results from pathfinding errors of corticospinal tract axons at the pyramidal decussation (Cohen et al., 1997).

Our observations support the view of Yamasaki and colleagues (1997) that the phenotypic variability of CRASH patients carrying the same mutation has been overestimated. The only exception is the development of hydrocephalus or macrocephalus. The severity of this defect is apparently determined by genetic factors other than L1.
3.4 The Phenotype of Heterozygous L1 Mutant Mice

3.4.1 Introduction

The neural adhesion molecule L1 is a cell recognition molecule of the immunoglobulin superfamily. The protein consists of six immunoglobulin-like domains, five fibronectin type III domains, a single-pass transmembrane domain, and a cytoplasmic domain. L1 is expressed by nerve cells in the CNS and by nerve cells and Schwann cells in the PNS. Functionally, L1 has been demonstrated to support migration of nerve cells, and to mediate elongation and fasciculation of axons (for detail and references, see chapter 1.5).

The gene encoding human L1 is located in the Xq28 region of the X-chromosome (Djabali et al., 1990). Clinical interest in L1 is related to the recent finding that mutations in the L1 gene result in severe neurological disorders (see chapter 1.5.3.7). Different X-linked syndromes, originally considered as distinct clinical entities, are all related to L1 mutations: HSAS (hydrocephalus as a result of stenosis of the aqueduct of Sylvius; Bickers and Adams, 1949; Willems et al., 1987); MASA (mental retardation, aphasia, shuffling gait, and adducted thumbs; Bianchine and Lewis, 1974; Schrander-Stumpel et al., 1990; Winter et al., 1989); SP1 (spastic paraplegia type-1; Kenwrick et al., 1986); and ACC (agenesis of corpus callosum; Fransen et al., 1995; Kaplan, 1983). Since L1 mutations are the cause of all these syndromes, they were lumped together and are now termed CRASH (corpus callosum hypoplasia, retardation, adducted thumbs, spastic paraplegia, and hydrocephalus; Fransen et al., 1995).

The clinical picture of CRASH syndrome is complex, and typical abnormalities of CRASH patients include mental retardation, aphasia, shuffling gait, adducted thumbs, enlarged ventricles, hypoplasia of the corpus callosum or corticospinal tract, and fusion of thalami and colliculi, with mental retardation and adducted thumbs being the most constant features (Kamiguchi et al., 1998a; Yamasaki et al., 1995; Yamasaki et al., 1997). The severity of the disease varies dramatically between patients with different L1 mutations, but also between patients carrying the same mutation (Yamasaki et al., 1997). For instance, families have been reported in which some members are affected by hydrocephalus whereas other members of the same family are not (Fried, 1972; Willems et al., 1987; Yamasaki et al., 1997). A simple genotype-phenotype correlation is thus lacking. However, a certain correlation between the type of mutation and the severity of the disease has been demonstrated recently (Yamasaki et al., 1997). Mutations resulting in truncations of the extracellular
domain of Ll cause most severe phenotypes, missense mutations in the extracellular domain result in intermediate phenotypes, and mutations in the cytoplasmic domain of Ll lead to relatively mild phenotypes (Yamasaki et al., 1997).

The Ll gene is located on the X-chromosome (Chapman et al., 1990; Djabali et al., 1990). Males carrying an Ll mutation are therefore affected by CRASH syndrome. In females, one X-chromosome is inactivated at random early during neural development. The nervous system of females heterozygous for an Ll mutation should therefore consist of cells expressing either wild-type Ll or mutated Ll. Surprisingly, however, obligate carrier females are considered as healthy, and only in a few cases a very mild phenotype has been reported (Kaepernick et al., 1994).

We have analyzed whether the nervous system of female mice heterozygous for an Ll mutation shows defects that are typically associated with mutations in the Ll gene. We also addressed the question whether the nervous system of heterozygous females consists of a mosaic of cells either expressing wild-type Ll or mutated Ll.

3.4.2 Material and Methods

3.4.2.1 Genotyping of Ll Mutant Mice

The genotype of Ll mutant mice was determined from mouse tail genomic DNA using polymerase chain reaction (PCR) as described in chapter 3.1.2.1. The primers used were the following: 1) for the wild-type Ll gene: 5' CAGTCATTGATCCTGGAGTGC3'and 5' GGTAGGCAGGAGATAAGGTCA3' and 2) for the mutated allele: 5' GGTAGGCAGGAGATAAGGTCA3' and 5' TGGGAGACAATAGCAGGCAT3' with a concentration of 8 pM/μl (Dahme, 1998).

3.4.2.2 Semithin Sections

Wild-type females, heterozygous females, and mutant male littermates of the Ll mutant line (Dahme et al., 1997) with a C57BL/6J genetic background in the age of 5-12 months were used for this study. For the preparation of the tissue, animals were deeply anesthetized and perfused through the left ventricle with 4% paraformaldehyde and 2% glutaraldehyde in PBS (pH 7.4). Parasagittal sections of the cerebellum were prepared, and the remaining brain including brain stem and a short segment of the cervical spinal cord was sectioned coronally with a vibratome. These sections were washed several times in PBS.
and then immersed in 1% OsO₄ in 0.1 M cacodylate buffer for 1-2 hours. After several washes with PBS, tissue was dehydrated in an ascending series of methanol and immersed in propylene oxide, and embedded in Epon. After polymerisation, 3 μm semithin sections were prepared from forebrain, cerebellum, and medulla of wild-type, heterozygous, and mutant mice, mounted on glass slides, and stained with toluidin-blue. The sections were analyzed with an Axiophot microscope (Zeiss). The number of Golgi cells was determined in parasagittal sections of the cerebellum, the corresponding area of the internal granular layer was measured using a Neurolucida image analysis system, and the density of Golgi cells was calculated. The area of the corticospinal tract was determined in frontal sections of the caudal medulla, using the Neurolucida system.

3.4.2.3 In situ Hybridization

Parasagittal sections from the cerebellar vermis of wild-type, heterozygous female, and mutant male mice of the L1 mutant line (Dahme et al., 1997) with a C57BL/6J genetic background were prepared and processed for in situ hybridization analysis as described in chapter 3.1.2.2. For each genotype, consecutive sections were alternatively incubated with either L1 or CHL1 digoxigenin-labeled riboprobes (Holm et al., 1996). L1- and CHL1-positive Golgi cells were counted, the area of the corresponding granule cell layer was determined using the Neurolucida image analysis system, and the density of L1- or CHL1-positive Golgi cells was calculated.

3.4.3 Results

3.4.3.1 Analysis of the Corticospinal Tracts and Lateral Ventricles of Heterozygous Females

The size of the corticospinal tract of L1 mutants is significantly reduced by more than 30% when compared to wild-type littermates (Dahme et al., 1997). To analyze whether heterozygous females are similarly affected, the size of the corticospinal tract was analyzed in these animals. A reduced size of the corticospinal tract of mutant males is already evident by macroscopic inspection of the ventral aspect of the medulla (not shown). Macroscopic inspection of the medulla of wild-type and age-matched heterozygous females, however, revealed no apparent difference in the size of the corticospinal tract between both genotypes (Fig. 20). Analysis of semithin sections revealed areas of the corticospinal tracts of 207,100 ± 37,705 μm² and 210,505 ± 45,230 μm² for wild-type and heterozygous female mice, respectively (mean ± SD, n = 16 for each
group; P=0.25; Fig. 20 and 21). The area of the corticospinal tract of an L1 mutant male was determined as a control and was reduced in size by about 40% when compared to wild-type males or wild-type or heterozygous females, confirming previous observations (Dahme et al., 1997).

**Figure 20:** The corticospinal tract of wild-type and heterozygous female mice. Analysis of cross sections of corticospinal tracts (CST) of wild-type (a) and heterozygous (b) female mice revealed no significant differences in size. The corticospinal tract is outlined by arrow heads. Scale bar 400 μm.

**Figure 21:** Size of the corticospinal tract in different genotypes. The size of the corticospinal tract was determined in wild-type females (wt), heterozygous females (het), and an L1 mutant male (mut). The corticospinal tract of the mutant mouse is significantly smaller (by 40%) than those of wild-type and heterozygous mice. The size of the corticospinal tract of wild-type and heterozygous females is not significantly different (P=0.25). Bars indicate average values (± SD, n=16 for wt, n=16 for het, and n=1 for mut).
The lateral ventricles of some L1 mutant males were significantly increased when compared to wild-type littermates (see chapter 3.3; Dahme et al., 1997). Dramatically enlarged ventricles were, however, only observed in mutant males derived from a mouse line that was maintained in a C57BL/6J genetic background (Dahme et al., 1997). Mutant males derived from a mouse line with a predominant 129/SvEv genetic background developed apparently normal lateral ventricles (Cohen et al., 1997; Dahme et al., 1997). However, a slight but significant enlargement of lateral ventricles has recently been demonstrated also for 129/SvEv L1 mutant mice using high resolution magnetic resonance imaging (Fransen et al., 1998a). The analysis of heterozygous females of the L1 mutant mouse line maintained in a C57BL/6J genetic background did not reveal significantly enlarged lateral ventricles when compared to wild-type littermates (n=16 for heterozygous and n=16 wild-type animals; Fig. 22).

Figure 22: Size of lateral ventricles of heterozygous and wildtype females. Carrier (heterozygous) females of the L1 mutant mouse line cross-bred into a C57BL/6J background have no enlarged lateral ventricles (a) when compared to wild-type littermates (b). Scale bars 1 mm.

Other defects which were usually observed for L1 mutant males include, for instance, a significantly reduced body weight; increased mortality during embryonic development; a reduced life span; lacrimous eyes that were further back in their sockets; or abnormally long hind-paw toe nails (Cohen et al., 1997; Dahme, 1998; Dahme et al., 1997). All these defects were not observed for female mice heterozygous for the L1 mutation (our unpublished observations), and we therefore conclude that heterozygous L1 females lack a detectable phenotype, typically observed for L1 mutant males.
Projects

The Density of Golgi Cells Expressing Wild-Type L1 Transcripts in Female Mice Heterozygous for the L1 Mutation

The L1 gene is located on the X-chromosome, and in females one X-chromosome is inactivated at random early during development. The apparent lack of a phenotype of females heterozygous for the L1 mutation thus suggests that only a portion of all normally L1-positive cell types has to express L1 to allow normal brain development. Alternatively, cells expressing the mutated L1 may be eliminated preferentially during the period of developmental cell death, resulting in a nervous system consisting mainly of cells expressing wild-type L1.

To discriminate between both possibilities, we studied the density of Golgi cells expressing L1 mRNA in the cerebellar cortex of wild-type and heterozygous females (Fig. 23). Cerebellar tissue from an L1 mutant male was used in addition to control the specificity of the L1 in situ hybridization signal, and L1-positive cells were not detected in the mutant (Fig. 23).

Qualitative analysis revealed that the density of L1-positive Golgi cells in the cerebellar cortex of heterozygous females appeared to be reduced when compared to wild-type females (Fig. 23). L1 and CHL1 transcripts are co-localized in cerebellar nerve cells (Dahme et al., 1997; Holm et al., 1996). A similar density of L1- and CHL1-positive Golgi cells in the cerebellar cortex of wild-type mice confirms this result (Fig. 23). In heterozygous females, however, the density of L1-positive Golgi cells is apparently reduced when compared to the density of CHL1-positive Golgi cells in the same animal which were visualized in consecutive tissue sections (Fig. 23).

Quantitative analysis confirmed a significantly reduced density of L1-positive Golgi cells in heterozygous females (34.8 ± 11.4 cells/mm²; mean ± SD, n = 9 for each genotype; P<0.01) when compared to wild-type females (71.5 ± 13.4 cells/mm²). Furthermore, we found a similar density (P=0.95) of L1-positive (71.5 ± 13.4 cells/mm²) and CHL1-positive (70.2 ± 11.1 cells/mm²) Golgi cells in the cerebellar cortex of wild-type mice (Fig. 24a). In heterozygous females, in contrast, the density of L1-positive Golgi cells (34.8 ± 11.4 cells/mm²) was significantly reduced when compared to CHL1-positive Golgi cells (60.8 ± 11.6 cells/mm²; n=9 for each genotype; P<0.01; Fig. 24a).
Figure 23: L1 and CHL1 transcripts in Golgi cells of the cerebellum of different genotypes. Wild-type (a,b) and heterozygous (c,d) L1 mice are analyzed for L1 (a,c) and CHL1 (b,d) mRNA expression in Golgi cells of the internal granular layer (igl) of the cerebellum. The density of L1 expressing Golgi cells is significantly decreased in heterozygous animals when compared to wild-type mice and to CHL1-positive Golgi cells in all genotypes. L1 mutant mice do not express detectable levels of L1 transcripts, and expression levels of CHL1 mRNA and density of positive cells in the mutant is similar as in wild-type mice. mol: molecular layer; scale bars 3 mm.

Analysis of toluidin-blue stained semithin sections confirmed a similar density of Golgi cells in the cerebellar cortex of heterozygous and wild-type mice (85 and 83 cells/mm², respectively; n=2 for each genotype; P=0.84; Fig. 24b).

Together, data demonstrate a normal density of Golgi cells in the cerebellar cortex of female mice heterozygous for the L1 mutation, and expression of wild-type L1 by about 50% of all Golgi cells in heterozygous females.
Figure 24: Density of Golgi cells in the cerebellum of heterozygous and wild-type L1 mice. (a) L1 and CHL1 expressing Golgi cells in the internal granular layer of the cerebellum were analyzed. Heterozygous female (hf) mice show a decreased density of L1-positive Golgi cells when compared to wild-type mice (wt) and when compared to CHL1 expressing Golgi cells of either genotype (n=9 for each genotype; P<0.01). (b) The density of Golgi cells in the cerebellar internal granular layer of heterozygous (hf) and wild-type (wt) L1 mutant mice was additionally analyzed in toluidin-blue stained semithin sections. The total number of Golgi cells is not significantly different between both genotypes (n=2 for each genotype; P=0.84).

3.4.4 Discussion

The L1 gene is located on the X-chromosome and has been mapped to Xq28 (Djabali et al., 1990). Different types of mutations in all regions of the L1 gene cause a severe disease in humans, termed CRASH (Fransen et al., 1995; Wong et al., 1995b). Affected patients have a low IQ, and may display spastic paraplegia, aphasia, and adducted thumbs. Characteristic defects of the brain include enlarged lateral ventricles, and hypoplasia or absence of the corpus callosum or corticospinal tract (reviewed in Fransen et al., 1995; Wong et al., 1995b).

In females, one X-chromosome is inactivated at random very early during neural development (Wong et al., 1995b). The nervous system of carrier females should therefore consist of a mosaic of cells expressing either wild-type L1 or mutated L1. Surprisingly, however, carrier females are considered as healthy
and only a few mildly affected women have been described (Kaepernick et al., 1994).

L1 mutant mice offer the possibility to study in detail whether the development of the nervous system of carrier females heterozygous for an L1 mutation is affected. L1-deficient males display a variety of defects, including reduced body weight, increased mortality, lacrimal eyes that are further back in their sockets, or abnormally long hind-paw toe nails (Cohen et al., 1997; Dahme et al., 1997). All these defects were not observed for L1 heterozygous females. Significantly enlarged lateral ventricles and a reduced size of the corticospinal tract are characteristic abnormalities of the brain of L1 mutant males. A detailed morphological analysis of the brain of heterozygous females revealed normal development of the ventricular system and of the corticospinal tract.

Our observations demonstrate that heterozygous female mice lack a detectable phenotype and support the view that heterozygous women are not affected by CRASH syndrome. Results suggest that about 50% of all normally L1-positive cells have to express wild-type L1 to allow normal brain development. Alternatively, cells expressing mutated L1 may be eliminated during the period of developmental cell death, resulting in a nervous system mainly composed of cells expressing wild-type L1.

Determination of the percentage of Golgi cells expressing wild-type L1 in the cerebellar cortex demonstrates that the nervous system of carrier females consists of a mosaic of cells expressing wild-type or mutated L1. The weak phenotype of a few carrier women (Kaepernick et al., 1994) may be due to an inactivation of the X-chromosome with the wild-type L1-allele in an unusually high percentage of cells. Analysis of one affected heterozygous woman supports this view (Vance Lemmon, personal communication).

Hypoplasia of the corticospinal tract in the L1 mutant (Dahme et al., 1997) is likely the result of pathfinding errors of corticospinal tract axons at the pyramidal decussation (Cohen et al., 1997). Normal development of the corticospinal tract in heterozygous L1 mutants may relate to the correct outgrowth of corticospinal axons expressing wild-type L1. These L1 positive axons may then be used by L1-negative axons as guidance cues and enable L1-negative corticospinal tract axons to reach their correct targets.
Mutations in the L1 gene may result in enlarged ventricles and eventually in macrocephaly. The developmental events leading to this abnormality are not yet understood, but it has been speculated that enlarged ventricles develop "ex vacuo" by loss of brain parenchyma (e.g., Fransen et al., 1998a; Kamiguchi et al., 1998a). A portion of cells expressing mutated L1 may fail to innervate their correct target or may fail to migrate to their correct locations in the brain. Degeneration of a large number of such cells may then result in formation of enlarged ventricles.

In heterozygous females, L1-negative nerve cells may use processes of L1-positive neurons as guidance cues to project to their normal targets similarly as has been proposed for corticospinal tract axons. Nerve cells often migrate as “tandems” or streams of cells from the site of origin to their final locations (e.g., Altman, 1972; Lois et al., 1996). L1-negative nerve cells may thus reach their proper locations in the brain by L1-independent interactions with normally migrating L1-positive nerve cells. Given that only a fraction of all normally L1-positive cells has to express wild-type L1 suggests that somatic gene therapy may be a promising approach to repair the phenotype of L1 mutant mice. Experiments are currently performed, aimed to transfer the wild-type L1 gene into neuronal cells of mutant male mice using a replication-defective adenoviral vector.
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REFERENCES


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### Abbreviations

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>aa</td>
<td>amino acids</td>
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<tr>
<td>ABGP</td>
<td>ankyrin-binding glycoprotein</td>
</tr>
<tr>
<td>aFGF</td>
<td>acidic fibroblast growth factor</td>
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<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
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<tr>
<td>BGP</td>
<td>biliary glycoprotein</td>
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<td>BIG-1</td>
<td>brain-derived immunoglobulin superfamily molecule 1, PANG (plasmacytoma-associated neuronal glycoprotein)</td>
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<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
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<tr>
<td>BMPs</td>
<td>bone morphogenetic proteins</td>
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<tr>
<td>BSA</td>
<td>bovine albumine serum</td>
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<tr>
<td>CAMs</td>
<td>cell adhesion molecules</td>
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<tr>
<td>CD</td>
<td>cluster of differentiation (defined by the leukocyte typing workshops)</td>
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<tr>
<td>CEA</td>
<td>carcinoembryonic antigen</td>
</tr>
<tr>
<td>CHL1</td>
<td>close homolog of L1</td>
</tr>
<tr>
<td>cis</td>
<td>in the same plane, e.g., on the same membrane</td>
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<td>CNS</td>
<td>central nervous system</td>
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<tr>
<td>CNTF</td>
<td>ciliary neurotrophic factor</td>
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<td>CRASH</td>
<td>corpus callosum hypoplasia, retardation, adducted thumbs, spastic paraplegia, hydrocephalus</td>
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<td>CST</td>
<td>corticospinal tract</td>
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<td>CTLA-4</td>
<td>cytolytic T lymphocyte-associated antigen 4</td>
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<tr>
<td>DAB</td>
<td>3,3'-diaminobenzidine-4HCl</td>
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<td>DAG</td>
<td>diacylglycerol</td>
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<td>DCC</td>
<td>deleted in colorectal carcinoma</td>
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<td>dorsal root ganglion</td>
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<td>extracellular matrix</td>
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<td>embryonic day</td>
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<td>Eph</td>
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<tr>
<td>F11</td>
<td>neural cell recognition molecule F11, contactin, F3, neuro-1 antigen</td>
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<td>ICAM</td>
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<td>Ig</td>
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<td>IGF</td>
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<td>i.p.</td>
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<td>NCAM</td>
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<td>Nr-CAM</td>
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### 6. APPENDIX

#### LICAM MUTATIONS (93 different mutations, found in 101 unrelated families)


Last update: March 22, 1999

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<th>Exon / Intron</th>
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8. **CURRICULUM VITAE**

**PERSONAL DATA:**

Name, First Name: Lang, Doris  
Date of Birth: May 15, 1964  
Place of Birth: Obererlinsbach, Switzerland  
Citizenship: Swiss

**EDUCATION:**

- **1971 - 1976**  
  Primary School, Obererlinsbach, SO, Switzerland

- **1976 - 1980**  
  Secondary School, Aarau, AG, Switzerland

- **1980 - 1984**  
  Gymnasium (Kantonsschule), Type E, Aarau, AG

- **1984**  
  Diploma (Maturitätszeugnis), Type E

- **1984 - 1986**  
  Studies of biochemistry, University of Zürich, Zürich, Switzerland

- **1987 - 1991**  
  Practical training and employment in a bank

- **1991**  
  English language study, International English Center, University of Colorado, Boulder, USA (TOEFL Test)

- **1992 - 1993**  
  Undergraduate studies in Molecular, Cellular and Developmental Biology (MCDB), University of Colorado, Boulder, USA

- **1993**  
  Bachelor’s of Arts (BA) in Molecular, Cellular and Developmental Biology

- **1993 - 1995**  
  Graduate studies in psychology, Behavioral Neuroscience Program, Dept. of Psychology, University of Colorado, Boulder, USA

- **1995**  
  Master’s thesis (May 12, 1995)  
  Advisor: Prof. E. Fifková, Title: Fine Structure of Hippocampal Dendrites in the Dentate Fascia of LS/SS-Mice after Chronic Ethanol Treatment

- **1995 - 1999**  
  Ph.D. graduate student and research assistant in biology, Inst. of Neurobiology, Swiss Federal Institute of Technology (ETH), Zürich, Switzerland

- **1999**  
  Ph.D. thesis defense (November 3, 1999)  
  Advisors: Prof. M. Schachner and Prof. M.E. Schwab
Publications:

Published:


Submitted:


In preparation:

Lang, D., Hillenbrand, R., Zhang, Y., Anderson, P.N., Schachner, M., and Bartsch, U., CHL1, a Cell Recognition Molecule Closely Related to L1, is Expressed at Elevated Levels by CNS Glial Cells upon Injury or Application of bFGF.

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