Functional analysis of the cell adhesion molecules L1, CHL1 and NCAM *in vivo*

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Project 3: Pathfinding errors of corticospinal tract axons in NCAM-deficient mice

Abstract

Cell recognition molecules of the immunoglobulin superfamily have been implicated in several processes of nervous system development, such as cell migration, axon outgrowth and fasciculation, myelination and synapse formation. They are characterized by the existence of at least one immunoglobulin like domain, enabling them to mediate cell adhesion in a calcium independent manner.

The neural adhesion molecule L1 is a member of the immunoglobulin superfamily and the founder of the L1 subfamily of cell recognition molecules. It performs important functions in the developing and adult nervous system. This view is confirmed by the fact that mutations in the human L1 gene cause a severe neurological disease, termed CRASH (acronym for: corpus callosum hypoplasia, retardation, adducted thumbs, spastic paraplegia, and hydrocephalus). X-linked hydrocephalus is certainly the most prominent symptom of CRASH syndrome. Mouse mutants deficient in L1 also develop enlarged ventricles. This study demonstrates that ventricular dilation in L1-deficient mice is not correlated with stenosis of the aqueduct of Sylvius nor with ultrastructural abnormalities of ependymal cells lining the lateral ventricles or the aqueduct. However, a few L1 mutant mice displayed severe hydrocephalus, characterized by a significant enlargement of the skull and an almost complete atrophy of the cerebral cortex. The aqueduct of these severely affected animals was completely closed. Since mutant animals from two independently generated L1-deficient mouse lines displayed a similar phenotype, severe hydrocephalus is considered as a specific consequence of L1-deficiency. However, results of the present study also indicate that severe hydrocephalus represents a secondary rather than a primary defect of the L1 mutation; the combined data suggest that deformations of the brain as a result of

massively enlarged ventricles secondarily cause stenosis of the aqueduct and subsequently high pressure hydrocephalus.

Another member of the L1 family of cell recognition molecules is the close homologue of L1 (CHL1). This protein is expressed by a variety of nerve cell types and subpopulations of glial cells *in vivo* and promotes elongation of neurites and survival of nerve cells *in vitro*. Here, it is demonstrated that glial cells up-regulate expression of CHL1 in response to an intraorbital crush of the adult mouse optic nerve. It is also demonstrated that a single intravitreal application of fibroblast growth factor-2 (FGF-2) increases expression of CHL1 in retinal astrocytes and Müller cells. Elevated expression of CHL1 by astrocytes in injured optic nerves and by astrocytes and Müller cells in FGF-2-treated retinas suggests a role of the protein in the lesioned central nervous system. Results also suggest that trophic factors might exert part of their biological functions by modifying expression of cell recognition molecules.

The functions of a third member of the Ig superfamily, the neural cell adhesion molecule (NCAM), were also investigated. NCAM is implicated in cell migration, myelination, synaptic plasticity, and elongation, fasciculation and pathfinding of axons. In the present study, NCAM-deficient mice were used to investigate the role of NCAM in the development of the corticospinal tract. A severe hypoplasia of the corticospinal tract in adult NCAM mutants is demonstrated. Anterograde tracing of the tract of early postnatal NCAM mutants revealed pronounced pathfinding errors of corticospinal axons. At the pyramidal decussation of mutant mice, some corticospinal axons either stayed ventrally and extended laterally or axons turned dorsally, but instead of growing to the contralateral dorsal column, a significant fraction of axons projected ipsilaterally. Moreover, corticospinal axons of NCAM mutants entered the pyramidal decussation significantly later than axons of wild-type littermates.

Together the findings of this study demonstrate the importance of L1 and NCAM for the normal development of the nervous system. They also suggest a critical role for CHL1 in the lesioned central nervous system.

Zusammenfassung

Zellerkennungsmoleküle der Immunglobulin-Superfamilie werden mit einigen Vorgängen während der Entwicklung des Nervensystems in Verbindung gebracht, wie zum Beispiel Zellmigration, Axonwachstum und Faszikulierung, Myelinisierung und Synapsenbildung. Sie sind characterisiert durch die Existenz mindestens einer Immunglobulin ähnlichen Domäne, die es ihnen ermöglicht Zelladhäsion auf eine Calcium unabhängige Weise zu vermitteln.

Das neurale Zellerkennungsmolekül L1 ist ein Mitglied der Immunglobulin Superfamilie und der Gründer der L1 Unterfamilie. Es führt wichtige Funktionen im sich entwickelnden und im adulten Nervensystem aus. Dieser Standpunkt wird bekräftigt durch die Tatsache, dass Mutationen im humanen L1 Gen eine schwere neurologische Krankheit verursachen, die CRASH genannt wird (ein Akronym für corpus callosum hypoplasia, retardation, adducted thumbs, spastic paraplegia, hydrocephalus). X-Chromosom gebundener Hydrocephalus ist sicherlich das markanteste Symptom des CRASH-Syndroms. Maus-Mutanten, denen das L1 Gen fehlt, entwickeln ebenfalls erweiterte Ventrikel. Diese Studie zeigt, dass erweiterte Ventrikel in L1 defizienten Mäusen weder mit einer Stenose des Aqaeductus Sylvius, noch mit ultrastrukturellen Abnormalitäten von Ependymalzellen entlang der lateralen Ventrikel und des Aquaeductes einhergehen. Dennoch wiesen einige L1-Maus-Mutanten einen schwerwiegenden Hydrocephalus auf, charakterisiert der durch eine signifikante Vergrößerung des Schädels und fast vollständige Atrophie des cerebralen Cortex. Der Aquaeduct dieser stark betroffenen Tiere war vollständig verschlossen. Da mutierte Tiere aus zwei unabhängig voneinander hergestellten L1 defizienten Maus-Linien ähnliche Phänotypen aufweisen, wird die schwere Form des Hydrocephalus als eine spezifische Folge der L1-Defizienz angesehen. Jedoch weisen die Ergebnisse dieser Studie auch daraufhin, dass die schwerwiegende Form

des Hydrocephalus eher einen sekundären als einen primären Defekt der L1-Mutation darstellt; die zusammengefassten Daten legen nahe, dass Verformungen des Gehirns verursacht durch die massiv vergrößerten Ventrikel sekundär eine Stenose des Aquaeductes und Hydrocephalus mit starkem Druck zur Folge haben.

Ein weiteres Mitglied der L1-Familie von Zellerkennungsmolekülen ist das „close homologue of L1“ (CHL1). Dieses Protein wird von einer Vielzahl von Nervenzellen und Subpopulationen von Gliazellen *in vivo* expremiert und fördert Neuritenwachstum und das Überleben von Nervenzellen *in vitro*.

Hier wird gezeigt, dass Gliazellen die Expression von CHL1 hochregulieren können als Folge einer intraorbitalen Quetschung des adulten optischen Nerven. Es wird weiterhin gezeigt, dass eine einzelne intravitreale Injektion des Fibroblastenwachstum fördernden Faktors 2 (FGF 2) die Expression von CHL1 in retinalen Astrozyten und Müllerzellen steigert. Die erhöhte Expression von CHL1 in Astrozyten verletzter optischer Nerven und in Astrozyten und Müllerzellen in FGF 2 behandelten Retinas legt nahe, dass dieses Protein im lädierten zentralen Nervensystem eine Rolle spielt. Die Ergebnisse deuten ebenfalls daraufhin, das trophische Faktoren möglicherweise einen Teil ihrer biologischen Funktionen ausführen, indem sie die Expression von Zellerkennungsmolekülen modifizieren.

Die Funktionen eines dritten Mitgliedes der Ig-Superfamilie, das neurale Zelladhäsionsmolekül (NCAM), wurden ebenfalls untersucht. Es wird in Verbindug gebracht mit Zellmigration, Myelinisierung, synaptischer Plastizität und Verlängerung, Faszikulierung und Wegfindung von Axonen. In dieser Studie, werden NCAM defiziente Mäuse verwendet, um die Funktion von NCAM während der Entwicklung des corticospinalen Traktes zu untersuchen. Es konnte eine schwere Hypoplasie des corticospinalen Traktes in adulten NCAM defizienten Mäusen nachgewiesen werden. Anterogrades Tracen des Traktes von früh postnatalen NCAM-Mutanten, zeigten

ausgeprägte Wegfindungsfehler corticospinaler Axone. An der Pyramiden-Kreuzung von Maus-Mutanten blieben einige corticospinalen Axone entweder ventral und weiteten sich nach lateral aus, oder die Axone wendeten sich dorsal, aber statt in den contralateralen dorsalen Funiculus, projezierte eine signifikante Fraktion der Axone ipsilateral. Darüberhinaus traten corticospinale Axone von NCAM-Mutanten signifikant später in die pyramidale Kreuzung ein als Axone von Wildtypgeschwistern.

Zusammengefasst weisen die Ergebnisse dieser Arbeit auf die Bedeutung von L1 und NCAM für die normale Entwicklung des Nervensystems hin. Sie deuten auch eine wichtige Funktion von CHL1 im lädierten zentralen Nervensystem an.

General Introduction

During development of the nervous system, neurons start to migrate and to extend axons in order to find their final functional position and to innervate their appropriate targets. These developmental processes are crucially dependent on cell-cell and cell-matrix interactions. Migrating cells and outgrowing processes orient themselves along short range and long range, attractive or repulsive guidance cues. These guidance cues can be expressed on the cell surface of for instance glial cells or pioneering axons, or they are secreted into the extracellular matrix.

One prominent group of molecules regulating cell-cell or cell-matrix are cell recognition molecules. There exist three main families of molecules, the integrins, the cadherins and the immunoglobulin superfamily (Ig superfamily). Characteristic for members of the Ig superfamily is the existence of at least one Ig-like domain, which allows them to mediate cell adhesion via a calcium independent mechanism. Three groups of subfamilies have been distinguished within the Ig superfamily: those molecules, that contain only Ig-like domains, molecules that posses Ig and fibronectin type III (FN III) like domains and a third group comprising molecules with a mixture of Ig-like and different domains (Brümmendorf et al. 1993).

This work will focus on the second subgroup of Ig superfamily members, specifically on members of the L1 subfamily, L1 and CHL1 and also on the neural cell adhesion molecule (NCAM).

The L1 family of cell recognition molecules

The L1 family is a small subfamily within the Ig superfamily of cell recognition molecules. The founding member is L1 in mouse and human and species homologues include L1.1 and L1.2 in zebrafish (Tongiorgi et al. 1995), neuroglian in invertebrates (Bieber et al. 1989), neuron-glial cell adhesion molecule (NgCAM) in

chicken (Grumet et al., 1984) and NILE in rats (Salton et al., 1983). Additionally three closely related proteins have been identified, which are the close homologue of L1 (CHL1) (Holm et al. 1996), NgCAM related cell adhesion molecule (NrCAM) (Grumet et al., 1991; Kayyem et al., 1992), and neurofascin (Volkmer et al., 1992).

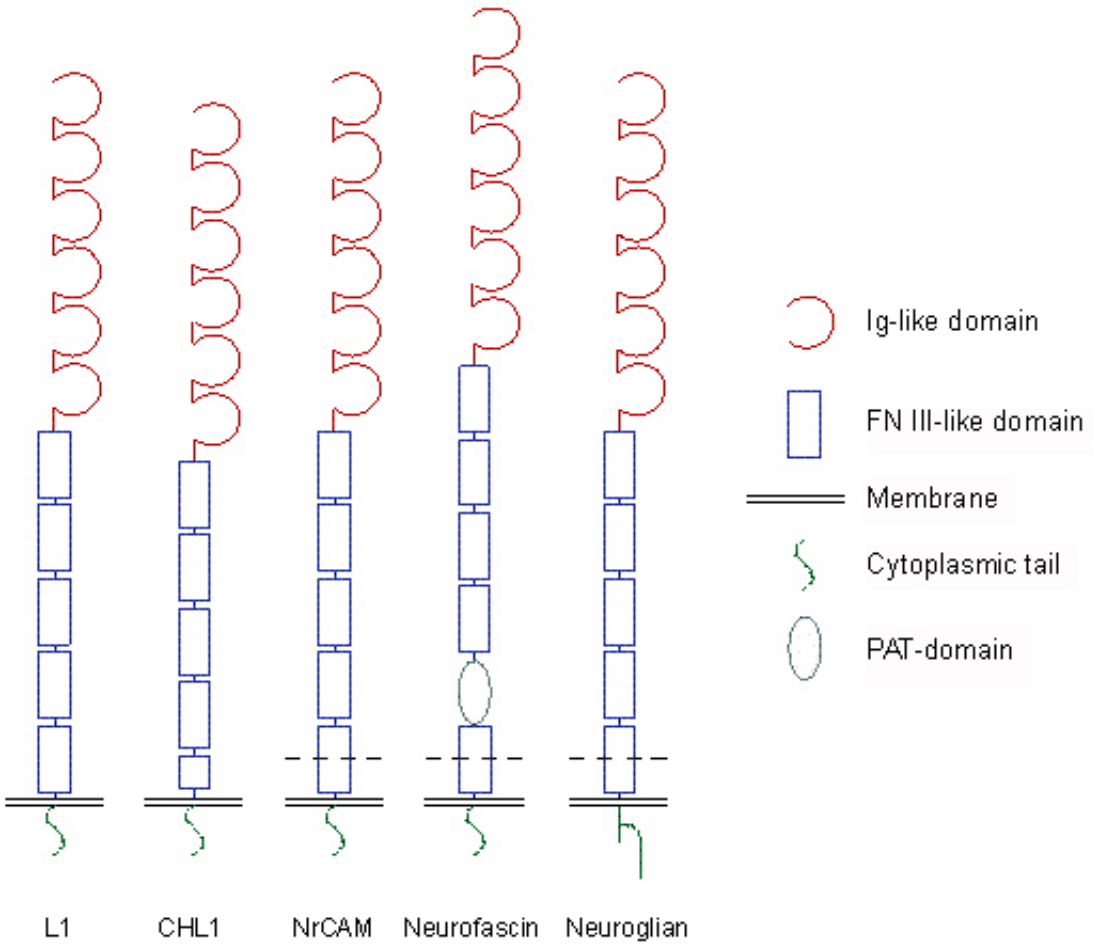

Figure 1: Modular structure of the different members of the L1 family.
The distinct modular structure of the L1 family members are displayed in this schematic drawing. The domains are specified in the list next to the scheme. PAT stands for proline-, alanine- and threonine rich domain.

All members of this family share a similar modular structure of six Ig-like domains, at least 4 FN III like domains, a transmembrane stretch and a highly conserved cytoplasmic tail (Fig. 1). Among the different molecules the similarity of the extracellular domain ranges from 26% to 43%. The intracellular domain is more

conserved, showing an average identity of 60%. Notably the intracellular domain between species homologues might show a homology of 99%, as is demonstrated for NrCAM of mouse and chicken (Holm et al 1996). Their high degree of conservation during evolution is indicative for the functional importance of these proteins.

Interestingly, the expression patterns of all L1 family members are very similar. Generally they appear relatively late during development, coinciding with the onset of axogenesis. In both the CNS and the PNS, their expression is mainly restricted to neurons, in particular to their axons (Hortsch 1996). While Schwann cells express most L1 molecules, NrCAM is also expressed by floorplate cells and CHL1 by astrocytes (Grumet et al., 1991; Stoeckli et al., 1995; Holm et al., 1996).

Several members of the L1 family can bind homophilically, i.e. one molecule binds to another of the same type (Kunz et al 1998, Kadmon et al. 1990, Mauro et al 1992). This might happen in *cis*, which means molecules interact homophilically on the same cell surface, forming clusters. It might also occur in *trans*, connecting two molecules on the surface of different cells. Homophilic interactions have been demonstrated to involve different Ig-like domains. For L1, for example, the second Ig-like domain has been identified as the homophilic binding site (Zhao et al., 1998). Additionally, there are also heterophilic binding partners known. Heterophilic binding partners include constituents of the extracellular matrix, such as laminin, neurocan or phosphacan. Some family members are also capable to bind to other members of the Ig-superfamily, like NCAM, or to integrins (Kadmon et al., 1990; Hortsch 2000). Finally, different members of the L1 family might interact with each other, as exemplified by the binding of NrCAM to neurofascin (Volkmer et al., 1996).

Binding mechanisms involved in neurite outgrowth have been studied extensively up to now, revealing different mechanisms that are important for this process. In *vitro*

studies on neurofascin have elucidated that this molecule promotes neurite outgrowth only, if presented in a substrate-bound form, pointing to the significance of its adhesive properties (Volkmer et al., 1996). L1 and CHL1 can promote neurite extention also when presented in soluble form, implying that in addition to adhesive functions also signal transducing events are necessary for cell recognition molecules to mediate neurite outgrowth (Hillenbrand et al., 1999). In this context it is interesting, that L1 molecules do not posses an intracellular catalytic domain. Thus binding partners accomplishing these criteria are essential. One putative candidate is the fibroblast growth factor receptor (FGF-R), since it displays an amino acid sequence within the extracellular domain that can also be recognized within the extracellular domains of L1, NrCAM and neurofascin (Doherty and Walsh 1996). The following model of FGF-R activation is suggested: after homophilic binding of L1 in *trans*, this complex binds in *cis* to the homologous sequence within the extracellular domain of the FGF-R**,** leading to its phosphorylation without binding of FGF. The phosphorylation of FGF-R subsequently activates a complex second messenger cascade, finally leading to an opening of calcium channels. Calcium dependent cytoskeleton modifiers then in turn regulate growth cone movement (Doherty and Walsh, 1996).

Additionally, L1 has been shown to be itself a substrate of phosphorylation. Serine phosphorylation, by p90srk, casein kinase II or ERK2, has an influence on the mobility of the L1 molecule within the membrane and on its internalization (reviewed in Crossin and Krushel 2000). This process is suggested to be required for activation of the mitogen activated protein kinase pathway, which is important during neurite outgrowth (Schaefer et al 1999; Schmid et al 2000).

In this context the phosphorylation of a tyrosine residue within the intracellular motif FIGQY is also of great interest, since this amino acid sequence is highly conserved

among almost all vertebrate L1 family members (Davies and Bennett 1994). It regulates the binding of ankyrin, a linker protein to the spectrin-based cytoskeleton situated beneath the plasma membrane (Jenkins et al., 2001). Ankyrin has been shown to be involved in two different aspects of nervous system development. First, it plays an important role in the formation of nodes of Ranvier. At the onset of myelination, neurofascin and NrCAM form clusters at sites of node formation. These clusters bind to ankyrin, and these stable complexes lead in turn to the recruitment of voltage gated Na-channels and Na/K-ATPase to the nodes, which is important for the generation action potentials at these specialized sites of myelinated axons (Lustig et al., 2001). Second, ankyrin is an important factor of axonal stabilization. In case of cell-cell contact mediated by hetero- or homophilic L1 binding, ankyrin accumulates at these sites and binds to spectrin. The concentration of cytoskeletal proteins, mediated by L1, leads to the stabilization of the axon itself, and also to the stabilization of L1 within the membrane, a process that is important for axonal fasciculation (Burden-Gulley et al., 1997; Scotland et al., 1998; Needham et al., 2001).

L1

The fact that mutations in the human L1 gene cause severe morphological syndromes in human highlights the importance of the protein for normal brain development. The human disease caused by L1 mutations is called CRASH, an acronym for corpus callosum hypoplasia, retardation, adducted thumbs, spastic paraplegia and hydrocephalus (Fransen et al., 1995). The estimated incidence of this syndrome ranges from one in every 25000 to one in every 60.000 male births (Halliday et al., 1986). The clinical picture varies strikingly among the affected patients even within the same family (Willems et al 1987). This variability refers to

the number and types of syndromes, but it also applies to the severity of each phenotype. Hydrocephalus, mental retardation and spasticity of the lower limbs are the most consistent pathological features of CRASH patients. The L1 gene is located at the locus Xq 28 within the X-chromosome. Up to date more than 140 different pathogenic mutations in this gene are known (L1CAM Mutation Web page: http://www.uia.ac.be/dnalab/l1 (Van Camp et al., 1996). A classification has been suggested relating intensity of the phenotype to the type and location of the mutation. Three different classes have been established. The first class comprises mutations within the intracellular domain, the second summarizes missense point mutations in the extracellular domain and the third class is assigned to missense or frameshift mutations that result in a premature stop codon, and thus in a truncation of the extracellular domain (Yamasaki et al., 1997). The severity is highest among patients suffering from class three mutations. This is expected, since due to the truncation of the extracellular part of L1 the molecule is no longer attached to the cell surface, which in turn abolishes its adhesive functions and the signaling mechanisms induced by L1. About 50% of patients affected by class three mutations did not survive the first year of life and about 93% suffered from severe hydrocephalus and grave mental retardation (Yamasaki et al., 1997).

The phenotype among patients affected by missense mutations in the extracellular domain of L1 varies extensively. About 32% of these patients did not survive the first year of life. About 26% of patients display no hydrocephalus, and moderate and severe forms of hydrocephalus are found in 26% and 48% respectively. The extent of mental retardation exhibited by these patients also varied from modest (73%) to grave (27%) (Yamasaki et al., 1997). The second class comprises mutations restricted to only short parts of the extracellular domain of L1. The mutations might be of varying importance for the functional properties of the molecule. Mutations of

surface residues, for instance, have a minor effect on L1 function, while mutations of key residues directly affect homophilic or heterophilic binding or they have an impact on the structural integrity of individual domains resulting in the loss of homophilic, heterophilic or both binding patterns (Fransen et al., 1998 a,b; De Angelis et al., 1999). In this context it is interesting that a point mutation of the cystein residue within the third Ig like domain results in a functional knock out. The molecule is normally translated, but due to the mutation, the protein is aberrantly folded and not transfered from the endoplasmatic reticulum to the Golgi apparatus, thereby failing to reach the cell surface (Rünker et al., in preparation). Remarkably, impaired trafficking of L1 to the cell surface has also been demonstrated for a variety of pathogenic mutations *in vitro*. (Moulding et al., 2000; De Angelis et al., 2002)

The modest expression of the disease is caused by mutations within the intracellular domain. Here signaling mechanisms are potentially affected, while the adhesive functions of L1 are likely to be intact. All patients of this class survive more than one year, a severe form of hydrocephalus was only found in 6% of the investigated cases and about 76% of the patients suffer from a moderate form of mental retardation (Yamasaki et al., 1997). Adducted thumbs, however, is a feature of the CRASH syndrome, which is very constantly expressed in all three classes of mutations (Kenwrick et al., 1996; Fransen et al., 1998; Yamasaki et al., 1997).

In order to investigate the molecular and cellular mechanisms that result in the phenotypic expression of this disease more effectively, knock out mice have been generated. One mutant mouse was generated by Dahme et al. in 1997 by insertion of neomycine/thymidine kinase cassette into the exon 8. The mutant still expressed a variant form of L1 but only at very low levels. The second L1 mutant was generated by replacing exon 13 and 14, encoding the sixth Ig-like domain, leading to a complete null mutation (Cohen et al., 1997). Both L1-defiecient mouse lines

displayed a similar phenotype, including dilated ventricles, hypoplasia of the corticospinal tract with pathfinding errors at the pyramidal decussation, motor incoordination, a reduced sensitivity to touch and pain, malformations of subsets of cortical dendrites and a reduced size of the corpus callosum (Cohen et al., 1997; Dahme et al 1997; Demyanenko et al., 1999; Fransen et al., 1998). One prominent feature of the mutant mouse generated by Dahme et al., was the development of a hydrocephalus, depending, however, on the genetic background of the animals. A severe form of hydrocephalus developed only when the mutants were maintained in a C57/BL 6J genetic background, suggesting the influence of unknown modifier genes that are involved in the expression of this phenotype.

Surprisingly, only a few, but not all, fiber tracts in the nervous system were affected in the mutant, although the importance for L1 in neurite extension and pathfinding has been demonstrated and postmitotic nerve cells in all brain regions express L1 (Cohen et al 1997; Dahme et al., 1997; Demyanenko et al., 1999). Furthermore, *in vitro* studies have shown a strong influence of L1 on the migration of cerebellar granule cells from the external to the internal granule cell layer (Lindner et al., 1983). Except of a moderate reduction of the size of one cerebellar lobe, the cytoarchitecture of the cerebellar cortex of L1 knock out mutants was not detectably affected (Fransen et al., 1998; Demyanenko et al., 1999). Given the high homology among members of the L1 family, and their similar functional properties and expression pattern, it is reasonable to assume that the loss of one molecule of the L1 family can be compensated by another one (Hortsch, 1996; 2000). Considering the development of the cerebellar cortex, this hypothesis is particularly relevant for NrCAM and L1. Both molecules, L1 and NrCAM are expressed by granule cells and have been implicated in granule cell migration from the external granule cell layer to the internal granule cell layer and also in the proliferation of granule cells (Lindner et

al., 1983; Faivre-Sarrailh et al., 1999; Sakurai et al., 2001). However, L1- and NrCAM knock out mutants had both a cerebellar cortex with a normal cytoarchitecture. The only defect observed in both knock out mutants was a reduced size of distinct cerebellar lobuli (Fransen et al., 1998; Demyanenko et al., 1999; Sakurai 2001). The analysis of double mutants deficient for both cell recognition molecules, L1 and NrCAM, however, revealed a severe cerebellar dysgenesis, with a strongly reduced thickness of the internal granule cell layer. Since in the double mutant not only the size of the cerebellar lobuli was massively reduced, but also the cytoarchitecture of the cerebellar cortex itself was severely affected, the phenotype of the double mutant does not simply result from the addition of the phenotypes of both single mutants. The phenotype of the double mutants rather suggests that L1 and NrCAM compensate for each other *in vivo* (Sakurai et al., 2001). Compensating mechanisms might also be relevant for L1 and CHL1, since both proteins show a considerably overlapping expression pattern in diverse brain regions and display similar functions *in vitro*. In the cerebral and cerebellar cortex for instance, both molecules are completely co-localized and both molecules enhance neurite outgrowth and survival of nerve cells *in vitro* (Holm et al., 1996; Chen et al., 1999; Hillenbrandt et al., 1999). Co-expression of L1 and CHL1 can also be observed in some of the major axon tracts, such as the optic tract, corpus callosum, fimbria, anterior commissure and the corticospinal tract (unpublished observations).

CHL1

Originally, a λgtl1 expression library for cDNA clones was screened with L1 polyclonal antibodies (Tacke et al., 1987), with the aim to find cDNA clones of L1. One of the isolated clones contained a partial cDNA sequence with 34% homology to L1. Subsequently, a full length cDNA clone was isolated, encoding a novel protein of

1209 amino acids with a calculated molecular mass of approximately 135 kD (Holm et al., 1996). This new molecule was then called close homologue of L1 (CHL1). The extracellular domain of CHL1 shows the strongest homology to chicken NrCAM with 39% identity, followed by L1 with 37 % identity, whereas the cytoplasmic domain is closest related to mouse NrCAM with 64% similarity, chicken NrCAM and mouse and rat neurofascin with 62% similarity respectively, followed by L1 with 57% identity (Holm et al., 1996).

Considering structural criteria, as the homology of the Ig and FNIII like repeats within the molecule itself as well as within the L1 family, the distance between the different domains and also the high degree of glycosylation, CHL1 displays all characteristics of the L1 family. Unique among members of the L1 family is a DGEA sequence, an additional integrin-binding motif within the sixth Ig-like domain of CHL1 (Holm et al 1996). However, an interaction of CHL1 with integrins or any other extracellular binding partners have not yet been demonstrated. For many members of the L1 family there is evidence that they can bind to laminin via the HNK-1 carbohydrate. This may also be true for CHL1, which is a carrier of this carbohydrate epitope (Holm et al., 1996). Notably homophilic binding has been excluded for CHL1 (Hillenbrand et al., 1999).

The expression of CHL1 starts around embryonic day 13 and increases up to postnatal day 7. Expression levels of CHL1 decrease with increasing age, but a significant amount of CHL1 is still expressed in the adult (Hillenbrand et al 1999). CHL1 immunoreactivity in newborn mice is mainly found on axons, as for instance in the anterior commissure, the corpus callosum, the optic nerve and the fimbria (unpublished observations). However at post natal day 7, a very strong and ubiquitously distributed CHL-1 immunoreactivity can be detected (unpublished observations), similar to the expression patterns of L1, which can be found on almost

all postmitotic neurons and also on Schwann cells. While the expression patterns of L1 and CHL1 overlap considerably there is one striking difference in that CHL1 transcripts, but not L1 transcripts, can also be found in astrocytes and oligodendrocyte precursor cells (Hillenbrand et al 1999).

The functional properties of CHL1 are not as extensively investigated as those of L1. In *vitro* studies have shown, that CHL1 is able to promote neurite elongation and neurite survival (Chen et al., 1999) Interestingly, recent findings suggest an important role of CHL1 in lesioned nervous tissue, as it was demonstrated that motor neurons, sensory neurons and Schwann cells up-regulate expression of CHL1 in response to peripheral nerve lesions (Zhang et al., 2000). Remarkably, adult neurons of the central nervous system are also able to up-regulate CHL1 expression after lesion when experimentally induced to re-grow their axons into peripheral nerve grafts (Chaisuksunt et al., 2000a,b). Elevated expression of CHL1 in neurons thus correlates with active axonal regeneration.

In order to gain further insights into the functions of CHL1, a CHL1 null mutant was generated by disruption of the ribosomal binding site, the translation initiation codon and the amino terminus including the signal sequence (Montag-Sallaz et al., in press). Similar to the L1 knock out mutant, CHL1 mutants displayed hydrocephalus when kept on a C57/BL6J genetic background. However, most brain regions like the thalamus, the cerebellum, the main fiber tracts and the retina were morphologically indistinguishable from those of wild-type mice. Up to now, only a few morphological abnormalities have been detected in CHL1 mutants. Mossy fibers of the hippocampus, for instance, show pathfinding errors within the CA3 region, in that a few thin bundles or single mossy fibers project through the pyramidal cell layer of the CA3 region, instead of being organized in clearly separated supra- and infrapyramidal bundles. Ectopically formed synaptic contacts at the cell bodies of the

pyramidal cells have been suggested by immunostainings with synaptophysin antibodies. Furthermore, the olfactory bulb is affected. Olfactory neurons in the CHL1 mutant establish contacts to more than one glomerulus, a condition never observed in wild-type mice. Additionally, some axons in the mutant abnormally pass through the glomerular layer and form contacts within the external plexiform layer (Montag-Sallaz et al., in press).

A striking finding in the CHL1 mutant is a significant up-regulation of NCAM 180 in the olfactory bulb, the cortex, the hippocampus and the amygdala, brain regions with high levels of CHL1 expression in the adult and characterized by a high degree of synaptic plasticity throughout life (Montag-Sallaz et al., in press). Both molecules, NCAM and CHL1, have been demonstrated to be involved in neurite outgrowth and pathfinding of hippocampal neurons. These findings demonstrate similar, and perhaps overlapping, functions of both molecules *in vivo*. Thus, the enhanced expression of NCAM in the CHL1 mutants might be indicative for compensatory functions of NCAM in the CHL1-deficient mutant.

NCAM

NCAM was the first cell adhesion molecule identified in the nervous system. It consists of five Ig-like domains and two fibronectin type III like domains (Fig. 2). From all members of the Ig-superfamily, it displays the highest homology to the myelin-associated glycoprotein (MAG) both with respect to sequence and overall structure. (Salzer et al., 1987).

The NCAM gene, which is situated on chromosome 9 in mice (D'Eustacio et al., 1985) and on chromosome 11 in human (Nguyen et al., 1986), gives rise to several different isoforms by alternative splicing of the primary transcript. The major isoforms have a size of 180, 140 and 120 kD, differing only in the size of their cytoplasmic

domain (Fig. 2). The 180 kD and the 140 kD forms are transmembrane molecules, whereas the 120kD form is attached to the membrane by a glycosyl-phosphaditylinositol (GPI) anchor (Gennarini et al., 1984 a,b).

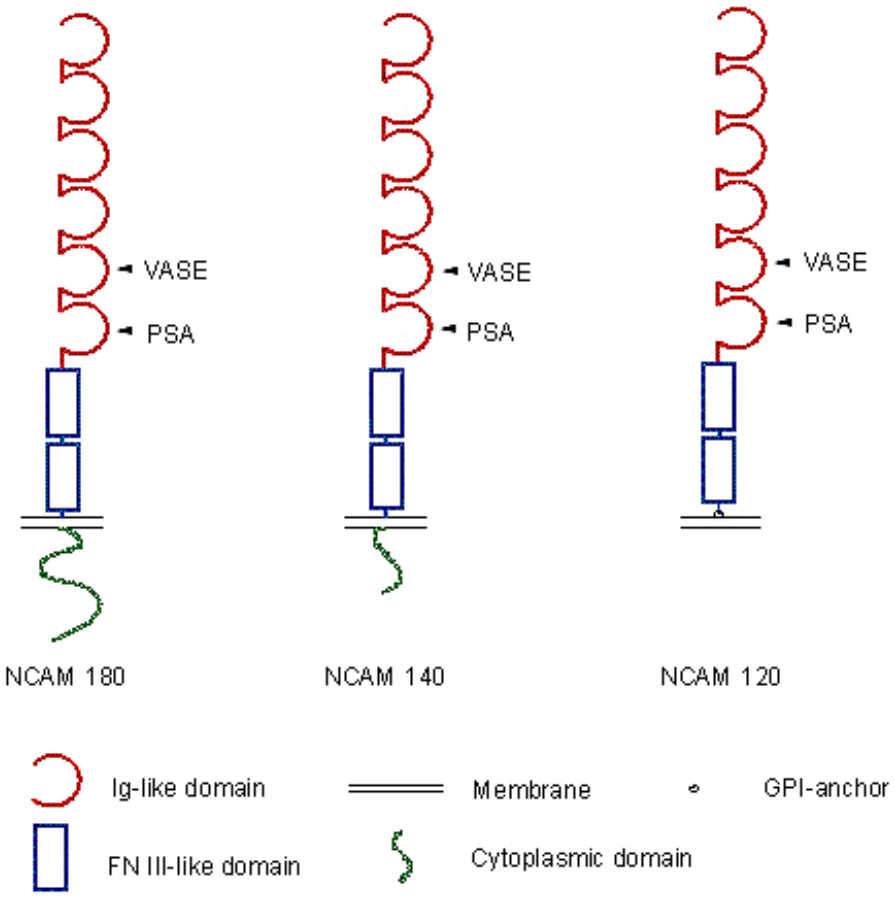

Figure 2: Modular structure of the major isoforms of NCAM
The schematic drawing displays the modular structure of the three major isoforms of NCAM. The different domains are described underneath the scheme. PSA stands for polysialic acid and VASE is an acronym for variable alternative spliced exon. Arrowheads indicate the attachment site of PSA and the insertion site of VASE.

Expression of NCAM starts very early during development. In Xenopus, for instance, first NCAM transcripts can be detected already 2 hours after neural induction (Balak et al., 1987). Generally NCAM is very ubiquitously expressed. It can be found on nearly all postmitotic neurons, on Schwann cells, oligodendrocytes, astrocytes and muscle cells (Moore and Walsh, 1986; Neugebauer et al., 1988; Seilheimer and

Schachner, 1988). Some cell types or subcellular structures specifically express one of the three main isoforms. The 180 kD form, for instance, is strongly associated with synapses. Its long cytoplasmic tail interacts with the spectrin-actin cytoskeleton and thereby stabilizes synapse formation (Pollerberg et al., 1987; Persohn et al. 1989). Astrocytes mainly express the 140 kD form and the 120 kD form is strongly expressed within the spinal cord and by oligodendrocytes and muscle cells (Chuong et al., 1984; Walsh and Doherty 1991).

An interesting feature of NCAM is a developmentally regulated modification of the molecule, which has an important impact on its functional properties. Two different mechanisms are known. The first occurs very early during development. After translation, a 2.8-polysialic polymer is attached to the fifth Ig like domain. This process is calcium dependent and is regulated by two different enzymes, designated ST8SiaII/STX and ST8SiaIV/PST (Ong et al., 1998). While all isoforms of NCAM might be sialylated, the 180kD form is the main carrier of polysialic acid (PSA) (Franceschini et al., 2001). The PSA polymer is negatively charged and it is strongly hydrated. Both, its size and its negative charge are believed to reduce NCAM-mediated cell-cell adhesion, but also cell-cell interactions mediated by other cell surface associated ligands, including L1 (reviewed in Rutishauser and Landmesser 1996; Kiss et al., 2001). By attenuating cell-cell contacts, the polysialylated form of NCAM is thought to be involved in dynamic processes, such as cell migration, axonal growth, pathfinding and synaptic plasticity (see Bruses and Rutishauer, 2001). After contact formation and establishment of axonal projections, the amount of NCAM-associated PSA decreases. However, PSA-NCAM remains expressed in adult brain regions exhibiting a permanent capacity for structural and synaptic plasticity, including the olfactory bulb, the hippocampus and the pituitary gland (Bonfanti et al., 1992;.Gubkina et al., 2001).

Coinciding with the decrease of PSA the expression of the second type of NCAM modification becomes pronounced, an alternatively spliced NCAM mRNA variant characterized by a 10 amino acid sequence insert within the fourth Ig-like domain, the so called variable alternative spliced exon (VASE) (Fig. 2). The VASE insert has been found in every major isoform of NCAM (Small and Akeson, 1990). Its presence correlates with a decreased capacity of NCAM to promote neurite outgrowth without, however, affecting adhesive properties (Lahrtz et al., 1997). At the beginning of neural development, only less than 3% of the NCAM transcripts contain this exon. With ongoing developmental progress the amount of VASE bearing transcripts increases up to 50% of all NCAM molecules in the adult, although at this time point, it is never found in brain regions characterized by synaptic and morphogenic plasticity, like the hippocamus and the olfactory bulb (Small et al., 1988).

Together, the shift of the functional properties of NCAM nicely correlates with the shift of its structural features, without changing the overall expression levels of NCAM. At the beginning of development dynamic processes like axon outgrowth, pathfinding, migration and synaptic plasticity are relevant, involving the PSA polymer. Later on maintenance of fasciculation and stabilization of synaptic contacts are of more importance, which correlates with an increase of the VASE insert bearing form of NCAM.

Interestingly, NCAM reveals several features that are very similar to those of L1, including the expression pattern and the binding patterns. For instance, both NCAM and L1 are expressed by subsets of postmitotic neurons, pre- and nonmyelinating Schwann cells and in certain neural tumors. (Kadmon et al. 1990) They also share certain binding partners like laminin, integrins and the FGF-receptor. NCAM binding to the FGF-receptor, for instance, leads to its phosphorylation initiating the same second messenger cascade as by L1 binding.(Walsh and Doherty, 1997). However,

NCAM is supposed to act differently from L1, since the PSA polymer attached to the NCAM molecule results in a strong enlargement of the whole molecule, and as a consequence decreases its ability of homophilic binding. Therefore, the molecule is distributed more loosely on the cell surface benefiting the dimerization of FGF-receptors and providing a better availability of its ligand. Which of this two mechanisms is responsible for the phosphorylation and thus for the initiation of the signal cascade is not yet elucidated. (Kiss et al., 2001).

NCAM was also demonstrated to interact directly with L1. For instance NCAM binds in *cis* to the carbohydrate core of L1 and by this interaction enhances the homophilic binding ability of L1 in *trans* (Kadmon et al., 1990; Horstkorte et al., 1993). This mechanism might in turn have an influence on the FGF-R activation and in consequence on neurite outgrowth.

The combined observations demonstrate that all three molecules L1, CHL1 and NCAM perform critical roles during the formation of the nervous system. The analysis of knock out mutants deficient in these proteins have indeed confirmed their pivotal function during normal brain development. The present study was performed to obtain further insights into the functions of the cell recognition molecules *in vivo* using genetically engineered and acutely manipulated mice.

Since hydrocephalus is the most prominent feature of CRASH syndrome in humans and L1 knock out mutants, this work attempted to understand the development of this defect. Originally X-linked hydrocephalus in humans was supposed to result from a stenosis of the aqueduct of Sylvius, a small channel connecting the third and fourth ventricle. Following the closure of this channel the cerebro-spinal fluid accumulates, resulting in high pressure hydrocephalus with massively dilated ventricles. However, not all patients with X-linked hydrocephalus displayed a stenosis of the aqueduct.

The aim of this study was therefore to investigate the cause of this pathology using the L1 deficient mouse as a model.

Since CHL1 is thought to play a critical role during axonal regeneration, this study attempted to further elucidate the expression patterns of CHL1 in the lesioned adult central nervous system. To this aim, two different experimental paradigms were used, intraorbital lesioning of the optic nerve and intravitreal application of fibroblast growth factor-2.

The third project of this study concentrates on the functional characterization of NCAM. Since this molecule is implicated in axonal outgrowth and since pathfinding errors within the hippocampus have been demonstrated in the NCAM knock out mutant, the role of NCAM in the development of a prominent axonal tract, the corticospinal tract was investigated. This tract is particularly suitable to study axonal pathfinding, since axons have to change their path considerably at the pyramidal decussation to reach their appropriate targets in the spinal cord, and have to cross from the ventral medulla to the contralateral and dorsal spinal cord. In order to elucidate whether NCAM is necessary for the correct formation of the tract, tracing studies of the corticospinal tract of young and adult NCAM knock out mice were performed.

Project 1: Severe Hydrocephalus in L1-Deficient Mice

Introduction

The neural cell adhesion molecule L1 is a transmembrane glycoprotein of the immunoglobulin (Ig) superfamily. The protein consists of six Ig-like domains, five fibronectin type III-like repeats, a single-pass transmembrane domain and a short cytoplasmic tail (Moos et al., 1988). L1 is abundantly expressed in the central and peripheral nervous system (Bartsch et al., 1989; Martini and Schachner 1986), and is also present in some non-neural tissues (Debiec et al., 1998; Kowitz et al., 1992). The protein supports migration of nerve cells (Lindner et al., 1983), elongation (Lagenaur and Lemmon 1987) and fasciculation (Fischer et al., 1986) of axons, survival of nerve cells (Chen et al., 1999), and has been implicated in synaptic plasticity (Lüthi et al., 1994). All these observations suggest important functions of L1 in the developing, adult, and injured nervous system (Brümmendorf et al., 1998; Hortsch 1996; Schachner 1994; Walsh and Doherty 1997).

A crucial role of L1 for normal brain development is highlighted by the fact that mutations in the human L1 gene, located on the X chromosome in Xq28, cause a severe neurological disease, termed CRASH (corpus callosum hypoplasia, retardation, adducted thumbs, spastic paraplegia, and hydrocephalus; (Fransen et al., 1995). The clinical picture of CRASH patients is complex and variable. Hypoplasia of the corticospinal tract or corpus callosum, adducted thumbs, mental retardation and hydrocephalus are among the symptoms of the disease (Brümmendorf et al., 1998; Fransen et al., 1995; Kamiguchi et al., 1998).

The X-linked hydrocephalus was at first described by Bickers and Adams in 1949. The affected patient they presented, displayed a severe hydrocephalus and a stenosis of the aqueduct of Sylvius, which is a small channel connecting the third and the fourth ventricle. Therefore this syndrome was called HSAS, an acronym for hydrocephalus due to a stenosis of the aqueduct of Sylvius. This X-linked form is the

most common genetic cause of hydrocephalus, appearing with an incidence of approximately one out of 30.000 male births (Halliday et al., 1986). The severity of the hydrocephalus depends strongly on the localization of the mutation in the L1 gene, which can differ within the same family (Willems et al., 1987).

To establish an animal model for CRASH syndrome, mouse mutants with null mutations in the L1 gene were generated independently in two laboratories (Cohen et al., 1998; Dahme et al., 1997). Similar to CRASH patients, L1-deficient mice displayed hypoplasia of the corticospinal tract (Dahme et al., 1997) and corpus callosum (Demyanenko et al., 1999). Mutant mice also developed enlarged ventricles (Dahme et al., 1997; Fransen et al., 1998a). However, a significant ventricular dilation was only observed in L1 mutants back-crossed into a C57BL/6J genetic background but not in mutants back-crossed into a 129 background, suggesting that modifier genes influence the severity of this L1-related defect (Dahme et al., 1997).

Here, it is reported that the significant enlargement of ventricles observed in many L1 mutants with a predominant C57BL/6J genetic background is not related to a diminution of the aqueduct of Sylvius. However, a few mutants of the published (Dahme et al., 1997) and a newly generated L1-deficient mouse line developed severe hydrocephalus, characterized by an enlargement of the skull and a complete occlusion of the aqueduct. This study suggests that, in these severely affected mutants, massively enlarged ventricles result in deformations of the brain which then cause a secondary stenosis of the aqueduct, ultimately leading to severe hydrocephalus.

Materials and Methods

The generation of L1-deficient mouse mutants by insertion of thymidine kinase and neomycin-resistance genes into the ninth exon of the L1 gene has been described in detail elsewhere (Dahme et al., 1997). The F1 generation of this mutant mouse line (C57BL/6J-129/SvEv hybrids) was back-crossed with C57BL/6J inbred mice, and wild-type and L1-deficient males from the second to tenth generation of such back-crosses were used in this study. We also used animals (C57BL/6J-129 hybrids of the F1 generation) from an independently established L1-deficient mouse line. Expression of L1 in this mutant was abolished by insertion of a tetracycline-controlled transactivator (tTA) (Gossen and Bujard 1992) into the second exon of the L1 gene (in the following, this mutant is termed L1/tTA knock-in mutant; Michael Kutsche and Melitta Schachner, unpublished data). The null mutation in L1/tTA knock-in mice was confirmed by immunoblot analysis of brain tissue. During the course of this study, we obtained 630 wildtype and 70 L1-deficient males from the breeding colony of the published mutant mouse line (Dahme et al., 1997), and 27 wild-type and 10 mutant males from the L1/tTA knock-in colony. Four mutants of the published line and one L1/tTA knock-in mutant displayed a significant enlargement of the skull (in the following defined as "severe hydrocephalus"). These severely affected animals, nine wild-type and 17 L1-deficient mice of the published line and one wild-type littermate of the L1/tTA knock-in mutant were further analyzed. All animals were aged between two and five months, and their genotype was determined using polymerase chain reaction.

Mice were deeply anesthetized and perfusion-fixed with 4% paraformaldehyde and 2% glutaraldehyde in phosphate buffered saline (pH 7.4). Brains were serially sectioned with a vibratome, sections were immersed in 2% OsO_4 in 0.1 M cacodylate buffer for 2 hours, dehydrated in an ascending series of methanol and embedded in Epon resin. Semithin sections were stained with toluidin-blue and analyzed with an Axiophot (Zeiss, Oberkochen, Germany). Ultrathin sections were counterstained with lead citrate and examined with an EM 10 (Zeiss).

Results

Mutations in the gene for the neural adhesion molecule L1 cause a variety of neuropathological abnormalities, both in humans and in mice. Hydrocephalus is certainly the most prominent defect in CRASH patients and L1-deficient mice, but how this abnormality develops is unknown. To better understand the development and the phenotypic variability of L1-related hydrocephalus, we studied L1 mutant mice from two independently generated L1-deficient mouse lines. For analysis, we selected mutant mice displaying a wide spectrum of this defect, ranging from apparently normal-sized ventricles to severe hydrocephalus.

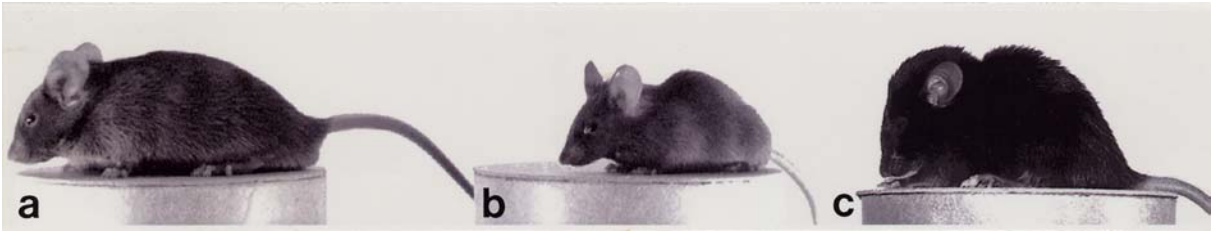

Figure 3: L1-deficient mice (b) are smaller than their wild-type littermates (a). A few mutants display severe hydrocephalus, characterized by a significantly enlarged skull (c). Note that animals in (a and b) and the animal in (c) are shown at different magnifications.

L1-deficient mice (Fig. 3b) were smaller than their wild-type littermates (Fig. 3a; (Cohen et al., 1998; Dahme et al., 1997)) and, when back-crossed into a C57BL/6J genetic background, eventually developed enlarged ventricles (Dahme et al., 1997). In the vast majority of L1-deficient mice, ventricular dilations were not accompanied by a detectable enlargement of the skull (Fig. 3b; but see below). Since a diminution of the aqueduct of Sylvius might be the cause of ventricular enlargement, we analyzed the size of the aqueduct in L1 mutants displaying either normal-sized (n=4), moderately (n=3) or massively dilated ventricles (n=10). Light microscopic inspection of serially sectioned brains revealed a patent aqueduct of Sylvius in all of these L1-deficient mice. In fact, there was no consistent difference in the aqueductal size between wild-type mice and L1-deficient littermates (compare Fig. 4 a´ and b´), irrespective of the degree of ventricular dilations in the mutants (for an animal with massively enlarged ventricles, see Fig. 4 b and b').

Totally four L1-deficient mice of the published L1-deficient mouse line (Dahme et al., 1997) and one mutant of the newly generated L1/tTA knock-in line developed severe hydrocephalus. In contrast to the L1 mutants described above, these animals were characterized by a significant enlargement of the skull (compare Fig. 3c and b). Histological analysis of brains of these severely affected mutants revealed massively enlarged ventricles (Fig. 4c), and a cerebral cortex which was markedly thinnened (Fig. 4c) or completely atrophied (not shown). The hippocampus was displaced from its medio-lateral to a ventro-dorsal orientation (Fig. 4c). shown).

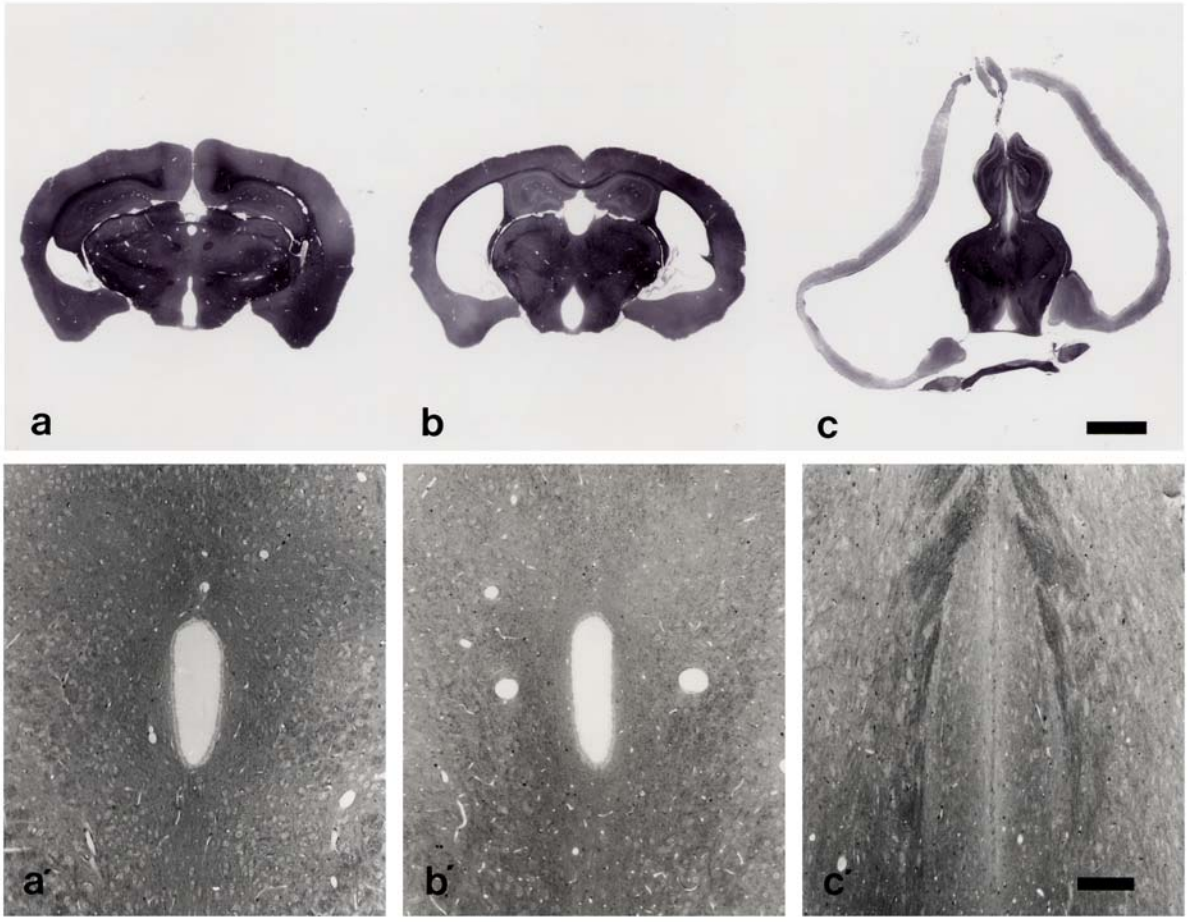

Figure 4: The size of ventricles and the aqueduct of Sylvius in wild-type mice and L1 mutants. The lateral ventricles of many L1-deficient mice with a C57BL/6J genetic background (b) are significantly enlarged when compared to wild-type littermates (a). Note the similar size of the aqueduct in the L1 mutant (b') and the wild-type littermate (a'). A few L1 mutants display severe hydrocephalus with massively enlarged ventricles (c). Note the thinnened cerebral cortex and the displacement of the hippocampus into a ventro-dorsal orientation. The aqueduct of these severely affected animals is completely closed and elongated in a dorso-ventral direction (c´). Bar in (c) for a-c: 2mm; in (c´) for a´-c´: 100µm.

Moreover, significant portions of the cerebellum were atrophied in all five mutants displaying severe hydrocephalus. While the principal cytoarchitecture of the few remaining cerebellar folia was indistinguishable from that of wild-type mice, cortical layers were significantly reduced in thickness, and contained regions of necrotic tissue (data not shown).

Importantly, light microscopic inspection revealed a complete closure of the aqueduct of Sylvius of all L1 mutants displaying severe hydrocephalus (Fig. 4c, c´). Ependymal cells lining the opposing walls of the aqueduct were pushed together, and formed a row of cells which was elongated in a dorso-ventral direction (Fig. 4c'). Complete occlusion of the aqueduct was confirmed at the ultrastructural level (Fig. 5b). Ependymal cells were only separated by a dense meshwork of their apical microvilli and cilia, and cells were elongated and appeared compressed (Fig. 5b). The general ultrastructural appearance of ependymal cells, however, was not detectably altered in L1-mutants when compared to wild-type littermates (compare Fig. 5a and b).

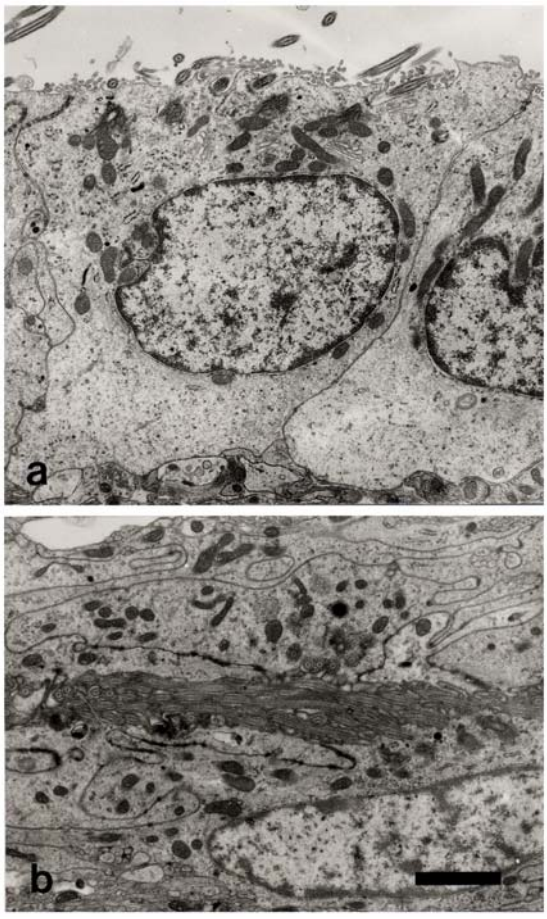

Figure 5: The ultrastructure of ependymal cells lining the aqueduct of wild-type mice (a) and L1 mutants displaying severe hydrocephalus (b). The aqueduct of the mutant is completely closed, with ependymal cilia being compressed between the opposing cells (b). Ependymal cells of the L1 mutant appear elongated but otherwise display a similar ultrastructure as cells of the wild-type mouse. Bar in (b) for a and b: 2µm.

Discussion

X-linked hydrocephalus is a highly variable symptom of CRASH syndrome. CRASH patients might either display a normal-sized ventricular system, mild ventricular dilations or a prominent enlargement of ventricles and macrocephaly (Yamasaki et al., 1997; Kenwrick et al., 1996). Much of this variability has been attributed to a significant influence of the type and location of a L1 mutation on the phenotypic expression of hydrocephalus (Fransen et al., 1998b; Michaelis et al., 1998; Yamasaki et al., 1997). However, intrafamilial variability of this symptom has also been reported (Kaepernick et al., 1994; Willems et al., 19987), indicating that the severity of hydrocephalus is additionally determined by secondary factors. In close analogy to CRASH patients, L1-deficient mice display variably enlarged ventricles (Dahme et al., 1997). Remarkably, significantly enlarged ventricles were only observed in mutant mice back-crossed into a C57BL/6J genetic background. When the same mutant mouse line was back-crossed into a 129 genetic background, in contrast, the ventricular system appeared normal as judged from histological investigations (Dahme et al., 1997). However, high resolution magnetic resonance imaging revealed slightly dilated ventricles in independently generated L1-deficient mice maintained in a 129 genetic background (Fransen et al., 1998a). Together, observations demonstrate that mutations in the L1 gene cause dilated ventricles and suggest that modifier genes influence the severity of this L1-related defect.

Since alterations in the shape and/or size of the aqueduct of Sylvius might cause increased pressure of the cerebrospinal fluid, and as a consequence ventricular dilations, the aqueduct has been extensively studied in L1-deficient mice. The aqueduct of the L1-deficient mutant originally published by Cohen and co-workers (Cohen et al., 1998) and maintained in a 129 background was described as being normal in size but altered in shape in one study (Fransen et al., 1998a), whereas another study reported a dilated aqueduct (Demyanenko et al., 1999). Here, the aqueduct of L1 mutants displaying massively enlarged ventricles are studied. Despite the severe phenotype of L1-deficient mice, no consistent difference in the

morphology of the aqueduct between wild-type mice and L1 mutants were found. Thus it is highly unlikely that morphological alterations of the aqueduct represent the primary cause of X-linked hydrocephalus.

Several alternative mechanisms have been proposed to cause enlarged ventricles in CRASH patients and L1 mutant mice. All these suggestions are based on the assumption that L1-related hydrocephalus develops *ex vacuo*, i.e. as a consequence of increased degeneration of neural cell types due to disturbed cell-cell or cell-matrix interactions. For instance, L1 has been shown to support migration of nerve cells, and dilation of ventricles might thus result from degeneration of aberrantly positioned neurons. Up to now, however, evidence for impaired neuronal migration in L1 mutant mice is lacking. Hydrocephalus might also result from impaired outgrowth and/or pathfinding of axons and subsequent death of those neurons which failed to innervate their appropriate targets. Interestingly, pathfinding errors of corticospinal axons at the pyramidal decussation have been described in L1-deficient mice (Cohen et al., 1998), and hypoplasia of the corticospinal tract of CRASH patients and L1-deficient mice might thus result from degeneration of pyramidal motoneurons which failed to innervate their appropriate target. It is of particular interest in the present context that the numbers of pyramidal and granule neurons in the hippocampus (Demyanenko et al., 1999) and of unmyelinated sensory axons in peripheral nerves (Dahme et al., 1997; Haney et al., 1999) are reduced in L1 mutants when compared to wild-type controls.

Four (5.7%) out of 70 L1-deficient males of the published line (Dahme et al., 1997) and one (10%) out of 10 L1/tTA knock-in mutants developed severe hydrocephalus, characterized by a significant enlargement of the skull. This low frequency of mutants with severe hydrocephalus might be related, at least in part, to an increased mortality of heavily affected L1-deficient mice during embryonic or early postnatal development. L1 is X-linked and L1-deficient animals did not produce offspring. Heterozygous females were therefore mated with wild-type males to obtain mutants. 50% of males from such crosses are expected to carry the mutated L1 allele.

However, only 11% of males from the published L1-deficient line, and 27% of males from the L1/tTA knock-in line as L1-deficient animals were identified.

Interestingly, we found that the aqueduct of all L1 mutants with severe hydrocephalus was completely closed. Since severe hydrocephalus with complete obstruction of the aqueduct was found in animals of two independently generated L1-deficient mouse lines, this defect is considered as a specific, although secondary consequence of L1 deficiency. This conclusion is based on the observation that the majority of L1-mutants with a C57BL/6J genetic background developed enlarged ventricles without displaying detectable morphological changes of the aqueduct (see above). The hypotheses is that, in the few severely affected mutants, massively enlarged ventricles caused deformations of the brain which in turn resulted in compression of the aqueduct and subsequently in high pressure hydrocephalus. X-linked hydrocephalus is also referred to as HSAS (hydrocephalus owing to stenosis of the aqueduct of Sylvius), since stenosis of the aqueduct has been described in several affected patients (Willems et al., 1987). Similar to L1-deficient mice, however, there are many patients displaying a patent aqueduct (Graf et al., 1998; Landrieu et al., 1979; Willems et al., 1987). These observations also indicate that aqueductal stenosis is the result rather than the cause of the symptom. Secondary aqueductal stenosis caused by compression of the midbrain by the expanding cerebral hemispheres has also been discussed for other forms of hydrocephalus with either known or unknown etiology. Such a pathogenetic sequence has been demonstrated, for instance, in a detailed study of mice carrying a mutation termed obstructive hydrocephalus (Borit and Sidman 1972).

Together, our data demonstrate an enormous variability of L1-related hydrocephalus in a mouse model for CRASH syndrome, and suggest a strong influence of modifier genes and secondary effects on the phenotypic expression of the symptom. The striking similarities between the phenotype of L1-deficient mouse mutants and CRASH patients further substantiate that L1-deficient mouse mutants represent an appropriate animal model for the disease.

Project 2: Altered Expression of CHL1 by Glial Cells in Response to Optic Nerve Injury and Intravitreal Application of Fibroblast Growth Factor-2

Introduction

Close homologue of L1 (CHL1; Holm et al., 1996) is a member of the L1 family of cell recognition molecules characterized by a common modular structure (Brümmendorf et al., 1998; Hortsch, 2000). CHL1 has a highly conserved intracellular domain and an extracellular part consisting of six immunoglobulin- (Ig) and five fibronectin type III- (FNIII) like repeats, the fifth FNIII repeat being only rudimentary. In the nervous system of mice, three isoforms of CHL1 have been described, a transmembrane form of 185 kD and two presumably proteolytically cleaved fragments of 165 and 125kD.

CHL1 promotes neurite elongation from cerebellar and hippocampal neurons when offered as a substrate-bound or soluble molecule *in vitro* (Chen et al., 1999; 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 cell surfaces has to be postulated. In support of this notion, expression of CHL1 in heterologous mouse L929 fibroblast or *Drosophila* S2 cell lines did not promote aggregation of cells, also indicating that CHL1 acts as a heterophilic cell recognition molecule (Hillenbrand et al., 1999). Substrate-bound or soluble CHL1 also promotes survival of cultured nerve cells (Chen et al., 1999).

In the central nervous system (CNS) of mice, expression of CHL1 becomes detectable at embryonic day 12 or 13, is strongest between embryonic day 18 and postnatal day 7, and decreases again with increasing age of the animal (Hillenbrand et al., 1999; Liu et al., 2000). Generally, there is a striking co-expression of CHL1

and L1 in many neuronal cell types, but distinct patterns of expression have also been described (Hillenbrand et al., 1999; Holm et al., 1996; Zhang et al., 2000). Most strikingly, CHL1, but not L1, is expressed by some CNS glial cells (Holm et al., 1996).

The gene encoding the human ortholog of CHL1, designated CALL (cell adhesion L1-like), has been mapped to 3p26.1 (Wei et al., 1998). Deletions of the short arm of chromosome 3 with breakpoints at 3p25-3p26 cause 3p- syndrome, a disease characterized by a variety of anomalies, including mental retardation (for references, see Angeloni et al., 1999). Mental retardation is also a characteristic feature of CRASH syndrome, a disease caused by mutations in the L1 gene (Fransen et al., 1995). Given the high degree of homology between CHL1 and L1 and the proposed functions of CHL1 for normal brain development (Hillenbrand et al., 1999), it has recently been hypothesized that mental retardation of 3p- patients might be related to haploinsufficiency in the CALL gene (Angeloni et al., 1999).

CHL1 might also perform important functions in the lesioned nervous system. This view is supported by the observations that (i) motor neurons, sensory neurons and Schwann cells up-regulate expression of CHL1 in response to peripheral nerve lesions (Zhang et al., 2000); and that (ii) expression of CHL1 is up-regulated by adult CNS neurons experimentally induced to regrow their axons into peripheral nerve grafts (Chaisuksunt et al., 2000a,b).

To obtain further insights into possible functions of CHL1 in the lesioned nervous system, we have studied the molecule´s expression in the injured optic nerve of adult mice. Moreover, we analyzed expression of CHL1 in the retina and optic nerve after a single intravitreal injection of FGF-2, a growth factor known to induce astrogliosis in the developing and adult brain (e.g. Eclancher et al., 1990; Lewis et al., 1992), and

to reduce death of axotomized retinal ganglion cells (Sievers et al., 1987; Weibel et al., 1994).

Materials and Methods

Animals

Adult (i.e. at least two months old) wild-type (C57BL/6J) and CHL1-deficient (129Ola-C57BL/6J) mice (Montag et al., 1997) were used in the present study. The genotype of mutant mice was determined using polymerase chain reaction. All animal experiments were approved by the local Animal Use Committee.

Optic Nerve Lesions and Intravitreal Application of FGF-2

Mice were deeply anaesthetized and the right optic nerve was crushed intraorbitally with watchmaker´s forceps for 15 seconds (Bartsch et al., 1995). The contralateral unlesioned nerve of each animal served as a control. For intravitreal application of FGF-2, a glass micropipette was inserted into the eye at the junction between sclera and cornea, 1µl of vitreous fluid was removed, and the same volume of FGF-2 (0.1µg/µl) was injected into the vitreous space. Intravitreal application of 0.9% NaCl was performed as a negative control. Untreated retinas were used as further controls. Animals with intraorbitally crushed optic nerves were sacrificed 2, 7, 14, 28 or 63 days after the lesion. Optic nerves from all post-lesion intervals were subjected to *in situ* hybridization analysis. Immunohistochemical and immunoblot analysis of injured optic nerves was performed 7 and 14 days after the lesion. Animals with intravitreally injected FGF-2 or NaCl were sacrificed after a survival period of 3, 7 or 14 days. *In situ* hybridization analysis of retinas was performed 3 and 14 days, immunoblot analysis 7 days, and immunhistochemistry 7 and 14 days after application of FGF-2 or NaCl.

In Situ Hybridization Analysis

Cryostat sections, 14µm in thickness, were prepared from experimental and control tissues and fixed in phosphate buffered saline (PBS; pH 7.4) containing 4% paraformaldehyde. *In situ* hybridization analysis with digoxigenin-labelled cRNA probes was performed as described (Bartsch et al., 1992). Preparation of cRNA probes specific to glial fibrillary acidic protein (GFAP), L1 and CHL1 has been described (Bartsch et al., 1994; Holm et al., 1996). In each experiment, some sections were incubated with the corresponding sense cRNA probes to control the specificity of the *in situ* hybridization signal.

Antibodies

For immunohistochemistry, we used monoclonal mouse antibodies to GFAP (Sigma, Deisenhofen, Germany), and polyclonal antibodies to L1 (Bartsch et al., 1989) and CHL1. For production of polyclonal CHL1 antibodies, the extracellular domain of mouse CHL1 fused to a human Fc tag was expressed in CHO cells. The secreted fusion protein was purified *via* a ProteinA column (Pharmacia) and used for immunization. For affinity purification, the IgG fraction of the crude serum was first loaded on a Sepharose 4B-precolumn, then on a human IgG column and finally twice on a mouse CHL1-Fc column. Secondary Cy3-conjugated goat anti-rabbit or goat anti-mouse antibodies were purchased from Dianova (Hamburg, Germany). Immunoblot analysis was carried out with polyclonal L1, GFAP (Bartsch et al., 1990) or CHL1 antibodies and monoclonal glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Chemicon, Temecula, CA) antibodies. Primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech, Braunschweig, Germany).

Immunohistochemistry

Cryostat sections from optic nerves and retinas were mounted onto poly-L-lysine-coated coverslips, air-dried and fixed in methanol for 10 minutes at -20C°. Sections were blocked in PBS containing 0.1% bovine serum albumine and incubated with primary antibodies overnight at 4°C. Primary antibodies were visualized with Cy3-conjugated secondary antibodies. As a negative control, sections were treated in the same manner except that incubation with primary antibodies was omitted. Lesioned optic nerves and FGF-2-treated retinas from CHL1-deficient mice were incubated with CHL1 antibodies to further control the specificity of the CHL1 antibodies.

Immunoblot Analysis

Unlesioned and lesioned optic nerves (7 or 14 days after an intraorbital crush) and NaCl and FGF-2-treated retinas (7 days after intravitreal application) were prepared for immunoblot analysis. In brief, tissues were homogenized in 40mM Tris-HCl (pH 7.4) containing 150mM NaCl, 5mM EDTA, 5mM EGTA, 1% (vol/vol) Triton X-100, 1mM phenylmethylsulfonylfluoride, 0.5mM iodoacetamide and 1mM dithiothreitol, and were agitated at 4°C on an end-over-end shaker. The non-soluble material was pelleted by centrifugation at 100,000g for 45 min at 4°C. Protein concentrations of supernatants were determined by the bicinchoninic acid-method according to the manufacturer's instructions (Pierce, Rockford, IL). Proteins were resolved by 8-10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose filters. Immunodetection was performed using polyclonal CHL1, L1 or GFAP and monoclonal GAPDH antibodies. Bound antibodies were detected with horseradish peroxidase-conjugated secondary antibodies and visualized by enhanced chemoluminescence according to the manufacturer's instruction (Amersham Pharmacia Biotech). For comparison of expression levels and sizes of

the different proteins, bound antibodies were removed and filters were reprobed with a different primary antibody.

Results

Expression of CHL1 in Lesioned Optic Nerves of Adult Mice

In the developing mouse optic nerve, CHL1 transcripts are expressed by glial cells located along the entire length of the nerve (Holm et al., 1996). However, in the adult optic nerve, CHL1-positive cells are restricted to its unmyelinated proximal (i.e. retina-near) portion, whereas expression in the distal myelinated segment of the nerve decreases below detection levels (Fig. 6a, c). Thus, expression of CHL1 transcripts in the adult optic nerve is restricted to a subpopulation of astrocytes which is also characterized by high levels of GFAP expression (see Fig. 6e).

After an intraorbital crush of the adult optic nerve, glial cells in the myelinated distal part of the nerve strongly up-regulated expression of CHL1 mRNA (Fig. 6b, d). Up-regulation of CHL1 transcripts in glial cells occurred rapidly, with the first CHL1-positive glial cells being detectable already two days after the lesion, the earliest time point investigated (data not shown). Numerous CHL1-positive glial cells were visible 7 days after the lesion (Fig. 6b). Increased expression of CHL1 was not restricted to cells located in the vicinity of the lesion, but was detectable in cells distributed along the entire length of the nerve until the optic chiasmn (Fig. 6b,d, and data not shown). Elevated expression of CHL1 mRNA in injured optic nerves was detectable as long as 63 days after the lesion, the latest time point investigated (Fig. 6d). In the retina, only a few cells expressed CHL1 transcripts (see below), and this pattern of expression was not detectably altered by the lesion at any of the post-lesion intervals investigated (compare Fig. 6a and c with Fig. 6 b and d).

In each experiment, expression of GFAP mRNA was studied in parallel with CHL1 to control the efficacy of the nerve crush. Compared to unlesioned control nerves (Fig. 6e), strong up-regulation of GFAP transcripts was observed in all lesioned nerves (Fig. 6f). L1 transcripts were not expressed by optic nerve glial cells, neither in

control nerves nor in lesioned nerves at any of the post-lesion intervals studied (not shown), in agreement with previous reports (Mohajeri et al., 1996). No signals were detectable when sections from control or lesioned optic nerves were hybridized with CHL1-, GFAP- or L1-specific sense cRNA probes (data not shown).

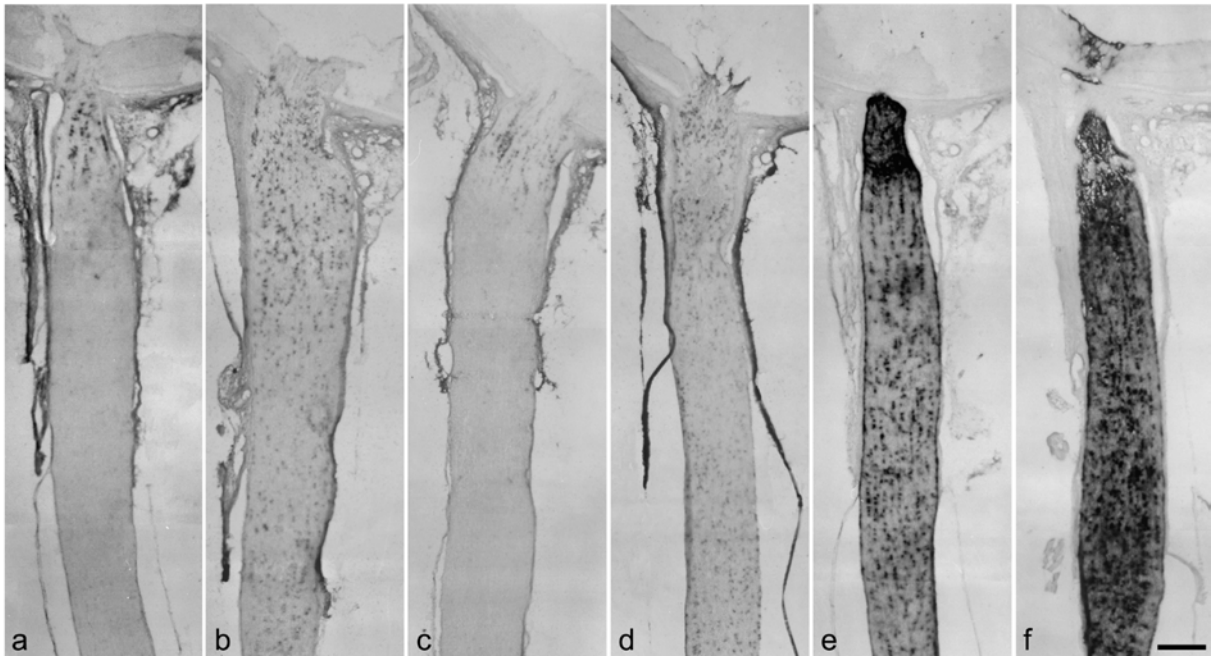

Figure 6: Expression of CHL1 and GFAP transcripts in control and lesioned optic nerves of adult mice. Expression of CHL1 transcripts in unlesioned optic nerves of adult mice is restricted to astrocytes located in the unmyelinated retinal end of the nerve (a). One week after an intraorbital nerve crush, CHL1-positive glial cells are also present in the myelinated distal segment of the nerve (b). Elevated levels of CHL1 mRNA remain detectable in the distal stumps of lesioned nerves for up to 63 days, the longest post-lesion interval investigated (d; the unlesioned contralateral nerve of the same animal is shown in c). Some sections of each experimental animal were hybridized with a GFAP-specific cRNA probe to control the efficacy of the lesion. GFAP transcripts are expressed in astrocytes throughout the entire length of the nerve, but are most strongly expressed in astrocytes located at the retinal end of the nerve (e). Expression of GFAP mRNA in astrocytes is significantly increased in response to a lesion (f, for a 7-day post-lesion interval). Scale bar: 500µm.

A polyclonal antibody against the extracellular domain of mouse CHL1 was used to study the expression of CHL1 in unlesioned and injured optic nerves at the protein level. Immunohistochemical analysis of unlesioned nerves revealed intense and

homogeneously distributed CHL1-immunoreactivity in the unmyelinated retina-near portion of the nerves (Fig. 7a).

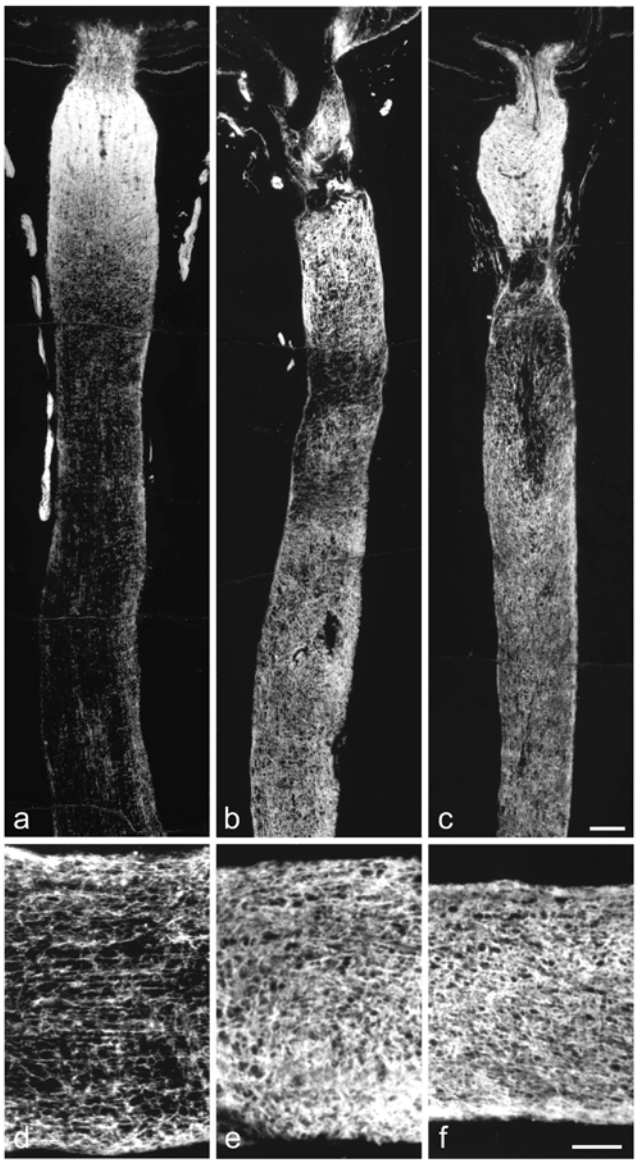

Figure 7: Expression of CHL1 protein in intraorbitally lesioned optic nerves. Intense and homogeneously distributed CHL1-immunoreactivity is present in the retinal end of non-lesioned optic nerves of adult mice (a). In the myelinated segment of control nerves, CHL1 protein is associated with unmyelinated retinal ganglion cell axons and processes of glial cells (a,d). The intensity and distribution of CHL1-positivity in the retinal segment of lesioned nerves is similar to that in control nerves, both 7 (b) and 14 (c) days after the crush. In the myelinated segment of injured nerves, expression of CHL1 protein is significantly elevated and the protein is more homogeneously distributed compared to non-lesioned nerve, both 7 (b,e) and 14 (c,f) days after the lesion. Scale bar in c (for a-c): 100µm; in f (for d-f): 50µm.

This result suggests CHL1-positivity of the unmyelinated proximal segment of retinal ganglion cell axons and of astrocytes located in this region of the nerve. Immunolabeling of the distal myelinated portion of the nerves was weak compared with their proximal unmyelinated segment. In the myelinated segment, CHL1 antibodies stained the few unmyelinated ganglion cell axons (Fig. 7a,d). Immunoreactivity of glial cell processes was also detectable (Fig. 7d). Accumulation

of CHL1 protein remained detectable in the unmyelinated retinal end of optic nerves 7 (Fig. 7b) or 14 (Fig. 7c) days after an intraorbital crush. In the myelinated segment of lesioned nerves, intensity of the immunostaining was increased and the protein was more uniformly distributed (Fig. 7b,c,e,f) compared with the contralateral unlesioned nerves (Fig. 7a,d). Incubation of unlesioned and intraorbitally crushed optic nerves from CHL1-deficient mice with CHL1 antibodies revealed no signals (data not shown), establishing specificity of this antiserum.

Altered Expression of CHL1 after Intravitreal Application of FGF-2

Cells expressing CHL1 transcripts were hardly detectable in untreated retinas (Fig. 8a). GFAP transcripts were restricted to cells located at the vitread margin, the typical location of retinal astrocytes (Fig. 8b). Intravitreal application of NaCl did not detectably alter expression of CHL1 (compare Fig. 8c and a) or GFAP (compare Fig. 8d and b) in retinas analyzed three (data not shown) or seven days after the treatment. However, three (data not shown) and seven days after a single intravitreal injection of FGF-2, *in situ* hybridization analysis revealed a massive up-regulation of both, CHL1 (Fig. 8e) and GFAP (Fig. 8f) transcripts when compared with untreated (Fig. 8a,b) or NaCl-treated retinas (Fig. 8c,d). Specifically, CHL1-positive cells in FGF-2-treated retinas became detectable at the vitread margin and in the inner nuclear layer (Fig. 8e). In comparison, application of FGF-2 resulted in elevated levels of GFAP mRNA in retinal astrocytes and induced expression of GFAP in Müller cells located in the inner nuclear layer (Fig. 8f). GFAP transcripts in FGF-2-treated retinas were not only detectable in the cell bodies of Müller cells, but were also diffusely distributed in the inner nuclear and plexiform layer (Fig. 8f), indicating the presence of GFAP mRNA in basal processes of these glial cells. In summary, up-regulation of CHL1 and GFAP transcripts was observed in retinal cell types with

similar locations, suggesting that FGF-2 induces expression of CHL1 in retinal astrocytes and Müller cells. Since intravitreal application of NaCl did not alter expression of CHL1 and GFAP, up-regulation of both molecules in FGF-treated retinas reflects a specific cellular response to FGF-2 rather than an unspecific reaction to the experimental manipulation (i.e. intravitreal injection).

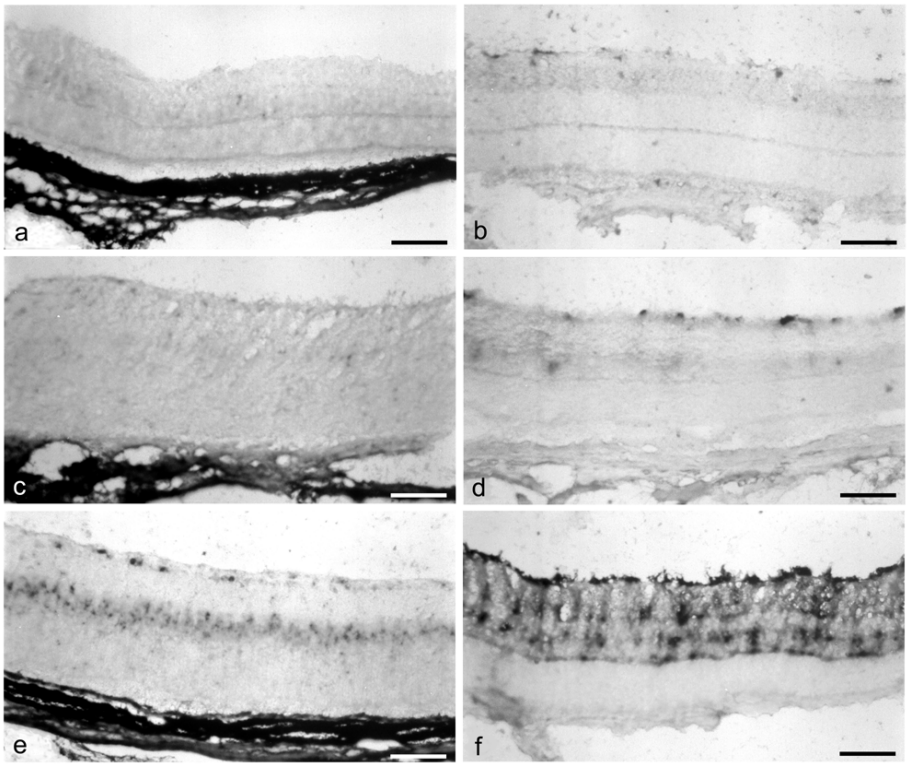

Figure 8: Expression of CHL1 and GFAP transcripts in control, NaCl-, and FGF-2-treated retinas of adult mice. CHL1-positive cells are virtually absent from untreated control retinas of adult mice (a). GFAP transcripts are restricted to retinal astrocytes located at the vitread margin of the retina (b). Intravitreal applications of NaCl did not detectably alter expression of CHL1 (c) and GFAP (d) compared to control tissues (a,b). Intravitreal application of FGF-2, in contrast, induces expression of CHL1 transcripts in cells located at the vitread margin of the retina and in the inner nuclear layer (e). Application of FGF-2 also results in elevated levels of GFAP mRNA in retinal astrocytes and induces expression of GFAP transcripts in cell bodies and basal processes of Müller cells (f). Scale bar: 300µm.

Exogeneous FGF-2 did not result in detectable changes of CHL1 or GFAP expression in the optic nerve (data not shown). However, both molecules are strongly expressed in astrocytes at the retinal end of the optic nerve, and effects of FGF-2 on the expression of CHL1 and GFAP in these glial cells might thus have

escaped analysis. No labeling was observed when control or experimentally manipulated retinas were incubated with GFAP- or CHL1-specific sense probes (data not shown).

Expression of GFAP and CHL1 in control, NaCl- and FGF-2-treated retinas was also studied at the protein level (Fig. 9). In agreement with the *in situ* hybridization data, expression of GFAP was restricted to retinal astrocytes located at the inner (i.e. vitread) side of the retina (Fig. 9a,d). Occasionally, immunoreactive Müller cell processes were observed. The intensity of GFAP-immunoreactivity in retinal astrocytes was not detectably altered after intravitreal application of NaCl, neither 7 (Fig. 9b) nor 14 (Fig. 9c) days after administration. In comparison, expression of GFAP was massively up-regulated in retinal astrocytes and Müller cells 7 (Fig. 9e) or 14 (Fig. 9f) days after a single intravitreal injection of FGF-2.

CHL1-immunoreactivity in untreated retinas was weak and mainly confined to the outer plexiform layer (Fig. 9g,j). Very faint immunostaining was also observed in the inner plexiform layer and in the nerve fiber layer (Fig. 9g,j). Specificity of these weak signals was confirmed by incubating control, NaCl- or FGF-2-treated retinas from CHL1-deficient mice with CHL1 antibodies (not shown). The pattern and intensity of CHL1-immunoreactivity detected in retinas 7 (Fig. 9h) or 14 (Fig. 9i) days after application of NaCl was not significantly different from that observed in untreated tissues. In contrast, expression of CHL1 protein was significantly increased after a single intravitreal injection of FGF-2, both 7 (Fig. 9k) and 14 (Fig. 9l) days after application. Elevated levels of CHL1 protein were detectable in the outer and inner plexiform layers and in the nerve fiber layer (compare Fig. 9k and l with Fig. 9j). Application of FGF-2 also induced weak labeling between cell bodies of the inner and outer nuclear layer. Finally, a narrow band of CHL1-immunoreactivity became

apparent above the external limiting membrane (Fig. 9k,l), indicative for immunoreactive microvilli of Müller cells.

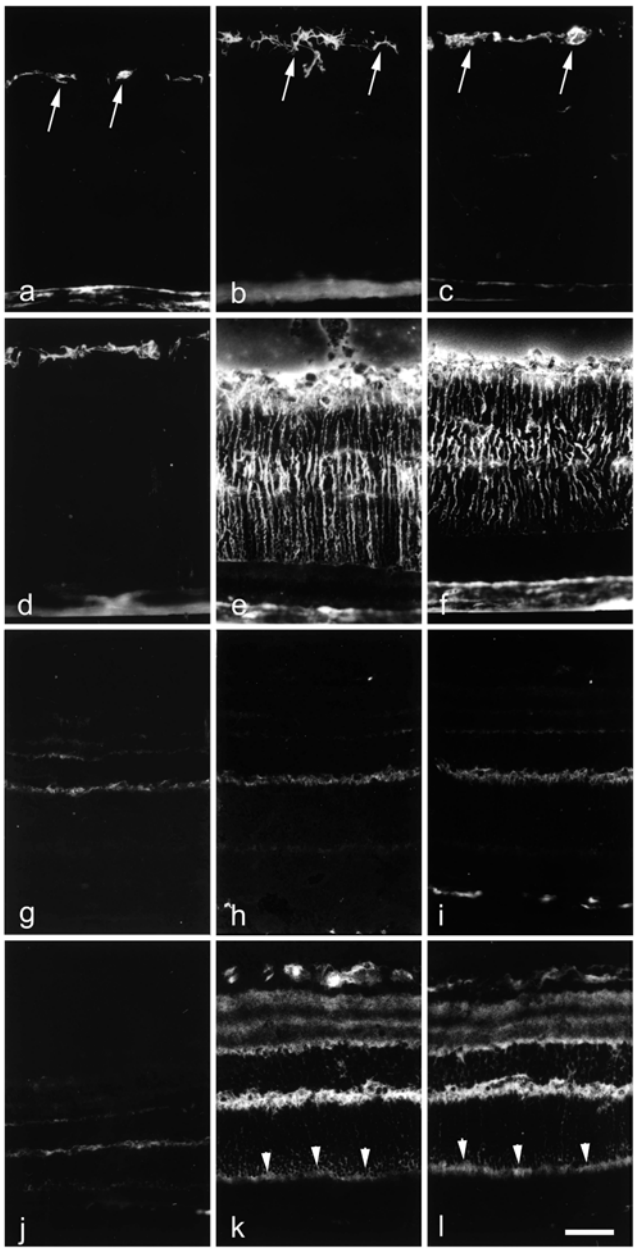

Figure 9: Expression of CHL1 and GFAP protein in control, NaCl-, and FGF-2 treated retinas of adult mice. GFAP-immunoreactivity in adult control retinas is restricted to retinal astrocytes located at the vitread margin (arrows in a). A similar distribution and intensity of GFAP-immunoreactivity is visible in retinas 7 (arrows in b) and 14 (arrows in c) days after intravitreal injection of NaCl. In comparison, GFAP-positivity of retinal astrocytes is massively elevated 7 (e) and 14 (f) days after application of FGF-2. In addition, GFAP in FGF-2-treated retinas becomes detectable in numerous radially oriented Müller cell processes (e,f). CHL1 protein in adult control retinas is mainly confined to the outer plexiform layer, but faint labeling is also detectable in the inner plexiform and nerve fiber layers (g). Pattern and intensity of the staining are not detectably altered 7 (h) or 14 (i) days after a single intravitreal injection of NaCl. In contrast, levels of CHL1 protein are significantly elevated 7 (k) and 14 (l) days after application of FGF-2. In FGF-2-treated retinas, CHL1 protein is strongly expressed in the outer and inner plexiform layers and in the nerve fiber layer (k,l). CHL1-immunoreactivity is also detectable in the inner and outer nuclear layer, and is associated with microvilli of Müller cells (arrowheads in k and l). Scale bar: 10µm.

Immunoblot Analysis of Lesioned Optic Nerves and FGF-2-Treated Retinas

Immunoblot analysis was performed to further study the expression of CHL1 and GFAP protein in both experimental tissues (Fig. 10). Expression of L1 was analyzed as a control. CHL1 protein was readily detectable in unlesioned nerves (Fig. 10a), in agreement with our immunhistochemical results. Compared to these controls,

intensity of CHL1-immunoreactivity was significantly increased 7 or 14 days after an intraorbital optic nerve crush (Fig. 10a). L1 protein was also detectable in unlesioned nerves (Fig. 10a), consistent with the presence of a few unmyelinated and L1-immunoreactive retinal ganglion cell axons in the adult mouse optic nerve (Bartsch et al., 1989). However, in striking difference to CHL1, expression of L1 protein was slightly down-regulated 7 days after the crush, and was hardly detectable 14 days after the lesion (Fig. 10a). Levels of GFAP protein in optic nerves isolated one week after the lesion were slightly elevated compared to non-injured control nerves. Expression of GFAP was significantly increased when nerves were analyzed two weeks after the crush (Fig. 10a). Levels of GAPDH were studied in the same tissues to control similar loading of proteins (Fig. 10a).

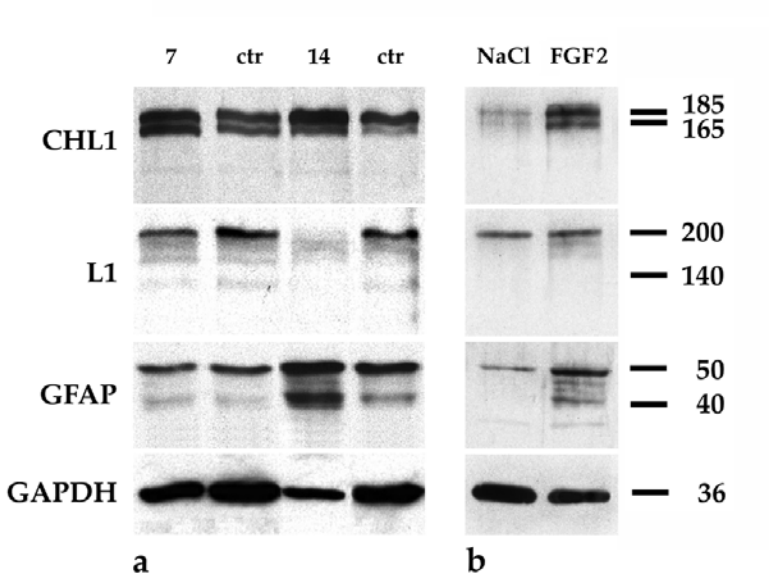

Figure 10: Immunoblot analysis of the expression of CHL1, L1 and GFAP in lesioned optic nerves and FGF-2-treated retinas of adult mice. Immunoblot analysis of intraorbitally crushed optic nerves 7 and 14 days after the lesion demonstrates significantly increased levels of CHL1 protein compared to the contralateral unlesioned nerves (a). Levels of L1 protein, in contrast, are progressively down-regulated one and two weeks after the crush (a). Analysis of retinas one week after a single application of FGF-2 reveals significantly elevated levels of CHL1 protein when compared to NaCl-treated retinas (b). Levels of L1 protein, in contrast, are similar in FGF-2- and NaCl-treated retinas (b). Expression of GFAP is up-regulated in lesioned optic nerves (a) and FGF-2-treated retinas (b) when compared to the corresponding control tissues. Expression of GAPDH was studied to control similar loading of proteins. Molecular weights (in kD) are indicated at the right hand margin.

Immunoblot analysis of retinal tissue one week after a single intravitreal application of FGF-2 revealed significantly increased levels of CHL1 when compared with NaCl-treated retinas (Fig. 10b). Levels of L1, in contrast, were similar in retinas exposed to either FGF-2 or NaCl (Fig. 10b). Amounts of GFAP protein were low in retinas exposed to NaCl, but markedly increased in FGF-2-treated tissue (Fig. 10b). GAPDH antibodies were used to control similar loading of proteins (Fig. 10b).

Discussion

CHL1 is a member of the L1 family of cell recognition molecules, additionally comprising L1, Nr-CAM and neurofascin. All these proteins are members of the Ig-superfamily and perform diverse functions in the developing or injured nervous system (Brümmendorf et al., 1998; Hortsch, 2000). L1 is certainly the most extensively studied member of this family and has been demonstrated to be critical for normal brain development in humans (Fransen et al., 1995) and mice (Cohen et al., 1998; Dahme et al., 1997). CHL1 has been identified as a heterophilic cell recognition molecule which supports neurite elongation and neuronal survival *in vitro* (Chen et al., 1999; Hillenbrand et al., 1999). Compared to other members of the L1 family, however, only little is known about the molecule's functions *in vivo*.

Recent studies suggest that CHL1 plays a role in the lesioned central and peripheral nervous system. For instance, spinal motoneurons and a subpopulation of dorsal root ganglion (DRG) neurons of adult rats up-regulated expression of CHL1 in response to a sciatic nerve crush. CHL1 was down-regulated in these nerve cells when functional regeneration had occurred, but remained elevated when functional regeneration was prevented by cutting or ligating the nerve (Zhang et al., 2000). Thus, increased expression of CHL1 by motoneurons and DRG neurons correlated with axonal regrowth, while down-regulation of the molecule appeared to require re-innervation of targets. Increased expression of CHL1 has also been observed in thalamic neurons after grafting a PNS explant. Interestingly, up-regulation of CHL1 occurred predominantly (and possibly exclusively) in those nerve cells which regenerated their axons into the peripheral nerve grafts (Chaisuksunt et al., 2000a). While the combined observations suggest a close correlation between elevated expression of CHL1 in neurons and axonal regrowth, there was no detectable up-regulation of CHL1 in a subpopulation of regenerating DRG neurons (Zhang et al.,

2000). Thus, CHL1 might be supportive but not absolutely required for successful axonal regeneration. It is also possible that axonal regeneration of some, but not all nerve cell types depends on CHL1 expression. CHL1 offered as a substrate or in soluble form supports neurite elongation by heterophilic interactions (Hillenbrand et al., 1999). CHL1 on the first regenerating axons might thus facilitate regrowth of later growing axons, or the 165 and 125kD fragments of CHL1 might support axonal regrowth in an autocrine or paracrine manner. CHL1 on growing axons might also interact with yet to be identified ligands, thereby transducing growth promoting signals to the nerve cells.

To obtain further insights into possible functional roles of CHL1 in the lesioned CNS, we studied the molecule's expression in the injured optic nerve of adult mice. Additionally, we analyzed the effect of exogeneous FGF-2, a growth factor known to induce astrogliosis and prevent death of axotomized neurons, on the expression of CHL1 in the adult mouse retina. Up-regulation of CHL1 expression by retinal nerve cells in response to an optic nerve crush was not observed, supporting the correlation between increased neuronal CHL1 expression and regrowth of injured axons (Chaisuksunt et al., 2000a,b; Zhang et al., 2000). However, in both experimental paradigms, we observed significantly increased expression of CHL1 in glial cells. In all experiments, there was a similar distribution of CHL1 and GFAP transcripts or protein, indicating that CHL1 in these experimental tissues is expressed by astrocytes.

Expression of tenascin-C, a cell recognition molecule of the extracellular matrix (for a review, see Bartsch, 1996), is also modulated by FGF-2. When FGF-2 was added to cultured astrocytes, levels of tenascin-C increased significantly (Mahler et al., 1996; Meiners et al., 1993). Interestingly, exposure of cultured astrocytes to FGF-2 reduced their ability to support neuronal adhesion, and evidence has been presented

that such astrocytes are less permissive substrates for growing neurites (Grierson et al., 1990; Meiners et al., 1993; Petroski et al., 1991). Tenascin-C and FGF-2 are both up-regulated in diverse brain regions after injury, as for instance around a stab wound of the cerebral cortex (Finklestein et al., 1988; Laywell et al., 1992). It is thus tempting to speculate that FGF-2 induces expression of tenascin-C, thereby modulating cell-matrix interactions in the lesioned CNS. There are several analogies between these data and our findings. We provide *in vivo* evidence that exogeneous FGF-2 up-regulates expression of another cell recognition molecule, CHL1, on retinal astrocytes and Müller cells. We also demonstrate that CNS glial cells up-regulate CHL1 after an optic nerve crush. FGF-2 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 injured optic nerve might, at least in part, be induced by elevated levels of endogeneous FGF-2.

The functional significance of the elevated expression of CHL1 by astrocytes in lesioned optic nerves and astrocytes and Müller cells in FGF-2-treated retinas remains to be elucidated. While substrate-bound and soluble CHL1 has been shown to potently promote neurite elongation (Hillenbrand et al., 1999), reactive astrocytes are generally considered as poor or non-permissive substrates for growing neurites (e.g. McKeon et al., 1991; Snow et al., 1990; Snow and Robson, 1995). However, reactive astrocytes express also other potent promoters of neurite elongation, like laminin (McKeon et al., 1991; Rogers et al., 1983; Tomaselli et al., 1990). Apparently, the growth promoting effect of such molecules on reactive astrocytes is masked by inhibitory components, such as chondroitin sulfate, heparan sulfate, dermatan sulfate, or keratan sulfate containing proteoglycans (McKeon et al., 1995). Interestingly, proteoglycans have been demonstrated to bind to permissive molecules, thereby reducing or neutralizing their permissive action (Dou and Levine,

1994; Friedlander et al., 1994; Smith-Thomas et al., 1995). In contrast to differentiated or reactive astrocytes, immature astrocytes support neurite elongation and axonal regrowth (e.g. Smith et al., 1986; 1990; Wunderlich et al., 1994). Since CHL1 is expressed by undifferentiated glial cells (Hillenbrand et al., 1999; Holm et al., 1996), the molecule might contribute to the permissive substrate properties of immature astrocytes and thus to the formation of axon tracts in the developing brain.

One may also speculate about other possible roles of CHL1 in the lesioned CNS. For instance, FGF-2 supports the survival of a variety of nerve cell types *in vitro* (e.g. Morrison et al., 1986; Unsicker et al., 1987) and of axotomized nerve cells, among them retinal ganglion cells, *in vivo* (e.g. Anderson et al., 1988; Sievers et al., 1987). CHL1 has also been shown to support neuronal survival *in vitro* (Chen et al., 1999). It is thus possible that FGF-2 exerts part of its neuroprotective effect by increasing the expression of other molecules with neurotrophic properties.

Endogeneous and exogeneous FGF-2 has been demonstrated to support axonal sprouting in the denervated hippocampus (Fagan et al., 1997; Ramirez et al., 1999). Several mechanisms have been suggested to explain this biological action of FGF-2, including a direct effect of FGF-2 on cholinergic axons or a FGF-2-induced expression of additional growth factors in astrocytes (Fagan et al., 1997). Results of the present study raise the additional possibility that FGF-2 mediates its biological activity, at least in part, by elevating the level of CHL1 on hippocampal glial cells.

Altogether, up-regulation of CHL1 expression by regenerating nerve cells (Chaisuksunt et al., 2000a,b; Zhang et al., 2000) and by glial cells in response to injury or application of FGF-2 (the present study) suggests a functional role of the protein in the lesioned nervous tissue. Experiments using CHL1-deficient mice (Montag et al., 1997) might help to elucidate the yet unknown function(s) of the molecule in the injured nervous system.

Project 3: Pathfinding errors of corticospinal tract axons in NCAM-deficient mice

Introduction

The neural cell adhesion molecule NCAM is a cell recognition molecule of the immunoglobulin (Ig) superfamily and exists in three major isoforms with 180, 140 and 120 kD generated by alternative splicing of a single gene product. NCAM is widely expressed in the developing and adult brain and mediates its functions by homophilic as well as heterophilic interaction with a variety of ligands (Walsh and Doherty, 1997; Kiss and Muller, 2001). A functionally important posttranslational modification of NCAM is the addition of a linear homopolymer of alpha-2,8-linked sialic acid residues (PSA) to the fifth Ig-like domain of NCAM. Two different enzymes regulate sialylation of NCAM, designated STSiaII/STX and STSiaIV/PST (Ong et al., 1998). Sialylation of NCAM is regulated independently of its expression. Highly sialylated NCAM is mainly expressed during neural development and persists in the adult brain in regions of neuronal plasticity (Seki and Arai, 1991). The prevailing view is that PSA attenuates cell-cell interactions mediated by NCAM or other cell surface or cell substrate ligands and thereby facilitates dynamic changes in the developing and adult brain (Kiss and Rougon, 1997; Bruses and Rutishauser, 2001).

Analysis of mutant mice deficient in the 180kD isoform of NCAM (Tomasiewicz et al., 1993) or the entire protein (Cremer et al., 1994) has demonstrated the importance of the molecule for the development of the nervous system. These studies revealed a crucial role of NCAM for chain migration of neuronal precursor cells, fasciculation and pathfinding of axons, and synaptic plasticity (Tomasiewicz et al., 1993; Ono et al., 1994; Muller et al., 1996; Cremer et al., 1997; 1998). Remarkably, these

functions of NCAM appear to be largely mediated by polysialic acid (PSA). Mutant mice deficient in the polysialyltransferase ST8SiaIV/PST-1 or wild-type animals treated with a PSA-specific endosialidase (EndoN) displayed defects highly reminiscent of those observed in NCAM-deficient mice (Ono et al., 1994; Becker et al., 1996; Muller et al., 1996; Seki and Rutishauser, 1998; Eckhardt et al., 2000). Enzymatic removal of PSA also affected fasciculation and pathfinding of axons in chicken (Tang et al., 1992; Yin et al., 1994; Monnier et al., 2001) and zebrafish (Marx et al., 2001), and interfered with the formation of collateral branches of corticospinal axons within the rat spinal cord (Daston et al., 1996).

Because of the critical role of NCAM in the formation of axon tracts, NCAM-deficient mice have been used to study the functions of the molecule during the development of a long axonal projection, the corticospinal tract (CST). It is demonstrated, that elongation and pathfinding of corticospinal axons is impaired in the absence of NCAM, resulting in a pronounced hypoplasia of the tract in the adult.

Materials and Methods

Animals

The generation of NCAM-deficient mice has been described (Cremer et al., 1994). NCAM and STSiaII/STX mutants and wild-type mice were maintained on a C57BL/6J genetic background, and their genotype was determined using polymerase chain reaction (Cremer et al., 1994, Fukuda et al unpublished data).

Light and Electron Microscopy and Morphometry

Adult NCAM mutants (three to eleven months old; n = 8) and age-matched wild-type littermates (n = 7) were deeply anaesthetized and fixed by perfusion with 4% paraformaldehyde and 2.5% glutaraldehyde in phosphate buffered saline (PBS; pH 7.3). Tissue was embedded in Epon resin, and semithin and ultrathin sections were prepared from the most caudal regions of the medullary pyramids. Semithin sections were examined with an Axiophot microscope (Zeiss, Oberkochen, Germany). The area of the CST was determined using the Neurolucida image analysis system (Microbrightfield, Colchester, UK). Ultrathin sections were examined with an EM10 electron microscope (Zeiss).

Anterograde Axonal Tracing

Adult NCAM mutants (three to nine months old) and age-matched wild-type mice were fixed by perfusion with 4% paraformaldehyde. The brains of STSiaII/STX mice (eight months old) were freshly prepared seperating the medulla from the telencephalon and fixed by immersion in 4% paraformaldehyde for at least one week. A small crystal of the lipophilic fluorescent dye 1,1´-dioctadecyl-3,3,3´,3´-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes, USA) was inserted unilaterally into the medullary pyramid approximately 1 mm rostral to the pyramidal

decussation. To prevent spreading of the tracer across the midline, the contralateral pyramid was carefully removed using fine microscissors (Fig. 11a). Brains were stored in 4% paraformaldehyde in the dark at 37°C for at least 4 weeks, and the medulla and cervical spinal cord were serially sectioned with a vibratome.

To anterogradely trace corticospinal axons during development, we used one-day-old mice (the day of birth being defined as postnatal day 0) from heterozygous NCAM breeding pairs. Animals were deeply anaesthetized and the skull was punctured three times with a 27G needle (Braun, Emmenbrücke, Switzerland). DiI was dissolved in dimethylformamide (Sigma, Deisenhofen, Germany), and about 1µl of tracer was applied at each injection point using glass micropipettes attached to a Multi-Channel Picospritzer (General Valve, Fairfield, NJ) (Fig. 11).

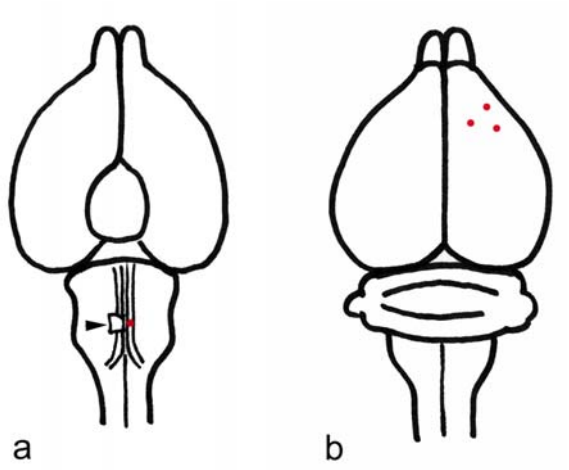

Figure 11:Schematic drawing of the tracer application site in adult and young animals
The scheme displays a ventral view of an adult brain in (a) and a dorsal view of a brain in (b). In adult animals, the spreading of the tracer is avoided by removing parts of the ventral pyramid contralateral to the application site of the DiI cristal (arrowhead). The DiI cristal is marked red. In young animals the tracer was injected through the skull at three different points of the motor-cortex (marked red, b).

Animals were sacrificed between the second and fifth postnatal day. Brains were fixed by immersion in PBS containing 4% paraformaldehyde and serially sectioned with a vibratome, starting at rostral levels of the medulla and ending at caudal levels of the cervical spinal cord.

Immunohistochemistry

Frontal and parasagittal vibratome sections were prepared from perfusion-fixed brains of neonatal and two-day-old wild-type and NCAM-deficient mice. Sections were blocked and incubated with polyclonal rabbit antibodies to NCAM (Bartsch et al., 1989) or monoclonal mouse antibodies 735 (Frosch et al., 1985), 12E3 (Seki and Arai, 1991) or 5A5 (Dodd et al., 1988), all of which recognize polysialic acid. In addition, some sections were incubated with polyclonal rabbit antibodies to the neural adhesion molecule L1 (Bartsch et al., 1989). Primary antibodies were visualized with Cy3-conjugated goat anti-rabbit or goat anti-mouse antibodies (Dianova, Hamburg, Germany).

Results

Hypoplasia of the corticospinal tract of adult NCAM-deficient mice

The CST originates from pyramidal neurons in layer 5 of the motor cortex. Corticospinal axons leave the cortex through the internal capsule and pass the basilar pons and the medulla. At the pyramidal decussation axons turn from ventral to dorsal, cross the midline and enter the spinal cord. In the medulla, corticospinal axons form the medullary pyramids at both sides along the ventral midline. At this level, the CST can be identified macroscopically. Macroscopic inspection of the medullary pyramids of adult NCAM-deficient mice and age-matched wild-type animals revealed a significantly reduced size of the tract in the mutants (not shown). Analysis of semithin sections prepared from the most caudal regions of the pyramids confirmed a significant hypoplasia of the CST of adult NCAM mutants when compared with wild-type animals (Fig. 12a,b).

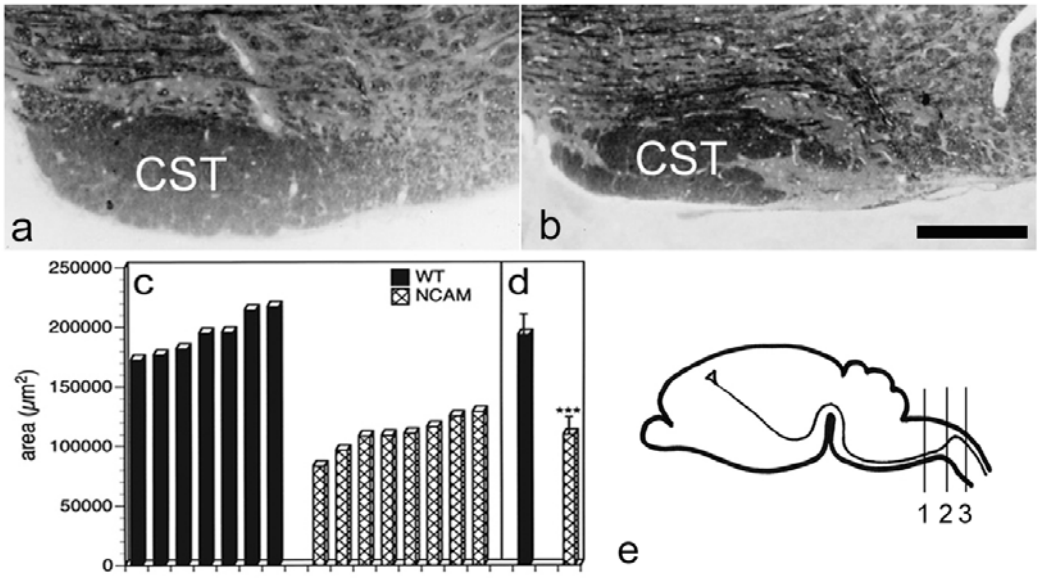

Figure 12: Hypoplasia of the corticospinal tract of adult NCAM mutants.
Frontal sections through the most caudal regions of the medullary pyramids of adult wild-type (a) and NCAM-deficient (b) mice reveal a significantly reduced size of the tract (CST in a and b) in the mutant. Morphometric analysis (c,d) of the CST in caudal regions of the medullary pyramids of 7 adult wild-type (filled bars) and 8 age-matched NCAM-deficient (hatched bars) mice confirms a statistically significant reduction in the size of the mutant CST (***: $p < 0.001$; Mann-Whitney U test). Bar in b (for a and b): 100µm.

Determination of the area of the CST of 7 adult wild-type and 8 age-matched mutant animals revealed average values of 193,275 ±17,639 μm^2 (mean ±SD) for wild-type and 109,774 ±14,786 μm^2 for NCAM-deficient mice (Fig. 12c,d). Thus, the size of the CST of adult NCAM mutants is reduced by more than 40% compared to wild-type controls ($p < 0.001$; Mann-Whitney U test).

NCAM has been implicated in myelination (see Bartsch, 1996, and references therein). To evaluate whether hypoplasia of the CST of NCAM mutants is related to defects in myelination (e.g. hypomyelination), we examined the ultrastructure of the tract at the level of the medullary pyramids. Myelin sheaths of NCAM mutants were intact and of normal thickness, axons were of normal size, and there was no evidence of hypomyelination of the mutant tract (data not shown).

Anterograde tracing of the CST of adult wild-type and mutant mice (n=15 for each genotype) with DiI and analysis of the tissue at the pyramidal decussation confirmed a significant hypoplasia of the tract in NCAM-deficient animals (Fig. 13). Defasciculation of the tract was not obvious, and corticospinal axons of all mutant mice turned dorsally at the pyramidal decussation, crossed the midline and entered the dorsal column.

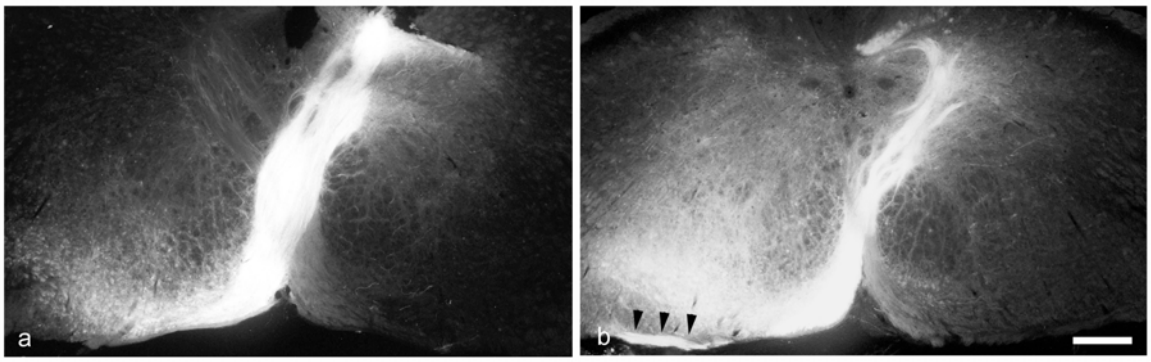

Figure 13: Anterogradly traced corticospinal tract axons of adult NCAM wildtype and knock out animals
Anterograde tracing of the corticospinal tract of NCAM wildtype mice revealed normally crossing axons growing from the ventral pyramid to the contralateral dorsal funiculus (a). NCAM ko mutants, in contrast, showed a significantly reduced size of the corticospinal tract (b). In some mutants (n=5) a ventral projection to the lateral side of the ipsilateral medulla was detected (arrow heads). Bar: 300µm

However, in some NCAM mutants (n=5) we detected a few corticospinal axons that remained ventral at the pyramidal decussation and, instead of growing to the contralateral side, projected to the lateral side of the ipsilateral medulla.

Pathfinding errors of corticospinal axons in young NCAM-deficient mice

Hypoplasia of the CST in adult NCAM mutants might result from pathfinding errors of a significant fraction of corticospinal axons during early development and the subsequent elimination of those aberrantly projecting fibers. To study whether pathfinding of corticospinal axons is impaired in the absence of NCAM, we performed anterograde tracing of corticospinal axons of early postnatal NCAM mutants and wild-type littermates.

In a first series of experiments, the tracer was applied at postnatal day one. Wild-type (n=11) and mutant (n=17) brains were analyzed three or four days later. In the medulla and at the pyramidal decussation, a prominent CST was visible in all wild-type animals. At the pyramidal decussation, corticospinal axons of all wild-type mice turned dorsally, crossed the midline and entered the dorsal column of the contralateral side (Fig. 14a,b). In NCAM-deficient mice, the size of the CST in the medulla and at the pyramidal decussation was reduced when compared with wild-type mice. In 16 out of 17 mutant mice, a substantial fraction of corticospinal axons displayed pronounced pathfinding errors at the pyramidal decussation. In 10 mutant mice, bundles of axons remained ventral and extended laterally instead of growing to the contralateral dorsal column (Fig. 14c,d). In 5 mutant animals, all corticospinal axons turned dorsally at the pyramidal decussation, but a substantial number of axons projected to the ipsilateral dorsal column instead of crossing to the contralateral side (Fig. 14e,f). Analysis of the dorsal columns of these animals revealed a prominent contralateral projection in wild-type mice (Fig. 14g), but a

bilateral projection in mutant mice (Fig. 14h). Finally, one mutant showed both an aberrant ventral and an aberrant ipsilateral projection of corticospinal axons, while one mutant showed no obvious pathfinding errors.

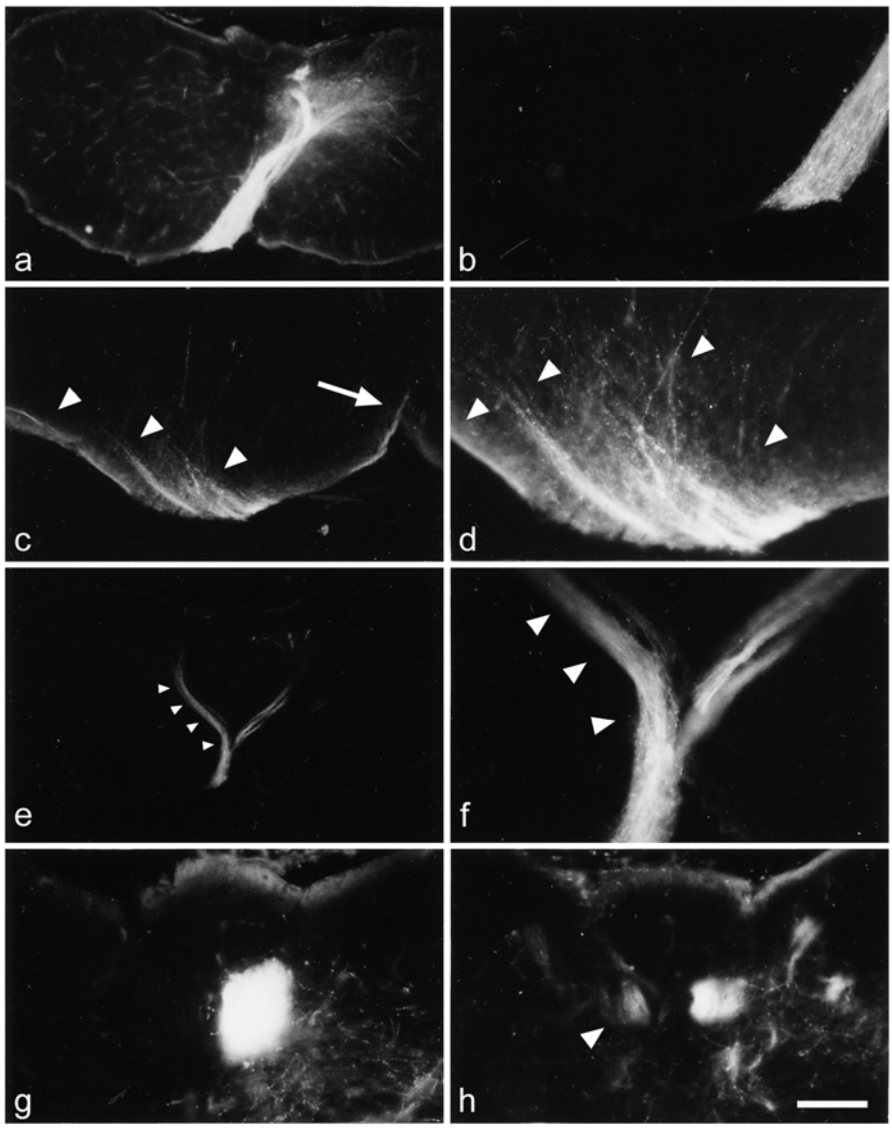

Figure 14: Pathfinding errors of corticospinal axons in young NCAM-deficient mice.
In wild-type mice (a) numerous corticospinal axons extend from the ventral pyramids to the contralateral dorsal column. Ipsilaterally or ventrally projecting axons are not detectable in these animals (a,b). In some NCAM mutants, a substantial number of corticospinal axons extends laterally at the ventral margin of the ipsilateral medulla (arrowheads in c and d) instead of crossing the midline (arrow in c) and extending into the contralateral dorsal column (d is a higher magnification of c). In other mutants, a significant portion of corticospinal axons fails to cross the midline and projects to the ipsilateral dorsal column (arrowheads in e; f is a higher magnification of e). In the dorsal column of wild-type mice, labeled axons are only detectable contralateral to the side of tracer application (g). Some mutant mice, in contrast, display a bilateral projection with a prominent contralateral and a smaller ipsilateral (arrowhead in h) projection. Animals in (a-d and g) and (e,f and h) were analyzed at postnatal day four and five, respectively. Bar in h (for a,e): 400µm, (b,d,f,g,h): 100µm, (c): 200µm.

Anterograde tracing of adult STSiaII/STX mice

The polysialyltransferase STSiaII/STX is the predominant enzyme regulating polysialylation during nervous system development in the embryo, whereas STSialV/PST remains expressed in the postnatal brain (Hildebrandt et al., 1998; Ong et al., 1998) A STX knock-out mouse was generated in the lab of Minoru Fukuda. Anterograde tracing of four STX-knock out mutants and four wildtype control animals revealed a significant hypoplasia of the corticospinal tract in the mutant. All axons were noramlly fasciculated and crossed from the ventral pyramid to the contralateral dorsal funiculus (Fig 15). The reduction of the thickness of the corticospinal tract is comparable to that of NCAM mutants, although a quantitative analysis has not yet been performed. Contrary to the NCAM mutant, no aberrantly growing fibers can be detected in adult STX knock out mice. While a detailed analysis of STX mutants, particularly during development, has still do be performed, these data strongly suggest that PSA, rather than the NCAM protein, is critical for the normal development of the corticospinal tract.

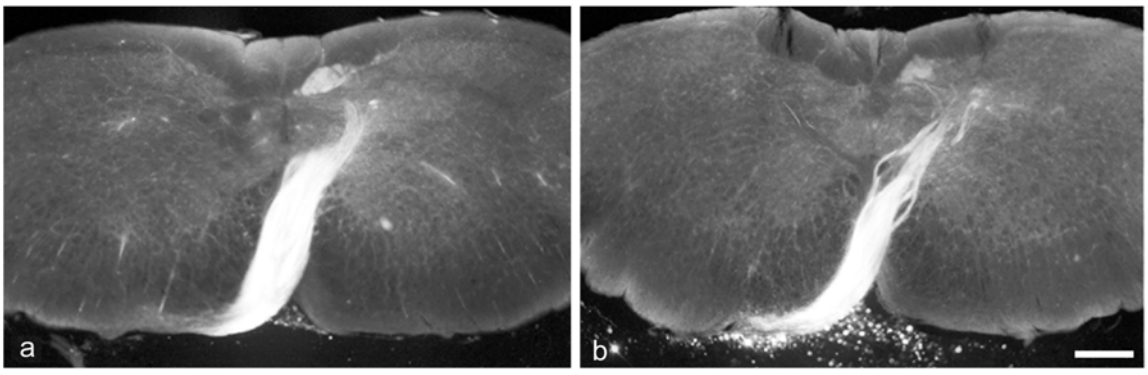

Figure 15: Anterograde tracing of adult STX wildtype and knock-out mutants.
Anterograde tracing of corticospinal tract axons of STX wildtype (a) and mutant mice (b) revealed a significant hypoplasia of the corticospinal tract. In contrast to the adlut NCAM knock-out mutant, the adult STX mutant displayed no aberrantly growing axons at the pyramidal decussation. Scale bar: 400µm

Delayed outgrowth of corticospinal axons in NCAM-deficient mice

NCAM has been demonstrated to promote neurite elongation *in vitro* (Walsh and Doherty, 1997). To evaluate whether this function is also apparent *in vivo*, we performed a second series of tracing experiments. DiI was applied at postnatal day one, and animals were analyzed one (4 wild-type and 4 mutant mice) or two days (1 wild-type and 2 mutant mice) later to determine whether formation of the CST is delayed in the absence of NCAM. Analysis of the CST at the pyramidal decussation in two-day-old wild-type mice (Fig. 16a) revealed the presence of a prominent axon tract that had crossed to the contralateral side and entered the dorsal column. In contrast, only a few axons had entered the pyramidal decussation in age-matched NCAM mutants (Fig. 16b). While these few axons had already turned to the dorsal and contralateral side, they had not yet entered the dorsal column. Labeled axons were not detectable in the dorsal column of mutant mice before postnatal day three (Fig. 16c). Thus, formation of the CST is significantly delayed in the absence of NCAM.

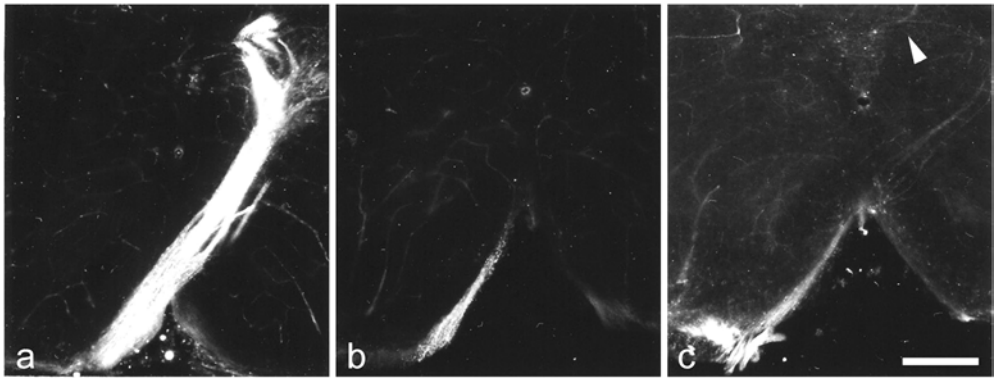

Figure 16: Formation of the CST of NCAM-deficient mice is delayed.
Corticospinal axons of two-day-old wild-type mice have crossed the midline and entered the contralateral dorsal column (a). In age-matched NCAM mutants, corticospinal axons have turned dorsally at the pyramidal decussation but have not yet crossed the midline (b). In three-day-old mutants, a few axons have entered the dorsal column (arrowhead in c). Bar in c (for a – c): 200µm.

Expression of NCAM and polysialic acid in the developing corticospinal tract

Expression of NCAM and PSA was studied in the CST of neonatal and two-day-old wild-type mice. The developing CST and the surrounding tissue was NCAM-immunoreactive at both developmental ages. However, NCAM-positivity in the CST was more intense than in the adjacent tissue and highlighted the developing pyramids in neonatal mice (not shown). Elevated NCAM-immunoreactivity was also detectable in the pyramids, the pyramidal decussation (Fig. 17a) and the outgrowing CST in the dorsal column of two-day-old animals. A similar spatio-temporal pattern of expression was observed when sections were incubated with the PSA-specific antibodies 735, 12E3 or 5A5. Intense PSA-immunoreactivity was associated with the developing pyramids of neonatal mice (not shown), and PSA antibodies highlighted the pyramids, the pyramidal decussation (Fig. 17b) and the CST in the cervical spinal cord of two-day-old mice.

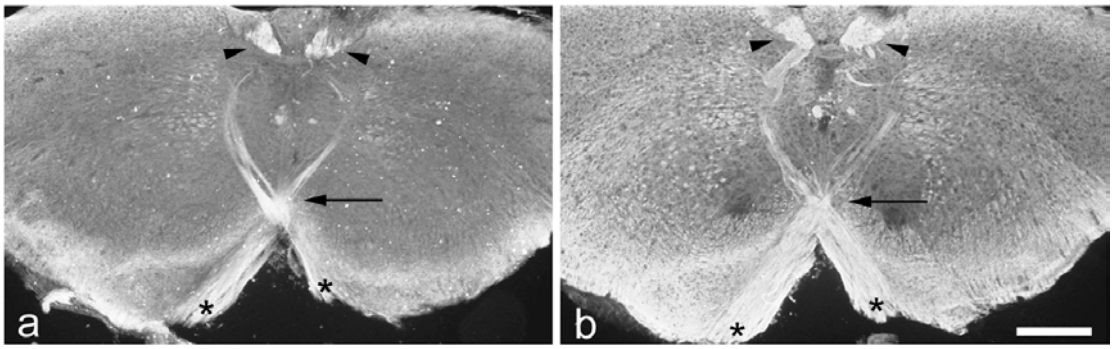

Figure 17: Localization of NCAM and polysialic acid in the developing corticospinal tract of mice.
Elevated levels of NCAM- (a) and PSA- (b) immunoreactivity highlight the corticospinal tract of two-day-old wild-type mice at the level of the pyramidal decussation. Intense NCAM- and PSA-positivity is associated with the tract as it turns from ventral to dorsal (asterisks in a and b), crosses the midline (arrow in a and b) and enters the dorsal column (arrowheads in a and b). Bar in b (for a and b): 300µm.

No immunoreactivity was observed when sections from NCAM-deficient mice were incubated with polyclonal NCAM- or monoclonal PSA-specific antibodies (not

shown). Furthermore, there was no obvious dysregulation of L1 expression in the CST of NCAM mutants (not shown).

Discussion

In the present study, we have used NCAM-deficient mice to analyze the functional roles of the molecule during the development of a long axon projection, the corticospinal tract. We observed severe hypoplasia of the tract in adult NCAM mutants, and pronounced pathfinding errors of corticospinal axons at the pyramidal decussation in early postnatal NCAM-deficient mice. Pathfinding errors were hardly detectable in adult mutants, suggesting that elimination of aberrantly projecting axons is the major cause of the hypoplasia of the tract in the adult.

Mice deficient in the neural adhesion molecule L1, a cell recognition molecule engaged in homophilic interactions that are enhanced by a carbohydrate-dependent cis-interaction with NCAM (Kadmon et al., 1990; Horstkorte et al., 1993), also show hypoplasia of the corticospinal tract (Dahme et al., 1997). Tracing experiments revealed pathfinding errors of L1-deficient corticospinal axons (Cohen et al., 1998) which are remarkably similar to those observed in NCAM mutants. In the search for molecular mechanisms causing these pathfinding errors, L1 has recently been demonstrated to associate with neuropilin-1 and, as a component of a Sema3A receptor complex, to convert Sema3A-mediated repulsive signals to growth cones of cortical neurons (Castellani et al., 2000). Pathfinding errors of corticospinal axons at the pyramdial decussation of L1-deficient mice might thus be related, at least in part, to a reduced sensitivity of L1-deficient axons to repulsive guidance cues expressed in the ventral spinal cord. Because of its association with L1, it is conceivable that NCAM may also confer repulsive guidance signals to growing axons.

While aberrantly projecting corticospinal axons persist in adult L1-deficient mice (Cohen et al., 1998), they were hardly detectable in adult NCAM mutants. It is therefore tempting to speculate that misrouted NCAM-deficient axons, in contrast to misrouted L1-deficient axons, fail to establish appropriate synaptic contacts and that

the elimination of these aberrantly projecting axons is the major cause of the severe hypoplasia of the CST in adult NCAM mutant mice. It is interesting in this context that enzymatic removal of PSA in neonatal rats delays formation of collateral branches from corticospinal axons and diminishes the extent of corticospinal axon branching within the spinal cord (Daston et al., 1996). Decreased axon branching might particularly interfere with the ability of aberrantly projecting corticospinal axons to appropriately innervate interneurons and motor neurons in the spinal cord of NCAM-deficient animals.

Defects in NCAM-deficient mice, including impaired chain migration of neuronal precursor cells within the rostral migratory stream or aberrant fasciculation and pathfinding of mossy fibers in the hippocampus is phenocopied in wild-type mice by the removal of polysialic acid (PSA) using the PSA-specific endosialidase N (EndoN; e.g.: Tomasiewicz et al., 1993; Cremer et al., 1994; 1997; Ono et al., 1994; Seki and Rutishauser, 1998). Removal of PSA also interferes with the fasciculation and pathfinding of peripheral or retinal axons in chick and zebrafish embryos (Landmesser et al., 1990; Tang et al., 1992; 1994; Yin et al., 1994; Monnier et al., 2001; Marx et al., 2001). Removal of PSA in other fiber tracts interferes with correct axonal pathfinding particularly at critical choice points. For instance, application of EndoN prevented defasciculation of outgrowing motor axons in the plexus region of the developing limb of chick embryos. Increased fasciculation interfered with the rearrangement of motor axons into appropriate muscle-specific nerve fascicles, ultimately resulting in aberrant axonal projections. Application of L1 antibodies reversed these effects (Tang et al., 1992; 1994). These observations indicate that negatively charged and extensively hydrated PSA mediates its functions by reducing homophilic and heterophilic adhesive interactions of NCAM or other cell substrate or cell surface ligands, thereby attenuating axon-axon or axon-substrate adhesiveness

and rendering axons more flexible to respond to guidance cues in their environment. NCAM-associated PSA might also be involved in the guidance of corticospinal axons. Expression of PSA in the developing CST of neonatal and early postnatal mice is in line with this hypothesis. In fact, intense and homogenously distributed PSA-immunoreactivity of the developing tract indicates expression of PSA on outgrowing corticospinal axons, as has been described for rats (Joosten et al., 1996). Of particular interest is the PSA-positivity of the CST at the pyramidal decussation, a region where corticospinal axons have to change their route of growth from a longitudinal into a transverse axis.

Analysis of mice deficient of the polysialyltransferase STX allowed investigation of the function of PSA during early developmental stages. Tracing experiments performed on STX knock out mutants revealed a hypoplasia of the corticospinal tract that appeared to be similar to that of the NCAM knock out mutant. These findings strongly suggest that the PSA homopolymer attached to the NCAM molecule is critical for the pathfinding of corticospinal axons at the pyramidal decussation.

However, a normal trajectory of CST axons in rats treated with EndoN shortly after birth (Daston et al., 1996) apparently argues against this hypothesis. It is possible that postnatal application of EndoN might be too late to uncover a function of PSA in the development of the CST. In rats, first corticospinal axons arrive at the pyramidal decussation shortly before birth and enter the upper levels of the cervical spinal cord at the day of birth (Joosten and Gribnau, 1989). Removal of PSA with Eno N during late embryonic development or analysis of young mice deficient in ST8SiaII/STX and possibly also in ST8SiaIV/PST-1 (Kiss and Rougon, 1997; Bruses and Rutishauser, 2001), will eventually provide more information about the molecular mechanisms that cause pathfinding errors of NCAM-deficient corticospinal axons at the pyramidal decussation.

Another finding of the present study is that corticospinal axons of NCAM-deficient mice arrive significantly later at the pyramidal decussation than corticospinal axons of wild-type mice. There are two possible explanations for this observation: either differentiation of pyramidal neurons in layer 5 of the cerebral cortex, the source of corticospinal axons, is delayed in the mutants, or the growth rate of NCAM-deficient corticospinal axons is slowed down. Since we are not aware of malformations of cortical layers in NCAM-deficient mice, we favor the latter hypothesis. NCAM has been shown to enhance neurite outgrowth *in vitro* by the activation of intracellular signaling cascades (Walsh and Doherty, 1997), and PSA has been demonstrated to accelerate neurite extension by reducing L1-mediated adhesion of neurites to the substrate (Zhang et al., 1992). Delayed outgrowth of corticospinal axons is, to our knowledge, the first observation of impaired axon elongation in NCAM-deficient mice. It remains to be investigated whether this defect is a direct or indirect consequence of NCAM deficiency.

Summary and Perspectives

Hydrocephalus is the most prominent feature of CRASH patients and also of the L1 knock-out mutant. In this study we demonstrated that a stenosis of the aqueduct of Sylvius is not the primary cause for the development of this pathology, neither in human, nor in the L1 mutant. Since L1 is implicated in cell migration and neurite outgrowth, it is reasonable to assume that L1 deficiency leads to impaired neuronal connections and subsequently to enhanced neural degeneration. The abnormal loss of nerve cells might then result in development of hydrocephalus *ex vacuo.* Thus, the mechanisms of the development of congenital X-linked hydrocephalus remain to be elucidated.

CHL1 has been implicated in axonal regeneration. Upregulation of CHL1 by astrocytes after an optic nerve lesion or by Müller cells and astrocytes after intravitreal application of FGF-2, also suggests a role of this protein in the lesioned central nervous system. The CHL1 knock out mutant will allow to investigate the function of CHL1 in the regeneration of nervous tissue in more detail. The FGF-2 knock out mouse will be a useful model to test whether upregulation of CHL1 after nerve lesion is in fact regulated by the expression of FGF-2.

Pathfinding errors of corticospinal tract axons in the NCAM knock-out mutant appear to be due to the loss of PSA, a large homopolymer attached to the NCAM molecule, rather than to the loss of the NCAM protein itself. Adult mice deficient in the polysialyltransferase STSiaII/STX show a similar hypoplasia of the corticospinal tract as adult NCAM mutants, confirming the importance of PSA for the normal development of this tract. A detailed developmental analysis of the outgrowth of corticospinal tract axons in young STX mutants will provide more information about the function of PSA during elongation and pathfinding of corticospinal axons.

Knock-out mutants provide useful *in vivo* models to investigate the specific functions of a molecule. The L1 knock-out mutant, for instance, serves as a good animal model for the CRASH syndrome in human. The mutant displays similar symptoms as found in human patients, such as hydrocephalus, motor impairments of the hindlimbs and hypoplasia of the corticospinal tract. Interestingly, not all brain regions where L1 is normally expressed, are affected in the knock-out mutant. Furthermore, cell types that have been shown to depend on L1 in *in vitro* experiments appear to develop normally in L1 mutants, as for instance cerebellar granule cells. These findings suggest compensatory mechanisms in the L1-deficient mouse.

In the cerebellum, NrCAM shows a striking co-localization with. Both molecules can be found on the processes of granule cells, and NrCAM, as L1, was demonstrated to be involved in the migration of these cells. Still, the cytoarchitecture of the cerebellar cortex of NrCAM mutants was not detectably affected, in analogy to the L1 mutant. The development of the cerebellar cortex was, however, severely impaired in double knock-out mutants lacking both L1 and NrCAM.

The cerebellum of the double knock out mice was smaller and the thickness of the internal granule cell layer was massively reduced, a phenotype that can not be simply explained by the addition of the phenotypes of the single mutants. These findings rather suggest that L1 compensates for the loss of NrCAM and vice versa. Only when both molecules are missing, the development of the cerebellum is severely impaired. These findings strongly confirm the hypothesis that homologous molecules are capable of compensating the loss of another molecule. It is interesting in this respect that in the cerebellum of NrCAM mutants, neurofascin binds to the (RPTP)β/ζ a function which is normally only demonstrated for NrCAM. This finding demonstrates that related molecules might compensate for functions, which they normally do not perform.

The upregulation of NCAM 180 in several brain regions of the CHL1 knock out mutant is another phenomenon that strengthen the hypothesis of compensatory mechanisms among cell adhesion molecules. However, the functional significance of elevated levels of NCAM in CHL1 remains to be elucidated.

In order to gain more insight into the co-operation of cell recognition molecules of the Ig superfamily, we have started to breed double knock out mutants, deficient in L1 and CHL1, L1 and NCAM or CHL1 and NrCAM. Gross morphological studies of the L1/CHL1 and the CHL1/NrCAM mutants and immunohistochemical studies using a variety of markers did, up to now, not reveal more severe phenotypes of the double mutants when compared to the respective single mutants. Further analysis of the double mutants, including acute perturbations, behavioural or electrophysiological investigations might help to understand the mechanisms of the interaction of the distinct members of the L1 family and NCAM more effectively.

Of potential interest is the observation that despite extensive breeding, we have not yet obtained any live L1/NCAM double mutants. It thus appears that these double mutants are severely affected, leading to death during embryonic development. Since such a sever phenotype would not be expected from a combination of the L1 and NCAM single phenotypes, this finding suggests compensatory functions for L1 and NCAM that are disrupted in the double mutant.

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Abbreviations

CALL	cell adhesion L1-like
CHL1	close homologue of L1
CNS	central nervous system
CRASH	corpus callosum hypoplasia, retardation, adducted thumbs, spastic paraplegia, and hydrocephalus
CST	corticospinal tract
DiI	1,1´-dioctadecyl-3,3,3´,3´-tetramethylin Docarbocyanine perchlorate
DRG	dorsal root ganglion
EM	electron microscope
FGF-2	fibroblast growth factor-2
FGF-R	fibroblast growth factor receptor
FN III	fibronectin type III
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFAP	glial fibrilary acidic protein
GPI	glycosyl-phosphaditylinositol
HSAS	hydrocephalus due to a stenosis of the aqueduct of Sylvius
Ig	immunglobulin
KD	kilo Dalton
MAG	myelin-associated glycoprotein
NCAM	neural cell adhesion molecule
NgCAM	neuron-glial cell adhesion molecule
NrCAM	NgCAM related cell adhesion molecule
PAT	proline-, alanine- and threonine rich domain
PBS	phosphate buffered saline
PNS	peripheral nervous system
PSA	polysialic acid
SD	standard deviation
STX	ST8SiaII/STX (polysialyltransferase)
tTA	tetracycline-controlled transactivator
VASE	variable alternative spliced exon

Publications

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Rolf B, Lang D, Hillenbrand R, Richter M, Schachner M and Bartsch U. Altered expression of CHL1 by glial cells in response to optic nerve lesions and intravitreal application of fibroblast growth factor-2. Submitted J Neurosci Res

Rolf B, Bastmeyer M, Schachner M and Bartsch U. Pathfinding errors of corticospinal axons in NCAM-deficient mice. Submitted J Neurosci

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