Distribution and functions of the Extracellular Matrix protein matrilin-2 in the nervous system of mice

Dissertation

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I. Introduction

1 Extracellular Matrix

Most cells are part of multicellular structures or tissues within the body. In epithelia, the cells are closely linked with each other, whereas in connective tissues the cells are loosely distributed within the extracellular matrix (ECM) (Fig. 1). These tissue types represent extremes and in most other tissues there are cells which are in close contact with some other cells, and cells which are predominantly embedded in the matrix. The ECM is secreted by the cells located within it. The composition of the ECM is often cell-type specific but the major protein components are shared by many tissues and include glyco-proteins, collagens and proteoglycans. Although the ECM has been considered to predominantly play a structural role, it is becoming clearer that the ECM is vitally important in determining the functional responses of cells to their environment. Thus, it is involved in determining developmental processes, cell migration, cell maturation and differentiation, cell survival, tissue homeostasis, tumor cell invasion and other pathological events. Cells express specific receptors on their surface for components of the ECM, which mediate these responses. Many comprehensive reviews have detailed the evidence supporting the role of the ECM in mediating different cellular processes, but much less information is available detailing the mechanisms by which the ECM can control these processes.

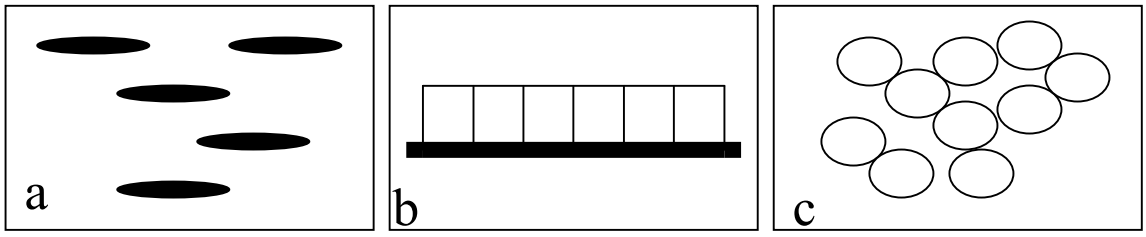


Fig. 1 Cellular interactions with neighboring cells and with the extracellular matrix: (**a**) connective tissue: sparse distribution of cells within plentiful ECM; (**b**) epithelial cells: tight connections between cells with only a very sparse matrix; the solid bar indicates the basal lamina underlying the cell sheet; (**c**) distribution of cells within many tissues, in which cells form interactions either with neighboring cells or components of the matrix.

1.1 Extracellular Matrix in peripheral nervous system (PNS)

The major cellular constituents of the mammalian PNS are neurons (axons) and Schwann cells. During peripheral nerve development Schwann cells actively deposit ECM, comprised of basal lamina sheets that surround individual axon-Schwann cell units and collagen fibrils. To interact with the ECM, Schwann cells express a number of integrin and non-integrin cell surface receptors. The expression of several Schwann cell ECM proteins and their receptors is developmentally regulated and, in some cases, depends on axonal contact. The peripheral nervous system ECM acts as an organizer of peripheral nerve tissue and strongly influences cell adhesion, migration, growth and differentiation and influences axonal growth during development and regeneration.

Two major subdivisions of the ECM are the basal lamina and the fibrillar matrix (Fig. 2). The fibrillar matrix is a thin sheet of ECM comprised of a cross-linked network of collagens and other molecules that encase individual axon-Schwann cell units and form contacts with the outer Schwann cell membrane. The fibrillar matrix consists of a collection of collagen-based fibrils that lie external to the basal lamina in mature nerves. The major producer of peripheral nerve ECM is the Schwann cell, but at least some aspects of ECM production and/or assembly are additionally regulated by neurons. The structure and composition of the peripheral nerve ECM changes during development to be able to promote the functional processes appropriate for each stage.

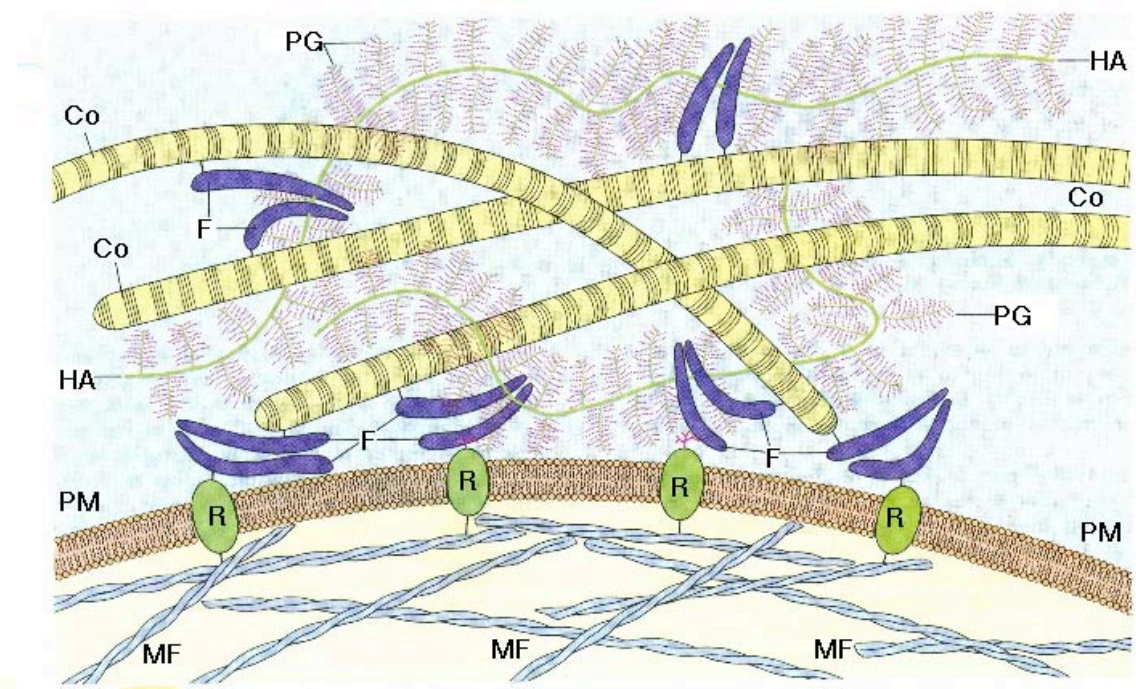


Fig. 2 The fibrillar matrix consists of collagen fibrills (Co), hyaluronic acid backbone (HA) connected with different proteoglycans (PG) and fibronectin (F). This structure interacts with plasmatic membrane (PM) via different receptors (R). MF-microfilaments.

1.1.1 Functions of peripheral nervous system ECM

Schwann cells adhesion and migration

A number of ECM proteins secreted by Schwann cells promote adhesion of these cells in vitro. These include laminin, fibronectin, collagen types I, IV, XVIII and p200 (McGarvey et al., 1984; Chernousov et al., 1996; Milner, 1997; Halfter et al., 1998). However, the intensity of adhesive activity of these proteins for Schwann cells differs. Laminin is considered to be the most active, followed by fibronectin and collagen type IV, V. (McGarvey et al., 1984; Milner, 1997; Chernousov et al., 2001). Fibronectin and laminin also promote migration of Schwann cells and stimulate their growth in cell culture (Baron-Van Evercooren et al., 1982; McGarvey et al., 1984; Anton et al., 1994; Milner, 1997).
Interestingly, not all ECM proteins promote adhesion or migration of Schwann cells. For instance, tenascin, which is abundantly expressed in the embryonic peripheral nervous system, does not support migration of Schwann cell precursors, but, moreover, actively inhibits migration of Schwann cells on fibronectin (Wehrle-Haller and Chiquet, 1993; Probstmeier et al., 2001). Another component of the peripheral nervous system ECM, fibrin, can also inhibit Schwann cell migration (Akassoglou et al., 2003). Likewise, decorin- and versican-like proteoglycans inhibit adhesion of Schwann cells to fibronectin, but not to laminin or collagen (Braunewell et al., 1995). This inhibitory effect is apparently specific for these proteoglycans and is not produced by perlecan (Braunewell et al., 1995). The physiological functions of such inhibitory activities are currently unknown.

Neurite outgrowth and axon fasciculation

Several peripheral nervous system ECM molecules including fibronectin, laminin, tenascin and collagen type IV promote outgrowth of neurites from peripheral neurons in vitro when coated as uniform substrates (Rogers et al., 1983; Lein et al., 1991; Wehrle-Haller and Chiquet, 1993; Anton et al., 1994).

The peripheral nervous system ECM also contains molecules with neurite-outgrowth-inhibiting activity. A 400 kDa chondroitin sulfate proteoglycan (CS-PG) synthesized by Schwann cells has been shown to inhibit the -neurite-outgrowth-promoting activity of laminin (Zuo et al., 1998). Glycoprotein tenascin-R and collagen type V inhibit neurite outgrowth from dorsal root ganglion neurons (Chernousov et al., 2001; Probstmeier et al., 2001). The inhibitory activity was attributed mainly to the chondroitin sulfate chains of the proteoglycan, since it was abolished by treatment with chondroitinase. The NG2 chondroitin sulfate proteoglycan, inhibits axon growth from cerebellular neurons and dorsal root ganglion (Ughrin et al., 2003).

Thus, the ECM that is deposited by Schwann cells during development appears to contain a complex mixture of permissive and non-permissive substrates. A function of this ECM, therefore, might be to facilitate directed axon growth. It would do this by promoting the growth of axons along appropriate pathways and inhibiting inappropriate growth, such as collateral sprouting.

Regulation of axonal fasciculation plays an important role in the precise patterning of neural circuits. The main roles in this process belong to neural cell adhesion molecules such as L1, NCAM, NgCAM, axonin1, fasciclin II, F3/contactin, and neurotrimin which normally promote axonal fasciculation in the peripheral and central nervous system (Cremer et al., 1997; Kunz et al., 1998; Wright et al., 1999; Chen et al., 2001; Falk et al., 2002; Wiencken-Barger et al., 2004). Also FEZ1 and FEZ2, which are the members of fasciculation and elongation protein zeta family were reported to be involved in axonal outgrowth and fasciculation (Fujita et al., 2004). It has been shown recently, that chondroitin sulfate proteoglycans ECM molecules promote axon fasciculation of neurons from dorsal root ganglion, that may be defasciculated upon contact with laminin (Snow et al., 2003). In co-cultures of dorsal root ganglion neurons and Schwann cells, collagen type V promotes axon fasciculation and association of axons with Schwann cells (Chernousov et al., 2001).

Myelination of axons by Schwann cells

A lot of evidence confirms interactions between Schwann cells and basal lamina ECM. It has been shown that these interactions are important for axon myelination. Co-culture grown in serum free medium without ascorbate fail to assemble ECM (Eldridge et al., 1987). The Schwann cells in these cultures proliferate but fail to myelinate axons. Myelination can be induced by addition of ascorbic acid, which stimulates assembly of ECM, or by addition of

exogenous basal lamina-type ECM components (Carey et al., 1986; Eldridge et al., 1987; Eldridge et al., 1989). Similarly, inhibition of basal lamina by addition of proteoglycan synthesis inhibitor blocks myelination (Carey and Todd, 1987).

The importance of basal lamina for the terminal differentiation of Schwann cells is also supported by the phenotype of the *dy/dy* mice, which have a mutation in the laminin-2 gene (Sunada et al., 1994; Xu et al., 1994). Schwann cells in spinal roots of these animals exhibit significant deficits in basal lamina assembly. Interestingly, these defects are much less severe in distal parts of the peripheral nervous system (Bradley and Jenkison, 1973; Bunge et al., 1982). This suggests that ECM proteins present in these tissues can compensate for the loss of laminin-2.

It is interesting to consider these findings with earlier studies of developing tadpole peripheral nerves, which revealed the lack of basal lamina structures around migrating but not myelinating Schwann cells (Billings-Gagliardi et al., 1974). Together, these findings suggest the possibility that appearance of basal lamina serves as a trigger to initiate the process of myelination.

1.2 ECM of central nervous system (CNS)

While ample evidence exists for the ECM in the peripheral nervous system, similar evidence for the central nervous system has been harder to obtain. Although the ECM of the brain has many components that can be found in the ECM of other tissues, there are some distinct differences in composition and some unique components that are only expressed in the brain. For example, the brain ECM contains relatively small amounts of fibrous proteins (e.g collagens or fibronectin) and high amounts of glycosaminoglycans, either bound to proteins, thus forming proteoglycans, or unbound in the form of hyaluronan.

The extracellular space surrounding many neuronal cells in the CNS is filled with a network of glycoproteins, proteoglycans and HA. Close to the membrane of such cells the ECM becomes denser, forming a basement membrane composed principally of collagens, glycoproteins - particularly tenascin-C and tenascin-R; CS-PGs and heparan sulphate proteoglycans (HS-PGs); HA; cell adhesion molecules and integrins.

1.2.1 Members of ECM and their functions in the CNS

Glycoproteins (Tenascins)

Tenascins (Tns) are very important ECM glycoproteins with a wide range of binding sites and functions (Hoffman et al., 1988; Steindler et al., 1989; Grumet et al., 1994; Husmann et al., 1995; Faissner, 1997). TNs are abundant in the basement membrane, and are produced by astrocytes during development, playing important roles in mediating axon-glia interactions (Steindler et al., 1989; Faissner and Kruse, 1990; Lochter et al., 1991). Currently, five members of the tenascin gene family (TN-C, -R, -X, -Y, and –W) have been identified in diverse species from zebrafish to humans (Bristow et al., 1993; Schachner et al., 1994; Hagios et al., 1996; Weber et al., 1998; Jones and Jones, 2000a). Of these, TN-C and TN-R have been reported to be expressed in the CNS (Jones and Jones, 2000b). TN-C has been studied in the greatest detail. It's mainly expressed by immature and reactive astrocytes (Kawano et al., 1995; Bartsch, 1996), but also expression in some nerve cell types has been demonstrated. These include immature neurons, such as granule cells in the hippocampus and motoneurons of the spinal cord (Kawano et al., 1995; Bartsch, 1996). TN-C inhibits neuronal plasticity in some restricted areas of the nervous system, such as the hippocampus (Ferhat et al., 1996; Nakic et al., 1998), cerebellar cortex, retina, optic nerve (Bartsch, 1996), and hypothalamus (Theodosis et al., 1997). TN-C is expressed as numerous alternatively spliced variants with various functions (Faissner et al., 1988; Stern et al., 1989; Chuong and Chen, 1991; Faissner, 1997). The same tenascin-C molecule may have either growth-inhibitory or growth-promoting effects towards different neurons within different contexts. A number of studies have demonstrated the neurite growth-inhibitory properties of tenascin in vitro (Pesheva et al., 1989; Crossin et al., 1990; Faissner and Kruse, 1990). Also growth-promoting effects have been ascribed to the alternatively spliced A-D and D5 domains (Meiners et al., 1999; Meiners et al., 2001).

TN-R expression appears to be restricted to the CNS. It is synthesized by oligodendrocytes during the process of myelination (Bartsch et al., 1993; Wintergerst et al., 1993). It is detectable at contact sites between unmyelinated axons and between myelin sheaths and is highly accumulated at the nodes of Ranvier (Bartsch et al., 1993). TN-R is also expressed by small subsets of CNS neurons, interneurons and motorneurons in spinal cord, retina, cerebellum and hippocampus (Fuss et al., 1993; Wintergerst et al., 1993; Weber et al., 1999). It is localized in perineuronal nets (PNs) that surround inhibitory interneurons (Celio and

Blumcke, 1994; Wintergerst et al., 1996). The lack of TN-R leads to abnormal distribution and shape of PNs (Weber et al., 1999).
The production of TN's is up-regulated in the glial scar after injury (Laywell et al., 1992). Tenascin is also known to interact with many CS-PGs in vitro (Grumet et al., 1994; Xiao et al., 1997; Xiao et al., 1998).

Proteoglycans

The CNS is rich in proteoglycans. These diverse molecules have a common buildup comprised of a core glycoprotein with glycosaminoglycan (GAG) sugar chains covalently attached. Each GAG consists of a simple, linear polymer of repeating disaccharide units, composed from two alternating monosacharides: usually one sugar is an uronic acid and the other is either N-acetylglucosamine or N-acetylgalactosamine. Different types of GAG may be created as a result of sulphation and epimerization modifications that are carried out on the sugars themselves following polymerization. The length of GAG chains may also vary from a polypeptide chain of 10 kDa to 400 000 kDa, and the core protein itself may have varying numbers of GAG chains attached - from one to well over 100, resulting in a diverse array of PGs with a range of functional complexities. Many of the functional properties of PGs are attributed to the attached side chains. Much of the interaction between PGs and cell-surface receptors or ECM proteins is thought to occur via binding sites on the GAG chains, although the core protein is also able to bind substrates (Bandtlow and Zimmermann, 2000). Sulphated GAG consists of CS, HS, dermatan sulphate (DS) or keratan sulphate (KS). The CS-PGs are represented by several types, including large aggregating proteoglycans such as aggrecan (Paulsson, 1987) and versican (Krusius et al., 1987) and the brain-specific proteoglycans neurocan (Grumet et al., 1993; Friedlander et al., 1994; Grumet et al., 1994; Oohira et al., 1994), brevican (Yamada et al., 1994a), NG2 (Levine and Card, 1987; Stallcup and Beasley, 1987) and phosphacan/DSD-1 (Grumet et al., 1993; Grumet et al., 1994; Maeda et al., 1994; Maurel et al., 1994). All of these CS-PGs are expressed in the CNS, are abundant within the ECM and interact with a variety of other matrix components including laminin, fibronectin (Schmidt et al., 1991), TN-C (Grumet et al., 1994), HA (Krusius et al., 1987; Paulsson, 1987; Doege et al., 1991) and collagen (Bidanset et al., 1992; Hedbom and Heinegard, 1993).
Proteoglycans have been associated with a variety of cellular processes including cell adhesion, growth, receptor binding, cell migration and barrier formation. Many *in vitro* studies have demonstrated that CS-PGs are inhibitory towards neurite outgrowth, either via

their CS chains or their core proteins (Dou and Levine, 1994; Friedlander et al., 1994; Smith-Thomas et al., 1994; Yamada et al., 1997; Niederost et al., 1999; Asher et al., 2000; Schmalfeldt et al., 2000). Additionally, both phosphacan and neurocan are able to bind with high affinity to the cell adhesion molecules N-CAM and Ng-CAM/L1 (Grumet et al., 1993; Friedlander et al., 1994), thus interfering with their interactions and indirectly inhibiting neurite outgrowth (Friedlander et al., 1994; Milev et al., 1994). Reactive astrocytes and/or OP cells within the glial scar have been shown to up-regulate their expression of various axon-inhibitory CS-PGs in vivo, in particular neurocan (Haas et al., 1999; McKeon et al., 1999; Asher et al., 2000), NG2 (Levine, 1994; Ong and Levine, 1999; Rhodes et al., 2003), phosphacan (Laywell and Steindler, 1991; McKeon et al., 1995) and versican (Asher et al., 2002; Jones et al., 2003; Tang et al., 2003).

1.2.2 Perineuronal nets (PNNs)

Structure and composition

PNNs were first described by Golgi and Cajal in the 1890s as reticular networks observed on the surface of neuronal cell bodies and proximal dendrites. They develop postnatally and finally appear as net-like features on the cell surface as a result of ECM materials deposited around synaptic endings and in the space between neurons and astrocytic processes. PNNs were originally visualized using iron- or silver-based histochemical stains. Most PNNs can be detected using lectins, such as the Wisteria floribunda agglutinin lectin, which recognizes N-acetylgalactosamine in CS-GAG chains (Hartig et al., 1992). Although it is not exactly known which cell types produce the PNNs, they are most commonly found around parvalbumin-containing GABAergic interneurons (Hartig et al., 1992; Bruckner et al., 1994; Hartig et al., 1994; Murakami et al., 1995b) and pyramidal cells (Hausen et al., 1996) in the cortex, and around projection neurons and large motorneurons of the brain stem and spinal cord (70-80% neurons in the cord have PNNs) (Asher et al., 1995; Murakami et al., 1995a; Takahashi-Iwanaga et al., 1998). Because glial cells in vitro can produce ECM similar to PNNs observed in vivo, even in the absence of neurons (Maleski and Hockfield, 1997), it's likely that astrocytes contribute at least in part to PNN formation. Although the structure and composition of PNNs has been studied intensively their function still remains obscure. It was demonstrated immunohistochemically (Asher et al., 1995) that aggrecan and a link protein,

two major cartilage constituents, are present in the perineuronal matrix of bovine spinal cord. Immunoreactivities were hyaluronate-dependent and could be abolished by pretreatment with the enzyme hyaluronidase. Aggrecan is a large proteoglycan belonging to the lectican family of CS-PGs, which also includes brevican, neurocan and versican. Whereas aggrecan and versican are present in a number of connective tissues (Mundlos et al., 1991; Yamagata et al., 1993; Glumoff et al., 1994; Popp et al., 2003), brevican and neurocan are usually found only in neural tissues (Rauch et al., 1992; Oohira et al., 1994; Yamada et al., 1994a; Seidenbecher et al., 1995; Watanabe et al., 1995). All four of these lecticans have since been detected in PNNs (Bignami et al., 1993; Koppe et al., 1997a; Koppe et al., 1997b; Bruckner et al., 1998; Matsui et al., 1998; Matthews et al., 2002), as has phosphacan/DSD-1 (Wintergerst et al., 1996; Haunso et al., 1999) and tenascin-R (Bruckner et al., 2000; Haunso et al., 2000). In a recent review (Yamaguchi, 2000) a model of PNNs has been proposed whereby long HA molecules bind CS-PGs (lecticans), which in turn bind tenascin and form net-like complexes between neurons and glia.

PNNs are not identical in composition: whereas PNNs in the rat deep cerebellar nuclei and spinal cord, for example, are rich in expression of phosphacan, sections of cortex demonstrate little phosphacan immunoreactivity (Fig. 3). Antibodies to the aggrecan-like antigen CAT-301 appear to label PNNs as frequently as the Wisteria floribunda lectin, whereas versican expression is more distinguishable around large motor neurons in the spinal cord and brainstem. The importance of differential expression patterns of CS-PGs in PNNs is unclear. Interestingly tenascin-knockout mice show disrupted PNNs (Weber et al., 1999; Bruckner et al., 2000; Haunso et al., 2000), whereas mice deficient in neurocan (Zhou et al., 2001) or brevican (Brakebusch et al., 2002) were anatomically and morphologically similar to wild-type mice, demonstrating that tenascin is a critical component of PNNs.

Previous and present hypothesis on the function of these nets and the strategies to understand their importance have followed in particular two major functional aspects:

- Mechanical stabilization of synaptic contacts via aggregating PGs (Hockfield et al., 1990), resulting in a permissive role for the structural plasticity in nervous system function (Fox and Caterson, 2002; Pizzorusso et al., 2002).
- Stabilization of permanently fast neuronal activity of net-associated neurons via the special hydrodynamic properties of PNs, that is a voluminous, strongly hydrated polyanionic matrix (Bruckner et al., 1993; Hartig et al., 1999). The low vulnerability of different types of net-associated neurons in Alzheimer's disease appears to be indicative of a neuroprotective effect of PNs (Bruckner et al., 1999; Schuppel et al., 2002). It is plausible that the nets could

play a neuroprotective role by buffering free Ca^{2+} in the extracellular space, which would "restrict" Ca^{2+} entry into the interneuron, and, thus, complement the parvalbumin-containing intracellular Ca^{2+} buffering system associated with interneurons expressing PNs. High buffering capacity of nets would also "soften" the depletion of extracellular Ca^{2+} during firing of PN-expressing interneurons, supporting their fast bursting and Ca^{2+} dependent adhesive intercellular interactions, mediated for instance by cadherins.

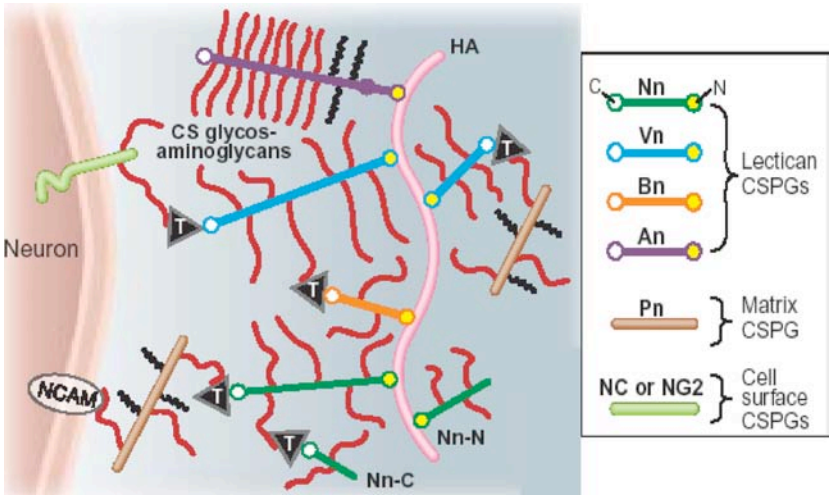


Fig. 3 The PN surrounds neuronal cell bodies and proximal dendrites in the central nervous system and is composed of several subclasses of CS-PGs. Members of the lectican subfamily of CS-PGs-neurocan (Nn), versican (Vn), brevican (Bn), and aggrecan (An)-are shown noncovalently associated with matrix HA (pink), through globular HA-binding domains at their amino termini (yellow circles). These associations result in large macromolecular aggregates of the PN. Specific noncovalent associations occur between the carboxyl-terminal globular domains (white circles) of some lecticans (such as neurocan and versican) and the matrix glycoprotein TN-R (T, triangles). TN, in turn, binds to CS-GAGs (red lines) on phosphacan (Pn) and other lecticans, as well as to cell surface CSPGs such as neuroglycan-C (NC) and NG2. Phosphacan can also bind to cell surface receptors such as neural cell adhesion molecule (NCAM) through its CS-GAG chains. Two splice variants of versican are depicted, as well as intact neurocan and its two proteolytic cleavage isoforms: neurocan-N (Nn-N) bound to HA, and neurocan-C (Nn-C) bound in the PN via interactions with TN-R and a CS-GAG (Fox and Caterson, 2002).

2 Nervous system regeneration

Neurons in the central and peripheral nervous system show various responses to axotomy, ranging from cell death or severe atrophy without axon regeneration to recovery with axon regeneration. Axons in the PNS frequently exhibit efficient and successful regeneration after injury, whereas CNS axons abortively sprout but fail to regenerate after injury.

2.1 Regeneration of axons in the PNS

Functional recovery from peripheral nerve injury and repair depends on a multitude of factors, both intrinsic and extrinsic to neurons. Neuronal survival after axotomy is a prerequisite for regeneration and is facilitated by an array of trophic factors from multiple sources, including neurotrophins, neuropoietic cytokines, insulin-like growth factors (IGFs), and glial-cell-line-derived neurotrophic factor (GDNF). Axotomized neurons must switch from a transmitting mode to a growth mode and express growth-associated proteins, such as GAP-43, tubulin, and actin, as well as an array of novel neuropeptides and cytokines, all of which have the potential to promote axonal regeneration. Axonal sprouts must reach the distal nerve stump at a time when its growth support is optimal. Schwann cells in the distal stump undergo proliferation and phenotypical changes to prepare the local environment to be favourable for axonal regeneration. Schwann cells play an indispensable role in promoting regeneration by increasing their synthesis of surface cell adhesion molecules (CAMs), such as N-CAM, Ng-CAM/L1, N-cadherin, and L2/HNK-1, by elaborating basement membrane that contains many extracellular matrix proteins, such as laminin, fibronectin, and tenascin, and by producing many neurotrophic factors. However, the growth support provided by the distal nerve stump and the capacity of the axotomized neurons to regenerate axons may not be sustained indefinitely. Axonal regeneration may be facilitated by new strategies that enhance the growth potential of neurons and optimize the growth support of the distal nerve stump in combination with prompt nerve repair.

The major events that occur within a nerve fascicle after injury are shown at Fig.4 and can be summarized as follows:

- axonal fragmentation and myelinolysis
- recruitment of haematogenously derived myelomonocytic cells into the endoneurium
- invasion of Schwann cell basal lamina tubes by macrophages which remove the cellular debris
- Schwann cell gliosis at the tip of the proximal stump and throughout the distal stump
- axonal sprouting and outgrowth of axons and Schwann cells from the proximal stump
- formation of bands of Büngner (columns of quiescent Schwann cells disposed within basal lamina tubes) throughout the distal stump
- penetration of bands of Büngner by regenerating axons

- establishment of axon-Schwann cell relationship and onset of myelination, in a proximo-distal sequence throughout the distal stump
- re-innervation of target structures and restitution of function

The number of Schwann cells within the endoneurium rises dramatically during the first two postoperative or posttraumatic weeks. They display a phenotype which resembles that of the normal adult non-myelinating cell, i.e. myelin genes are downregulated, whereas neurotrophic factors and ECM molecules (laminin, fibronectin, collagens) that are important for fasciculation, axonal prolongation and Schwann cells migration are strongly upregulated. Molecules in the basal lamina and endoneurium constitute a distinct class of neurite-promoting molecules and are synthesized and secreted by Schwann cells and fibroblasts of the peripheral nerve. These include laminin, tenascin, heparan sulfate proteoglycan, types IV and V collagens and variable amounts of fibronectin (Bunge et al., 1989; Bunge et al., 1990). Axonal contact is required for elaboration of the basal lamina by Schwann cells (Bunge et al., 1986; Bunge et al., 1989; Bunge et al., 1990). However, some components of the basal lamina, such as laminin, continue to be synthesized under denervated condition (Cornbrooks et al., 1983). In addition, the basal lamina in the distal nerve stumps remains intact for 3-4 weeks following injury (Neuberger and Cornbrooks, 1989). Over a longer period the Schwann cell basal laminae become fragmented, and laminin mRNA and protein levels decrease, if regeneration does not occur (Bignami et al., 1984; Roytta et al., 1988; Giannini and Dyck, 1990; Doyu et al., 1993). These changes are likely to contribute to the poor axonal regeneration and target reinnervation after delayed nerve repair (Fu and Gordon, 1995).

In contrast to many in vitro studies that have demonstrated neurite outgrowth on basement membrane alone or oncomponents of the basement membrane (Letourneau et al., 1988; Bixby and Harris, 1991; Carbonetto, 1991; Reichardt and Tomaselli, 1991; Bixby, 1992), only few studies have examined the role of the basal lamina in nerve regeneration in vivo (*see* Laminin). These studies have demonstrated an essential role for laminin and possibly TN-C in providing an essential substrate for axonal regeneration. In addition to their roles in providing a substrate for growth, ECM molecules bind to and regulate the activity and stability of several growth factors, most notably FGF and TGF-β (McCaffrey et al., 1989; Rifkin and Moscatelli, 1989).

Laminin

Laminin is present in the nervous systems that are able to regenerate and absent in those that are not, indicating that laminin is a prerequisite for successful axonal regeneration (Hopkins et

al., 1985; Liesi, 1985). For example, the presence of laminin in the basal lamina of the peripheral nerve is correlated with its ability to regenerate and may account for growth of CNS axons, which normally do not regenerate, through peripheral nerve grafts (David and Aguayo, 1981; Liesi, 1985; David et al., 1995).

A critical role of laminin in peripheral nerve regeneration has been demonstrated in experiments in which sectioned peripheral nerves are bridged by artificial or natural grafts in which the content of basal lamina proteins is experimentally controlled. Several experiments have demonstrated that regenerating axons fail to cross silicone or collagen grafts unless laminin is present (Madison et al., 1985; Williams, 1987; Yoshii et al., 1987; Bailey et al., 1993; Kauppila et al., 1993). Additionally antibodies against laminin or integrin receptors drastically interfere with regeneration (Toyota et al., 1990; Wang et al., 1992). In vitro, neurite growth cones prefer laminin to other growth-promoting substrates, such as fibronectin (Gaya-Gonzalez et al., 1991), which is also expressed in the endoneurial tube, but the expression of fibronectin is more diffuse than the distribution of laminin (Bunge et al., 1986). Consistent with this finding, neither exclusion of fibronectin from nerve grafts in vitro nor antifibronectin antibodies greatly affect axonal regeneration across the graft (Wang et al., 1992; Bailey et al., 1993).

TN-C

TN-C is normally confined to the ECM around the node of Ranvier and the perineurium (Daniloff et al., 1989; Martini et al., 1990). After injury, TN-C is expressed along the entire length of the distal nerve stump, the distal end of the proximal nerve stump, and the bridge between the proximal and distal nerve stumps (Martini et al., 1990). Its immunoreactivity is associated with the basal lamina of Schwann cells (Martini et al., 1990). In vitro, TN promotes neurite outgrowth (Wehrle and Chiquet, 1990; Lochter et al., 1991; Husmann et al., 1992). In vivo, immunohistochemistry reveals a close association of regenerating axons with TN-positive regions in the distal nerve stump (Martini et al., 1990). The spatial and temporal expression of TN together with its growth-promoting effects in vitro suggests a possible involvement in axonal regeneration.

Expression of TN is closely related to Wallerian degeneration and Schwann cell proliferation. Nonmyelinating Schwann cells reach their peak of proliferation a day earlier than myelinating Schwann cells (Clemence et al., 1989) and are also the first Schwann cells to reexpress TN-C, followed by myelinating Schwann cells (Fruttiger et al., 1995). It is possible that IL-1 and TGF-β released from invading macrophages play a role in the upregulation of TN in Schwann

cells, since both cytokines enhance TN-C expression in other cell types (Pearson et al., 1988; McCachren and Lightner, 1992). Nevertheless, the sustained expression of TN in chronically denervated nerve stumps and its downregulation by regenerating axons suggest that loss of axonal contact is a sufficient signal to induce TN expression (Martini et al., 1990; Martini, 1994) as part of the repertoire of changes in gene expression associated with conversion of myelinating Schwann cells to the nonmyelinating phenotype.

In summary, the dramatic molecular changes that occur in the distal nerve stump after injury promote the formation of a conducive environment for axonal regeneration. Loss of contact between Schwann cells and axons together with chemical signals from invading macrophages induce or at least contribute to these phenotypic changes. In vivo experiments, particularly those that utilize artificial and neural grafts, have demonstrated the essential role of Schwann cells and their basal lamina in supporting regenerating axons.

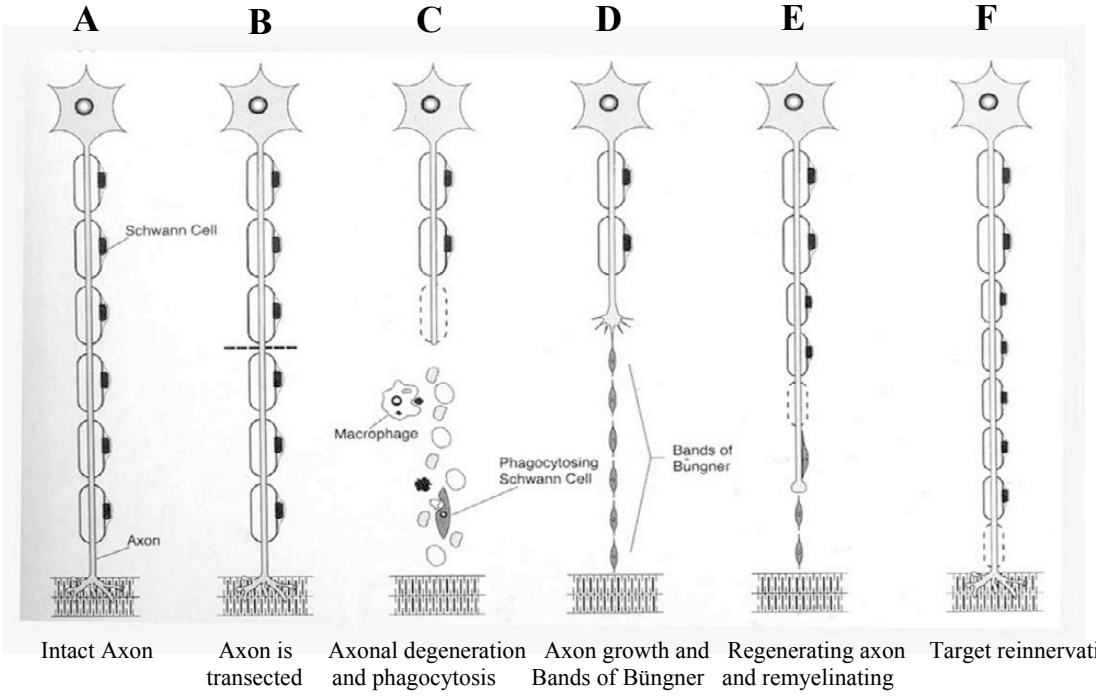


Fig. 4 Effects of axotomy in the PNS. (**A**) An intact motor axon is myelinated by several Schwann cells and projects to muscle. (**B**) The nerve is transsected at a single site. (**C**) The proximal axonal segment undergoes limited degeneration to the previous node of Ranvier. Myelin breakdown occurs. The distal axon segment begins to undergo Wallerian degeneration. The axon becomes granular, then disintegrates, and is phagocytosed by resident and recruited macrophages as well as activated Schwann cells. (**D**) Following Wallerian degeneration of the distal axonal segment, the remaining Schwann cells divide and align longitudinally within basal lamina

tubes. Growth cones from regenerating axons extend along Schwann cell column, growing along the Schwann cell membranes and basal lamina. Schwann cells then begin to wrape regerating axons as the growth cones extends along Schwann cell columns. (**E**) As regenerating axons reinnervate the target, Schwann cells continue to ensheath or myelinate, forming substantially shorter internodal distances than found in the unlesioned state.

2.2 Regeneration of axons in the CNS

It is now well established that most axons within the adult vertebrate CNS fail to regenerate following injury (Clemente and Van Breemen, 1955; Puchala and Windle, 1977; Reier et al., 1988). One reason for this unability to regenerate might be the delay in clearance of axonal and myelin debris. Unlike Schwann cells, whose phagocytic activity after axonal injury has been described both *in vitro* (Fernandez-Valle et al., 1995) and *in vivo* (Stoll et al., 1989; Reichert et al., 1994; Liu et al., 1995), oligodendrocytes do not exhibit phagocytic activity (Lampert and Cressman, 1966; Bignami and Ralston, 1969). Another reason is the formation of the so-called glial scar composed of astrocytes and connective tissue that constitutes a structural barrier for growing axons (Windle and Chambers, 1950; Windle et al., 1952; Clemente and Windle, 1954). Recent studies indicate that the astrocytic scar does not prevent axon growth simply by a mechanical mechanism (Reier et al., 1983; Davies et al., 1996).
Both tenascins and certain proteoglycans are upregulated following injury to the CNS (Laywell and Steindler, 1991; McKeon et al., 1991; Laywell et al., 1992; Brodkey et al., 1995; McKeon et al., 1995) or the spinal cord (Pindzola et al., 1993; Zhang et al., 1995). Similarly, chondroitin sulfate proteoglycans persist in the ECM of the CNS following injury (Pindzola et al., 1993; Levine, 1994; Fitch and Silver, 1997). Thus, ECM molecules produced in the CNS in response to injury provide a substrate that is inhibitory to axon regeneration *in vitro*, and in addition with their spatial and temporal appearance after CNS trauma suggest a causative role for the inability of CNS regeneration.

3 ErbB3 receptor and it's ligand neuregulin-1 are essential for development of the peripheral nervous system

Neural crest cells constitute a transient and migratory cell population that generates the majority of the peripheral nervous system including neurons and Schwann cells. Tyrosine kinase receptors (c-Ret, ErbB3/ErbB2, and c-Kit) and their ligands have been implicated in

the control of the development of neural crest cells (Wehrle-Haller and Weston, 1997). The *neuregulin-1* gene encodes different isoforms of an EGF-like growth and differentiation factor that is also known as NDF, heregulin, GGF, ARIA, or SMDF (Holmes et al., 1992; Falls et al., 1993; Marchionni et al., 1993; Ho et al., 1995). Neural crest cells and various other cell types expressing the neuregulin-receptors respond to neuregulin-1 by growth and differentiation (Lemke, 1996; Burden and Yarden, 1997). Biochemical and genetic data indicate that the functional neuregulin receptors are ErbB3/ErbB2 or ErbB4/ErbB2 heterodimers (Plowman et al., 1993; Carraway and Cantley, 1994; Sliwkowski et al., 1994; Tzahar et al., 1994; Beerli et al., 1995; Horan et al., 1995; Riese et al., 1995; Wallasch et al., 1995). Gene targeting experiments in mice have revealed that distinct receptor combinations are essential for different developmental events. Thus ErbB2 and ErbB4 receptors cooperate in transmission of neuregulin-1 signals in the heart, whereas ErbB2 and ErbB3 cooperate in neural crest cells (Gassmann et al., 1995; Lee et al., 1995; Meyer and Birchmeier, 1995; Erickson et al., 1997; Riethmacher et al., 1997). Three distinct neural crest cell derivatives, neurons of cranial ganglia, sympathethic neurons and Schwann cell precursors, were reported to strongly depend on neuregulin-1 (Lee et al., 1995; Meyer and Birchmeier, 1995; Erickson et al., 1997; Riethmacher et al., 1997). Interestingly the initial development of motor and DRG sensory neurons is not affected in erbB3 deficient mutants but at later stages most motor neurons (79%) and DRG sensory neurons (82%) undergo cell death (Riethmacher et al., 1997). Similar results were obtained with rescued erbB2 mutants (Woldeyesus et al., 1999) and additionally both mutants show severe defasciculation of axon bundles. It is important to note that the effects on neuronal survival in these mutants are not cell autonomous. The neurons that die, do not express ErbB2 or ErbB3. Rather, these effects are due to the absence of Schwann cells and amazingly these neuronal losses are more extensive than those seen in knockout of the TrkA, TrkB, or TrkC neurotrophin receptor genes, or neurotrophin genes themselves (Klein, 1994). Thus, the neuronal survival is strictly dependent on the survival of Schwann cells (Fig. 5).

As was shown by DNA-microarray recently, Matrilin-2 is a differentially expressed gene in dorsal root ganglion (DRG) cultures of ErbB3-/- embryos (E12.5) as compared to wildtype cultures *(data unpublished*). Thus, Matrilin-2, being expressed by embryonic Schwann cells, when they migrate, proliferate and differentiate, could play a role in these processes.

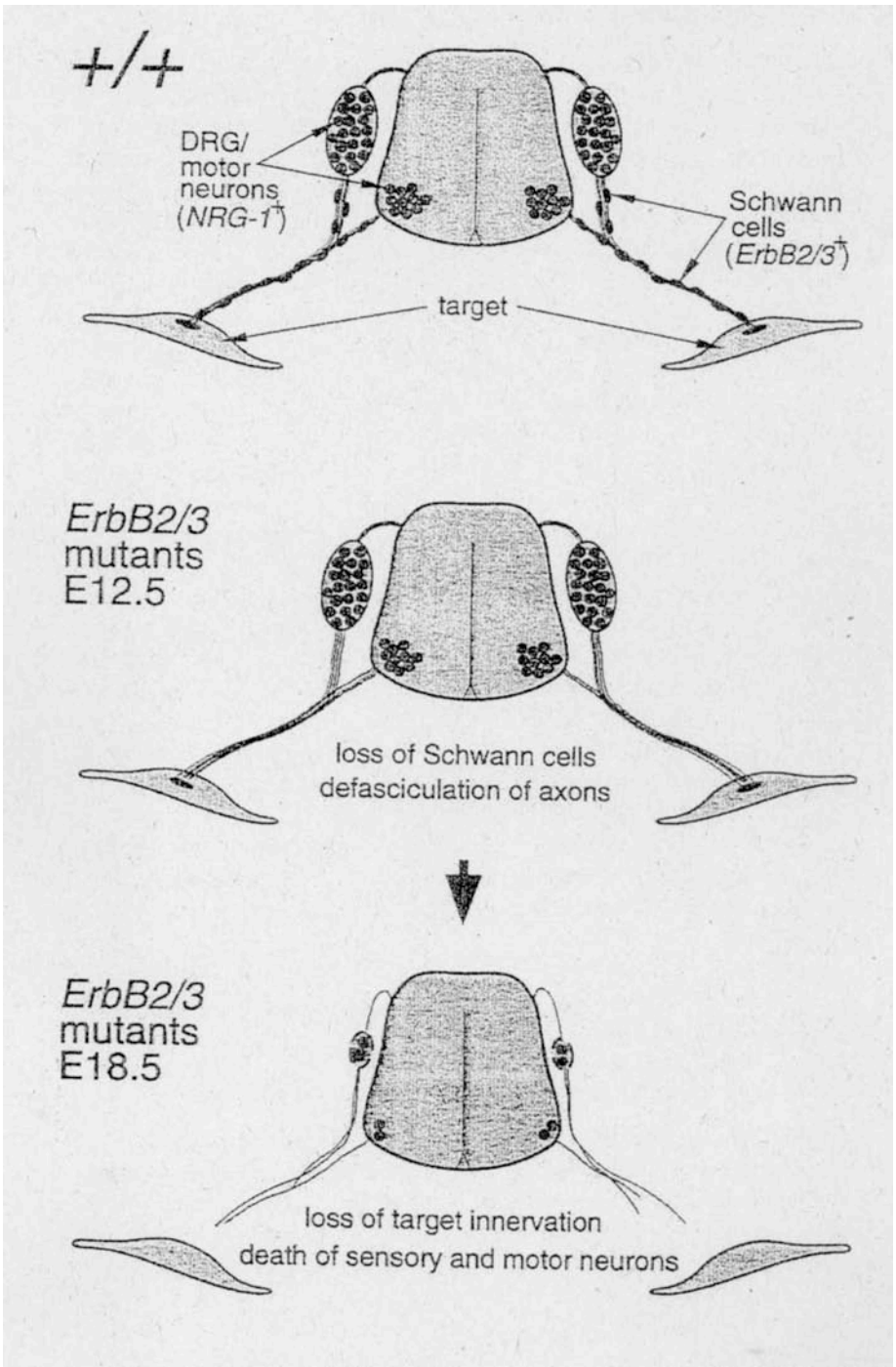


Fig. 5 Survival of neurons dependent on the survival of Schwann cells.

4 Matrilins are oligomeric extracellular matrix proteins

The matrilin family has four members that all share a common structure consisting of von Willebrand factor A domains, epidermal growth factor-like domains and a coiled coil -helical module. Matrilin-1 (also known as cartilage matrix protein, CMP), and matrilin-3 are abundant in cartilage (Paulsson and Heinegard, 1982; Wagener et al., 1997; Klatt et al., 2000)

while matrilin-2 and –4 show a broader tissue distribution (Deak et al., 1997; Wagener et al., 1998; Piecha et al., 1999; Klatt et al., 2001). Matrilins are associated with cartilage proteoglycans and are components of both collagen-dependent and collagen-independent fibrils.

4.1 Structure

The domain structures of the matrilins are given in a schematic form in Fig. 6. The vWFA domains fall into two groups with the vWFA1 module always occurring towards the N-terminal and the vWFA2 module closer to the C-terminus. The vWFA2 module has been deleted in matrilin-3 and the vWFA1 module has been deleted in one splice variant of mouse matrilin4. vWFA domains are found not only in the matrilins, but also in a large number of other extracellular proteins such as von Willebrand factor, collagens type VI, VII, XII and XIV, complement factors B and C2, the H2 and H3 subunits of the inter-trypsin inhibitor, the α-chains of seven integrins and in a cochlear protein (Colombatti and Bonaldo, 1991; Lee and McCulloch, 1997).

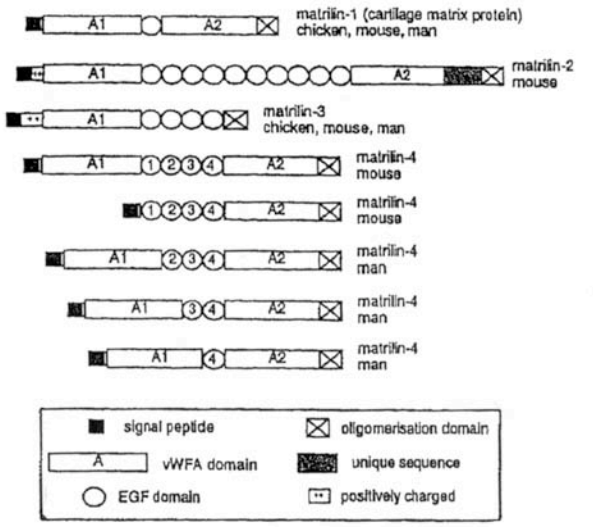


Fig. 6 Comparison of the domain structures of matrilins. Mouse matn4 occurs in two and human matn4 in three alternatively spliced forms. In addition to the domains discussed in the text, matn2 and matn3 contain a positively charged domain between the signal peptide and the vWFA1 domain and matn2 contains a unique domain that has no sequence homology to any other known protein. (Deak et al., 1999).

The number of EGF repeats, that are not of the Ca2+-binding type, varies between one in matrilin-1 and 10 in matrilin-2. Although they show an overall structural similarity to epidermal growth factor there is no evidence that they retain growth factor activity. It is more likely that they serve as spacers between vWFA domains, which show ligand binding activities in many other proteins.

All matrilins can form homo-oligomers by the C-terminal heptad repeats folding into a coiled-coil -helix. The homo-oligomer of matrilin-1 consist of three subunits (Paulsson and Heinegard, 1981), while for matrilin-2, -3 and –4 multiple assembly forms are detected in tissue extracts and cell culture media (Piecha et al., 1999; Klatt et al., 2000; Klatt et al., 2001). Additionally, biochemical and biophysical methods showed that matrilins can form hetero-oligomers via the proteins' coiled-coil domain (Frank et al., 2002).

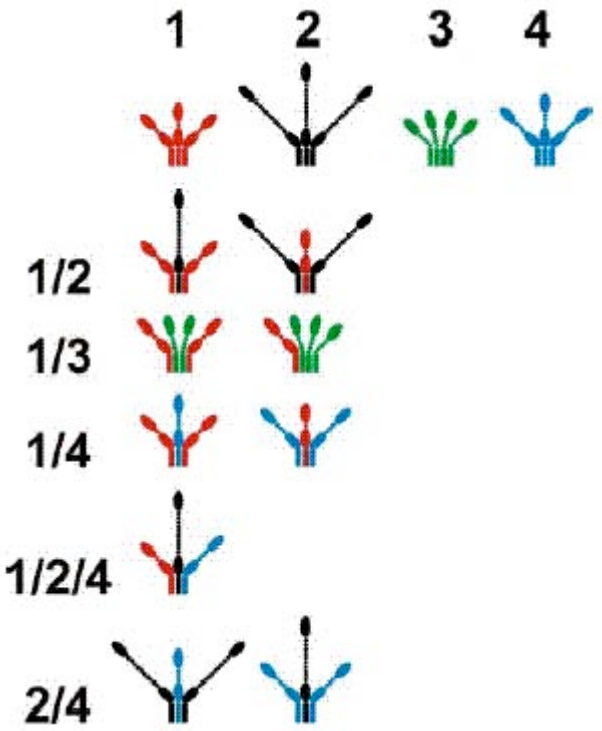


Fig. 7 Possible matrilin homo- and hetero-oligomers. *1*, matn1; *2*, matn2; *3*, matn3; *4*, matn4. (Piecha et al., 2002)

4.2 Functions of matrilins

It is thought that matrilins play a role in mediating interactions between major components of the extracellular matrix such as collagens and proteoglycans (Hauser et al., 1996). The three forms abundant in cartilage (matrilin-1, -3, and –4) are indeed associated with native collagen type VI microfibrils extracted from rat chondrosarcoma tissue (Wiberg et al., 2003). In this tissue the matrilins are bound to the small leucine-rich repeat proteoglycans biglycan and decorin, which, in turn, interact with the N-terminal globular domains of the collagen VI molecules. When located at the periphery of the microfibrillar complex, matrilins mediate interactions with aggrecan or collagen II. Matrilin-3 is strongly upregulated in human osteoarthritic cartilage (Pullig et al., 2002), and a missense mutation in the human matrilin-3 gene was recently found to coincide with hand osteoarthritis in a group of patients in the

icelandic population (Stefansson et al., 2003). Interestingly, matrilin-1 and –3-deficient mice (Aszodi et al., 1999; Ko et al., 2004), created by gene targeting, do not show any obvious skeletal malformations nor other phenotypical changes.

Matrilin-2 is the largest known matrilin with a molecular mass of 106800 Da. Matrilin-2 is expressed in a variety of tissues, including both loose and dense connective tissues as well as subepithelial basement membranes in the skin and the digestive tract. a weak expression of matrilin-2, mainly restricted to the epiphyseal growth plate, has been observed in cartilage (Piecha et al., 1999; Segat et al., 2000; Klatt et al., 2002). Some ECM ligands for matrilin-2 in supramolecular extracellular assemblies have been identified. Surface plasmon resonance and electron microscopy showed interactions between matrilin-2 and different types of collagens, fibrilins-1, -2, laminin and fibronectin (Piecha et al., 2002).

Thus, matrilin-2 is a widespread extracellular matrix component that can interact with itself and with other both collagenous and non-collagenous matrix molecules. This interaction repertoire together with its oligomeric structure suggests that matrilin-2 may function as an adapter or mediator molecule for interactions between other matrix macromolecules during the assembly of an extracellular matrix. It has the potential to serve such functions in and between basement membranes, microfibrils and in ECM. Finally, matrilin-2-deficient mice were generated recently, and similarly to matrilin-1 and –3-deficient mice these mutants do not show any obvious abnormalities during embryonic or adult development (Mates et al., 2004). Because of this lack of phenotypes in all matn mutants a compensatory upregulation mechanism has been discussed. However, in matn2 mutants, as well as in matn1 and matn3 mutants, no upregulation of other family members has been observed (Aszodi et al., 1999; Ko et al., 2004; Mates et al., 2004).

II. Aims of the study

The ECM has been demonstrated to serve a lot of functions during development and also maintenance of different tissues. The matrilin family members are components of the ECM whose precise roles have not yet been elucidated. A differential gene expression analysis has revealed that two members of the matrilin family, matn2 and matn4, are expressed in the peripheral nervous system. Therefore, the aims of this study have been

- to investigatate the expression patterns of different matrilins in the peripheral nervous system in great detail using different approaches,
- to analyse the roles of matrilin-2 in cell migration, axon outgrowth, axon fasciculation using purified matrilin-2 protein and matrilin-2 deficient mouse mutants (see below)
- to study possible roles of matrilin-2 in peripheral nerve regeneration using different *in-vitro* and *in-vivo* approaches.

During my present study I have found that matrilin-2 is also expressed in the mouse central nervous system, e.g. the hippocampus. Therefore, additional aims have been

- to submit the expression patterns of matn2 in mouse hippocampus a careful examination
- to analyze the roles of matrilin-2 in axon outgrowth and neuritogenesis using hippocampal neuronal culture,
- to elaborate on a potential involvement of matrilin-2 in hippocampal synaptic plasticity, synaptogenesis and neuritogenesis in CNS.

Matrilin-2 deficient mice were generated recently. However, these mice show no obvious abnormalities during embryonic or adult development (Mates et al., 2004). As subtle mutations are sometimes overlooked we analyzed those mutants in great detail with a special emphasis on the peripheral and central nervous system and cells derived from them. First I wanted to find possible abnormalities in CNS and PNS of matrilin-2 deficient mice using histological and immunohistological methods. Second I tested different matrilin-2 deficient neuronal populations in cell culture for aberrant behavior.

III. Materials

1. Chemicals

All chemicals were obtained from the following companies in p.a. quality: GibcoBRL (Life technologies, Karlsruhe, Germany), Macherey-Nagel (Düren, Germany), Merck (Darmstadt, Germany), Serva (Heidelberg, Germany) and Sigma-Aldrich (Deisenhofen, Germany).
Restriction enzymes were obtained from New England biolabs (Frankfurt am Main, Germany) and MBI Fermentas (St. Leon-Rot, Germany). Molecular weight standards were obtained from GibcoBRL. DNA purification kits were purchased from Life Technologies (Karlsruhe, Germany), Pharmacia Biotech (Freiburg, Germany), Macherey & Nagel and Qiagen (Hilden, Germany). Plasmids and molecular cloning reagents were obtained from Clontech, Invitrogen, Pharmacia Biotech, Promega, Qiagen and Stratagene. Cell culture material was ordered from Nunc (Roskilde, Denmark), GibcoBRL, Invitrogen or Sigma-Aldrich.

2. Solutions and buffers

(in alphabetical order)

Acetylation buffer	3.125	ml	Triethanolamin
(In Situ)	675	µl	Aceticangydrid
	200	ml	H_2O
Anaesthesition solution	1	g	2,2,2 Tribrom-ethanol
(surgery)	620	µl	2-methyl-2-butanol
	79	ml	H_2O
Antibody solution	0,2	% (w/v)	BSA in PBS
(Immunocytochemistry, immunostaining)	0.01	% (w/v)	Triton X 100
Blocking buffer	2	% (w/v)	BSA in PBS
(Immunocytochemistry)	0,1	% (w/v)	Triton X 100

Blocking buffer	2	% (w/v)	BSA in PBS
(Immunohistochemistry)	0,01	% (w/v)	Triton X 100
Blocking buffer	5	% (w/v)	instant milk powder
(Western Blot)			in TBS/PBS
Blotting buffer	25	mM	Tris
(Western Blot)	192	mM	Glycine
	10	%	Methanol
Buffer 1	1	% (w/v)	BSA in TBS
(Binding assay)	1	mM	$CaCl_2$
	1	mM	$MnCl_2$
	1	mM	$MgCl_2$
	0.05	% (v/v)	Tween 20
Denhards (50x)	50	g	Ficoll 400
	50	g	Polyvinilpyrrolidon
	50	g	BSA
	1	l	Water
Embryo lysis buffer	10	mM	Tris PH 8,9
	50	mM	KCl
	3	mM	$MgCl_2$
	0,45	% (v/v)	Nonidet P40
	0,45	% (v/v)	Tween 20
Developing solution	2	% (w/v)	Sodium Carbonate
(Silver Staining)	0.04	% (v/v)	Formaldehyde
DNA-sample buffer (5x)	20	% (w/v)	glycerol in TAE buffer
(DNA-gels)	0,025	% (w/v)	orange G
Digestion solution PH 7.4	800	mg	NaCl

(for cell culture)	37	mg	KCl
	99	mg	Na_2HPO_4
	595	mg	HEPES
	35	mg	$NaHCO_3$
	100	ml	H_2O
Ethidiumbromide	10	µg/ml	ethidiumbromide in 1xTAE
Giemsa solution	8	ml	Giemsa
(In situ)	200	µl	1M $NaPO_4$-buffer PH 6
	200	ml	H_2O
Fixation solution	50	% (v/v)	Methanol
(Silver Staining)	5	% (v/v)	Acetic Acid
	45	% (v/v)	H_2O
Fixing solution	20	ml	Methanol
(Coomassie Staining)	79	ml	H_2O
	1	ml	o-Phosphoric acid 85%
Hybridization buffer	50	% (v/v)	Formamid (deionizied)
(In Situ)	10	mM	Tris-HCl, pH 7.5
	10	mM	$NaHPO_4$, pH 6.8
	5	mM	EDTA
	2	x	SSC
	150	µg/ml	Salmon Sperm DNA
	150	µg/ml	Yeast tRNA
	10	% (v/v)	Dextran sulfate
	100	ml	H_2O (DEPC)
Ligation buffer (10x)	200	mM	Tris-HCl, pH 7,9
	100	mM	$MgCl_2$
	100	mM	Dithiothreitol (DTT)
	6	mM	ATP

Master mix (1 probe)	4	µl	10xMEN
(Nothern blot)	6	µl	Formaldegyde 37%
	20	µl	formamid
	1	µl	Ethidium bromide
	0.1	µl	Bromophenol blue
MEN buffer (10x)	41.9	g	Mops
(Nothern blot)	4.1	g	natriumacetat
	3.72	g	EDTA
	1000	ml	H_2O
Phosphate buffered saline	150	mM	NaCl
(PBS)	20	mM	Na_3PO_4 pH 7.4
Phosphate buffered saline	150	mM	NaCl
Tween (PBS-T)	20	mM	Na_3PO_4 pH 7.4
	0.05	% (v/v)	Tween 20
Pre-hybridization buffer	6	x	SSC
(Northern blot)	2	x	Denhards
	0.1	% (v/v)	SDS
RNase buffer	0.5	M	NaCl
(In situ)	10	mM	Tris pH 7.5
	1	mM	EDTA pH 8.0
Roti-Blue staining solution	20	ml	Methanol
(Coomassie Staining)	20	ml	Roti-Blue 5 x concentrate
	60	ml	H_2O
Running Gel 10%	3.92	ml	deionized water
(Protein-gels)	5.26	ml	1M Tris pH 8.8
	0.14	ml	10% SDS

	4.70	ml	30%Acrylamide–Bis 29:1
	70.0	µl	10% APS
	7.00	µl	TEMED
Sample buffer (5x)	0.312	M	Tris-HCl pH 6.8
(Protein-gels)	10	% (w/v)	SDS
	5	% (w/v)	β-Mercaptoethanol
	50	% (v/v)	Glycerol
	0.13	% (w/v)	Bromphenol blue
Sample buffer	0.02	M	Tris-HCl, pH 8.0
(store proteins)	0.028	M	NaCl
	0.02	% (v/v)	NaN_3
SDS running buffer (10x)	0.25	M	Tris-HCl, pH 8.3
(Protein-gels)	1.92	M	glycine
	1	M	SDS
Silvering buffer	0.1	% (w/v)	$AgNO_3$
(Silver Staining)			
SSC (20x)	3	M	NaCl
	0,3	M	Natriumcitrat, PH 7.0
Stabilizing solution	20	g	Ammonium sulphate
(Coomassie Staining)			up to 100 ml H_2O
Stacking Gel 5%	3.77	ml	deionized water
(Protein gels)	0.32	ml	1 M Tris pH 6.8
	0.05	ml	10% SDS
	0.83	ml	30%Acrylamide – Bis29:1
	25.0	µl	10% APS
	7.00	µl	TEMED

Stopping solution *(Silver staining)*	5	% (v/v)	Acetic acid
Sensitizing solution *(Silver Staining)*	0.02	% (w/v)	Sodium thiosulfate
Solution I *(DNA mini prep)*	50 25 10	mM mM mM	Glucose Tris-HCl, pH 8.0 EDTA
Solution II *(DNA mini prep)*	0.2 1	M % (w/v)	NaOH SDS
Solution III *(DNA mini prep)*	3	M	Kalium acetat PH 4.2
Storage solution *(Silver Staining)*	1	% (v/v)	Acetic acid
Stripping buffer *(Western blots)*	0.5 0.5	M M	NaCl acetic acid
TAE (50x) *(DNA-gels)*	2 100	M mM	Tris-Acetat, pH 8,0 EDTA
TE (10x)	0,1	M	Tris-HCl, pH 7,5
Tris Buffered Saline (TBS)	10	mM	Tris-HCl, pH 8.0
Washing buffer *(Coomassie Staining)*	25 75	ml ml	Methanol 99.8% H_2O
Washing buffer I *(In Situ)*	5 50	x % (w/v)	SSC Formamid

	1	mM	DTT
Washing buffer II	2	x	SSC
(In Situ)	50	% (w/v)	Formamid
	1	mM	DTT
Washing buffer I	2	% (w/v)	SSC
(Northern blot)	0.1	% (w/v)	SDS
Washing buffer II	0.5	% (w/v)	SSC
(Northern blot)	0.1	% (w/v)	SDS

3. Bacterial media

(Media were autoclaved and antibiotics were supplemented prior to use)

LB-medium pH 7,4	10	g/l	Bacto-tryptone
	10	g/l	NaCl
	5	g/l	yeast extract
LB/Amp-medium	100	mg/l	ampicilin in LB-Medium
LB/Amp-plates	20	g/l	agar in LB-Medium
	100	mg/l	ampicillin

4. Bacterial strains

XL1-Blue MRF Stratagene

Δ(mrcA) 183Δ(mrcCB-hsdSMr-mrr)173 end A1 supE44 thi-q recAq gyrA96 relA1 lac[F´proAB $lacl^{q}$ZΔm15 Tnf10 (Tet^{r})]

5. Mammalian cell lines

CHO-K1	*Chinese Hamster Ovary* Dehydrofolatereductase deficient hamster cell line
S16	Rat Schwann cell line
EBNA-293	human embryonic kidney cell line

6. Cell culture media

Media were prepared from a 10X stock solution purchased from Gibco GBL

CHO-cell Medium	Glasgow MEM (GMEM) (with nucleotides, L-Glutamine) supplemented with 10 % (v/v) fetal calf serum (FCS) 50 U/ml Penicilline/Streptomycine 2 mM L-Glutamine
S16-cell Medium	D-MEM (with GlutaMAX) supplemented with 10 % (v/v) fetal calf serum (FCS) 50 U/ml Penicilline/Streptomycine
EBNA293-cell medium	D-MEM/F12 (1:1) (Mixture) suplemented with 10 % (v/v) fetal calf serum (FCS) 50 U/ml Penicilline/Streptomycine 2 mM L-Glutamine
Sato medium	D-MEM supplemented with 5µg/ml bovine insulin 50µg/ml human transferrin 100µg/ml BSA V 6.2ng/ml progesterone

	16µg/ml putrescine 5ng/ml sodium selenite 400ng/ml T3 (tri-Iodothyroxine) 400ng/ml T4 (thyroxine) 4mM L-Glutamine 50 U/ml Penicilline/Streptomycine
DRG-explant medium	D-MEM/F12 (1:1) (Mixture) suplemented with 60ng/ml progesterone 16µg/ml putrescine 340ng/ml T3 (tri-Iodothyroxine) 400ng/ml T4 (thyroxine) 38ng/ml sodium selenite 0.35% (v/v) BSA (Path-O-Cyte 4) 2mM L-Glutamine
Neuronal medium (first day) *(primary dissociated hippocampal culture)*	Eagle MEM supplemented with 5mg/ml D-Glucose 100µg/ml Transferrin 25µg/ml insulin 2mM Glutamax 5µg/ml gentamycin 10% (v/v) Horse serum
Neuronal medium	Neurobasal medium A supplemented with 2% (v/v) B27 supplement 2mM L-Glutamine

7. Molecular weight standards

1kb DNA ladder	14 bands within the range from 200-10000 bp (Gibco)
Precision Plus Protein™	10 µl of the ladder were loaded on the SDS-PAGE gel

8. Plasmids

pcDNA3.1/pcDNA3.1-myc-His	Invitrogen Mammalian expression vectors for transfection of eukaryotic cells.
pBluescript-SKII (+/-)	Stratagene Vector for transformation of bacterial strains
pGem-T Easy	Promega Vector for easy cloning of the PCR fragments

9. Antibodies

9.1 Primary antibodies

Normal	Working dilution		Host species	Type	Source
	Immunocyt.	Western blot			
NF160	1:200		mouse	monoclonal	Sigma
NF200	1:200		rabbit	polyclonal	Sigma
Sox10	1:5		mouse	monoclonal	Michael Wegner
GFAP	1:400		mouse	monoclonal	Sigma
GFAP	1:100		rabbit	polyclonal	Sigma
Myc (9E10)	1:500	1:5000	mouse	monoclonal	Upstate

MBP	1:200		rat	monoclonal	Chemicon
Matrilin-2	1:100	1:300	rabbit	polyclonal	Raimund Wagener
Matrilin-4	1:200	1:1000	rabbit	polyclonal	Raimund Wagener
WFA	1:200		W.Floribunda, biotin-conjugated	lectin	Sigma
Nidogen-1	1:40		rat	monoclonal	Raimund Wagener
p75	1:30		goat	polyclonal	Santa Cruz

9.2 Secondary antibodies

Name	Host species	Working dilution	Source
Mouse IgG, Cy2	goat	1:200	Dianova
Mouse IgG, Cy3	goat	1:500	Dianova
Rabbit IgG, Cy5	goat	1:200	Dianova
Cy3™ conjugated streptovidin	goat	1:200	Dianova
Rabbit Alexa Fluor 488	goat	1:200	MoBiTec
Rabbit IgG, Peroxidase-conjugated	goat	1:5000	Dianova
Rat IgG, Cy3	goat	1:400	Dianova
Goat IgG, Cy2	donkey	1:200	Dianova

IV. Methods

1. Protein-biochemical methods

1.1 SDS-polyacrylamide gel electrophoresis

Separation of proteins was performed by means of the discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using the Mini-Protean III system (BioRad). The size of the running and stacking gel were as follows:

Running gel: height 4.5 cm, thickness 0.75 mm
8 % or 10 % acrylamide solution
Stacking gel: height 0.8 cm, thickness 0.75 mm
5% (v/v) acrylamide solution
10 or 15-well combs

After complete polymerization of the gel, the chamber was assembled as described by the manufactures protocol. Up to 25 µl sample were loaded in the pockets and the gel was run at constant voltage at 80 V for the first 15 min and then for the remainder of the time at 120V. The gel run was stopped when the bromphenolblue front had reached the end of the gel. Gels were then either stained or subjected to Western blotting.

1.2 Western Blot-analysis

1.2.1 Electrophoretic transfer

Proteins were transferred from the PAA-gel onto a nitrocellulose membrane (Protran Nitrocellulose BA 85, 0,45 µm, Schleicher & Schüll) using a Mini-Transblot-apparatus (BioRad). After equilibration of the SDS-PAA-gel in bloting buffer for 5 min, the blotting sandwich was assembled as described in the manufactures protocol. Proteins were transferred electrophoretically at 4°C in blotting buffer at constant voltage (90 V for 120 min or 35 V

overnight). The prestained marker BenchMark™ (Gibco BRL) was used as a molecular weight marker and to monitor electrophoretic transfer.

1.2.2 Immunological detection of proteins on Nitrocellulose membranes

After electrophoretic transfer, the membranes were removed from the sandwiches and placed in glass vessels with the protein-binding side up. Membranes were washed once in TBS and incubated in 10 ml blocking buffer for 1 h at room temperature. Afterwards, the primary antibody was added in the appropriate dilution either for 2 h at RT or overnight at 4°C. Exessive primary antibody was removed by washing the membrane 5 x 5 min with TBS followed by an incubation with the appropriate secondary antibody for 1 h at RT. The membrane was again washed 5 x 5 min with TBS and then the immunoreactive bands were visualized using the enhanced chemiluminescence detection system (ECL), (see below).

1.2.3 Immunological detection using enhanced chemiluminescence

The horseradish peroxidase (HRP) coupled secondary antibody bound to the membrane was detected using the enhanced chemiluminescence detection system (Pierce). The membrane was soaked for 1 min in detection solution (1:1 mixture of solutions I and II). The solution was removed and the blot was placed between to saran warp foils. The membrane was exposed to X-ray film (Biomax-MR, Kodak) for several time periods, starting with a 5 min exposure.

1.3 Coomassie staining of polyacrylamide gels

The colloidal Coomassie staining of polyacrylamide gels was performed with the Roti-Blue kit (Carl Roth GmbH + Co). After SDS-PAGE, the gels were fixed in fixing solution for 60 min and subsequently incubated with Roti-Blue staining solution for 2-15 h with constant agitation. The gels were then incubated in destaining solution until the background was gone and the gel appeared nearly transparent.

1.4 Silver staining of polyacrylamide gels

After SDS-PAGE, gels were fixed with acetic acid/methanol solution for 30 min, intensively washed and quickly rinsed with freshly prepared thiosulfate solution. Afterwards, gels were silvered for 30 min at 4^0C, washed and developed with formaldehyde/sodium carbonate solution. When a sufficient degree of staining had been obtained the reaction was stopped with 5% acetic acid.

1.5 Determination of protein concentration (BCA)

The protein concentration assay was determined using the BCA kit (Pierce). Solution A and B were mixed in the ratio of 1:50 to give the BCA solution. 10 µl of the sample were then mixed with 200 µl BCA solution in microtiter plates and incubated for 30 min at 37°C. A BCA standard curve was co-incubated ranging from 0.1 mg/ml to 2 mg/ml protein concentration. The extinction of the samples was determined at 560 nm in a microtiter plate reader.

1.6 Protein purification using Ni-NTA sepharose

(see the QIAexpressionist™ QIAGEN)

The 6xHis-tagged proteins were purified as described in the manufactures protocol. The purifications were done under native and denaturing (2M Urea) conditions. Routinely, cell supernatants were filtrated throw 45 µm filter. The pH was adjusted to pH 8 and Ni-NTA sepharose was added and mixed. The suspensions were incubated overnight at 4°C. After sedimentation the supernatants were decanted and sepharose was washed several times with washing buffer (10mM imidazole in PBS pH 8). Then the proteins were eluted by the addition of elution buffer (200mM imidazole in PBS pH 8). The wash and elution fractions were subsequently analysed for their protein contents.

2. Molecular biology

2.1 Bacterial strains

2.1.1 Maintenance of bacterial strains

Strains were stored as glycerol stocks (LB-medium, 25% (v/v) glycerol) at –70°C. An aliquot of the stock was streaked on an LB-plate containing the appropriate antibiotics and incubated overnight at 37°C. Plates were stored up to 6 weeks at 4°C.

2.1.2 Production of competent bacteria

XL1-Blue bacteria were streaked on LB-plates containing the appropriate antibiotics and grown overnight at 37°C. Single colonies were picked and used for inoculation of 10 ml of an overnight culture. 1 ml of the overnight culture was added to 100 ml of pre-warmed LB broth containing antibiotics and shacked until an optical density of OD_{600} of 0.5 was reached (approximately 90-1230 min). The culture was cooled on ice, transferred to sterile round-bottom tubes and centrifuged at low speed (4000 x g, 5 min, 4°C). The supernatants were discarded and the cells were resuspended in cold TBF1 buffer (30 ml for a 100 ml culture). The suspension was kept on ice for additional 90 min. Then the cells were collected by centrifugation (4000 x g, 4°C), the supernatant was discarded again and the cells resuspended in 4 ml ice-cold TBF2 buffer. Aliquots of 100 µl were prepared, frozen in dry ice-ethanol mix and stored at –80°C.

2.1.3 Transformation of bacteria

To 100 µl of competent XL1-Blue either 50-100 ng of plasmid DNA or 20 µl of ligation mixture were added and incubated for 30 min on ice. After a heat shock (2 min, 42°C) and successive incubation on ice (3 min), 800 µl of LB-medium were added to the bacteria and incubated at 37°C for 30 min. Cells were then centrifuged (10000 x g, 1 min, RT) and the

supernatant removed. Cells were resuspended 100 µl LB medium and plated on LB plates containing the appropriate antibiotics. Plates were incubated at 37°C overnight.

2.2 Plasmid isolation of E. coli

2.2.1 Plasmid isolation from 3 ml cultures (Minipreps)

3 ml of LB/Amp-Medium (100 µg/ml ampicillin) were inoculated with a single colony and incubated over night at 37°C with constant agitation. Cultures were transferred into 2 ml Eppendorf tubes and cells were pelleted by centrifugation (12,000 rpm, 1min, RT). Cells were resuspended in 100µl solution I, after this, 150µl of solution II was added to the pellet and mixed by inverting the tubes 5-10 times. The tubes were incubated for 5 min (RT) before solution III was added and mixed again by inverting the tubes 5-10 times. The tubes were put on ice, before they were centrifuged for 10min (max speed) to remove cell debris. 1ml of 96% ethanol was added to the DNA containing solutions followed by a centrifugation for 20 min (max speed, 4°C). The pellets were washed by 70% ethanol and centrifuged for 10 min (max speed, 4°C). After this the pellets were dissolved in 50µl TE buffer.

2.2.2 Plasmid isolation from 150 ml-cultures (Maxipreps*)*

(See JETstar Maxiprep kit)

For preparation of large quantities of DNA, the JETstar Maxiprep kit was used. A single colony was inoculated in 150 ml LB/amp (100 µg/ml ampicillin) medium and grown at 37°C for 12-18 h with constant agitation. Cells were pelleted in a Beckmann centrifuge (8,000g, 10 min, 4°C) and DNA was isolated as described in the manufactures protocol. Finally, the DNA pellet was resuspended in 600 µl of TE buffer and the DNA concentration was determined.

2.3 Enzymatic modification of DNA

2.3.1 Digestion of DNA

For restriction, the DNA was incubated with the recommended amount of appropriate enzymes in the recommended buffer for 2 h. Restriction was terminated by addition of sample

buffer and applied on an agarose gel. If two enzymes were incompatible with each other, the DNA was digested successively with the enzymes. The DNA was purified between the two restrictions using the phenol/chloroform extraction and ethanol precipitation.

2.3.2 Dephosphorylation of Plasmid-DNA

After restriction the plasmid DNA was purified and SAP buffer (Boehringer Ingelheim) and 1 U SAP (scrimps alkine phosphatase) per 100 ng plasmid DNA were added. The reaction was incubated at 37°C for 2 h and terminated by incubation at 70°C for 10 min. The plasmid DNA was used for ligation without further purification.

2.3.3 Modifying sticky ends to blunt ends

To fill-in 3´recessed ends Klenow Enzyme was used, which has DNA dependent 5´-3´polymerase with 3´-5´exonuclease activity catalyzing the addition of mononucleotides from dNTP to the 3´OH terminus of a primer/template DNA.

Template DNA	1 µg DNA
Nucleotides (final concentration)	1 mM of desired dNTP each
10x Filing buffer	2 µl
Klenow	1 unit
H_2O	Add up to 20 µl
Incubation	15 min at 37°C

2.3.4 Ligation of DNA-fragments

Ligation of DNA fragments was performed by mixing of 50ng vector DNA with different amounts of insert DNA, depending on the size of the insert. For sticky ends:

- when vector DNA and insert DNA are similar in length: a molar ratio of 1:3 (vector versus insert DNA) was used.
- when vector DNA and insert DNA are not similar in length: a molar ratio of 1:1 or 1:2 (vector versus insert DNA) was used.

- blunt ends: a molar ratio of vector DNA to insert DNA of 1:5 was used.

1 µl of T4-Ligase and 2 µl of ligation buffer (both Boehringer Ingelheim) were added and the reaction mix was brought to a final volume of 20 µl. The reaction was incubated either for 3 h at room temperature or overnight at 16°C. The reaction mixture was used directly for transformation without any further purification.

2.4 Purification of DNA fragments

(GFX PCR DNA and Gel Band Purification kit, Amersham Biosciences)

DNA fragments were purified according to the manufactures protocol. The DNA was eluted from the column by addition of 10-50 µl TE buffer or H_2O. The DNA-concentration was determined using the undiluted eluate.

2.5 Phenol/chloroform extraction

An equal volume of a neutralized phenol/chloroform solution (1:1) was added to the aqueous DNA containing samples and vortexed for 1-2 min (longer for larger volumes) resulting in an emulsion. After centrifugation at 16,000xg (RT) for 3-5 min, the aqueous layer that was on top after centrifugation was carefully transferred to a new tube, avoiding any flocculent material at the interface. Then an equal volume of chloroform was added, the solution was mixed and centrifuged as described above. The last step was repeated one more time, the aqueous/top layer was transferred to a new tube, 3M NaAc (1/10 part of the volume of the transferred solution) and ethanol (2 parts), was added and vortexed. The mix was centrifuged for 20 min (max speed, 4°C). The pellet was washed by 70% ethanol and centrifuged again for 10 min (max speed, 4°C), after this the pellet was shortly air dryed and then dissolved in TE buffer.

2.6 Total RNA isolation from mammalian cells

Total RNA was purified using the silica-gel-membrane technology as adopted in Qiagen's Rneasy system. The manufacturer provided all buffers. Samples, up to $5x10^7$ cells per sample,

were homogenized in 400µl buffer RLT with 4µl β-mercaptoethanol by pipetting. The purified total RNA was assessed by spectrophotometry and agarose gel electrophoresis. Total RNA samples were stored at –80°C.

2.7 Genomic DNA isolation from mouse tail and tissues

Tissue's fragments and tips of mouse tails (0.2cm) were lysated by adding Embryo lysis buffer (*Solutions and buffers*) containing proteinase K and incubated overnight at 55°C under constant agitation. The samples were centrifuged for 5 min (16,000xg, RT). For routine genotyping of mice, 2µl of the supernatants were subjected to standard PCR reactions.

2.8 Polymerase chain reaction (PCR)

The *in-vitro* amplification of DNA fragments was usually performed in the PTC-200 thermal cycler. Routinely, PCR reactions were set up by adding the following ingredients to a 0.2ml thin-walled tube: the template DNA, the primers flanking the region (s) to be amplified, dNTPs, buffer, and Taq-DNA polymerase. Primer sequences were selected "manually" or electronically determined with the Primer3 algorithm. Annealing temperatures can be determined in the same program. Routinely, reactions were performed with volumes of 20-µl to 50-µl. *Taq* DNA polymerase was used for general PCR reactions, and *PfuTurbo* was used to amplify DNA that was later on used for further cloning steps, because the *Pfu* enzyme contains a proof-reading activity that is absent from Taq-polymerase.
The PCR was performed with the following protocol:

1) Initial denaturating	95°C	1-3 min
2) Denaturating	95°C	1 min
3) Annealing	T_a	1 min
4) Synthesis	72°C	1 min/ 1kb DNA
5) Termination	72°C	10 min
6) Cooling	4°C	

The amplification procedure (steps 2-4) was repeated 39 times. If two primers had different annealing temperatures, the lower one was used. Afterwards, the PCR reactions were analysed

by gel electrophoresis. For routine genotypying the gels were documented and interpreted while PCR products for further cloning procedures were purified using the GFX PCR DNA and Gel Band Purification kit.

2.9 First strand synthesis, RT-PCR

For reverse transcription *in-vitro*, an RNA-dependent DNA polymerase activity and a hybrid-dependent exoribonuclease (Rnase H) activity of the reverse transcriptase enzyme (RT) were utilized to produce single stranded cDNA from RNA. Routinely, SuperScript™ Double-Stranded cDNA Synthesis Kit was used to produce first strand cDNA from 50-1000 ng of total RNA as a starting material. After denaturating total RNA samples with oligo-dT primer for 2 min at 70°C, the following reactions were set up by adding all further components by a master-mix. First strand synthesis was achieved by a 2-h incubation at 42°C.

1	µl	dT-primer (10µM)
1	µl	dNTP (10mM)
4	µl	5xFirst-strand reaction buffer
2	µl	DTT (0.1M)
1	µl	R-nase inhibitor
1	µl	SuperScript™ II RT
Up to 20	µl	DEPC-treated H_2O

First strand cDNA was stored at –20°C or directly subjected to PCR reactions.

2.10 TA cloning

TA cloning of PCR products containing an additional adenosine at their 3'-ends was performed with the pGEM-T easy system (Promega). The procedure to clone PCR fragments into the pGEM-T vector simply followed the manufacturer's instructions.

2.11 DNA Gel-electrophoresis

DNA fragments were separated by horizontal gel electrophoresis in chambers (BioRad) using agarose gels. Agarose gels were prepared by heating 1-2 % (w/v) agarose (Gibco) in 1xTAE

buffer, depending on the size of DNA fragments to be separated. The ethidiumbromide was added in the gel (7 µl to 100ml gel solution). The gel was covered with 1xTAE buffer and the samples (Mix of DNA solution and sample buffer) were pipetted in the gel pockets. The gel was run at constant voltage (10V/cm gel length) for an appropriate time. Finally gels were documented using a UV-light imaging system.

2.12 Extraction of DNA fragments from agarose gels

(GFX PCR DNA and Gel Band Purification kit, Amersham Biosciences)

For isolation and purification of DNA fragments from agarose gels, ethidiumbromide-stained gels were illuminated with UV-light and the desired DNA fragment was excised from the gel with a clean scalpel and transferred into an Eppendorf tube. The fragment was isolated following the manufactures protocol. The fragment was eluted from the column by addition of 10-50 µl TE buffer or H_2O. The DNA-concentration was determined using the undiluted eluate.

2.13 Determination of DNA concentrations

DNA concentrations were determined spectroscopically using an Amersham-Pharmacia spectrometer. The absolute volume necessary for measurement was 50 µl. For determining the concentration of DNA preparations (III 1.2), the eluate was diluted 1:50 with water and the solution was pipetted into a 50µl cuvette. Concentration was determined by measuring the absorbance at 260 nm, 280 nm and 320 nm. Absorbance at 260 nm had to be higher than 0.1 but less than 0.6 for reliable determinations. A ratio of A_{260}/A_{280} between 1,8 and 2 indicated a sufficient purity of the DNA preparation.

2.14 DNA-Sequencing

(Step-by-Step protocols for DNA-sequencing with Sequenase-Version 2.0, 5th ed., USB, 1990)

DNA sequencing was performed by the sequencing facility of the ZMNH. Customers had to provide 1 µg of DNA diluted in 7µl ddH_20 and 1 µl of the appropriate sequencing primer (10 pM).

2.15 Purification and labelling of nucleic acid probes

2.15.1 Random primer DNA labelling

CDNA probes used for Northern blot analysis were [^{32}P]-dCTP-labelled with Prime-IT® RmT random primer labelling kit (Stratagene). 25-50 ng of the designated probes were added to LMT agarose in a total volume of 42 µl and mixed by pipetting. The reactions tubes were boiled for 5 min at 95°C and then centrifuged briefly at RT. 5µl of [α^{32}P]-dCTP and 3µl of magenta DNA polymerase (4 U/µl) were added to each of the reaction tubes and mixed. The reactions were incubated for 10 min at 37°C. For removal of unincorporated nucleotides ProbeQuant™ G-50 micro columns (Amersham Biosciences) were used as described in the manufacturers protocol. Directly before applying the probes to prehybridized blots, the molecules were denatured by boiling for 5 min.

2.15.2 Template purification and generating RNA probes by in-vitro transcription

To produce highly specific antisense-probes, devoid of vector-specific sequences, approximately 2-5 µg of plasmids containing T3, T7 or SP6 RNA polymerase promoter sites were digested with appropiate restriction endonucleases overnight at positions located 3` of the to be transcribed strand of DNA. The linearised templates were purified by phenol/chloroform extraction, precipitated and finally the pellets were dissolved in DEPC-H_2O to make a final concentration of approximately 1µg/µl. Before the actual radioactive labelling an in-vitro transcription using unlabelled nucleotides was performed using the following components:

1	µl	DNA (0.5-1µg)
2	µl	10xTranscription buffer
0.5	µl	RNase inhibitor
2	µl	NTP (10mM)
1	µl	RNA polymerase (T3/T7/SP6)

Up to 20 µl DEPC-H_2O

To generate radioactively labelled probes for in situ hybridization, 50 µCi α-[^{35}S]-UTP (2.5 µl) and 50 µCi α-[^{35}S]-CTP (2.5 µl) were used for each reaction with a final volume of 20 µl. After DNase treatment (3 µl RNase free DNase, 1 µl tRNA, 20 µl $MgCl_2$, 70 µl DEPC-H_2O were added to 20 µl of the reaction) for 10 min at 37°C, the probes were purified using ethanol precipitation. For that purpose, a mix containing 107 µl ammonium acetat (7.5M), 190 µl DEPC-H_2O and 1 ml ethanol was added into the reaction tubes. The tubes were shortly put into liquid nitrogen and then centrifuged for 10 min at 4°C. The pellets were washed by a mix of 145 µl DEPC-H_2O, 54 µl ammonium acetat (7.5M) and 500 µl ethanol and centrifuged as described above. The precipitate was dried at RT and diluted in a mix containing 30 µl DEPC-H_2O, 30 µl formamid and 1 µl DTT. To monitor the specific activity of each radioactive probe 1 µl was diluted in 3 ml of scintillation fluid and measured using a scintillation counter.

2.16 Analysis of nucleic acids by hybridization

2.16.1 Northern blot analysis

Denaturing electrophoresis of total RNA samples (2.6) and capillary blotting onto a Hybond^{TM}N$^{(+)}$ membrane were performed following standard procedures (Sambrook, J. et al., 1989). Briefly, total RNA, 15-20 µg in a volume of 20µl was mixed with 30µl master mix (see Materials). Next the RNA was denatured by incubation at 65°C for 10 min. Samples were then loaded on an agarose gel (1.5% agarose, 1xMEN buffer, 16.6% formaldehyde (37%). After the gel had run for an appropriate time a capillary blot was set up and the RNA transferred overnight using 20xSSC as a transfer buffer. The membrane was rinsed in 2xSSC to remove agarose and the RNA was crosslinked to the air-dried membrane by UV radiation (0.07 J/cm^2). The membrane was incubated in pre-hybridization buffer for 20-30 min at 65°C. A radioactively labelled cDNA probe was denatured for 5 min at 95°C and added into pre-hybridization buffer. Overnight hybridization was carried out at 65°C. After washing the membrane for 2x20 min with washing buffer I, 2x20 min with washing buffer II at 60°C it was wrapped in plastic foil and exposed to an imaging plate (Fujifilm) for 4 h at RT. The result was analysed using Fla-3000 scanner (Fujifilm).

2.16.2 RNA in situ hybridization using radiolabelled RNA probes

Cryostat sections, usually 10 µm thick, were prepared from murine tissues and mounted on silanized slides (Marienfeld). Sections were fixed in PBS containing 4% paraformaldehyde for 20 min at RT and washed twice for 5 min in PBS. Thereafter, free amino groups were acetylated in acetylation buffer for 10 min at RT, washed once in PBS, dehydrated in an ascending series of ethanol, and air-dried. Radioactive probes were diluted in hybridization buffer to a concentration of 100.000 cpm/µl, denatured for 5 min at 95°C, cooled by ice, and finally 1/10 of volume of DTT was added. 20 µl of probe was added onto each slide and carefully covered by coverslips (before this, coverslips were washed in Sigmacote, ethanol and air dried). The slides were incubated for 12-16 h at 55°C in moist chambers. After hybridization, slides were washed under gentle agitation for 20 min at 60°C in washing buffer I, and for 30 min at 60°C in washing buffer II, then twice for 10 min at 37°C in RNase buffer, once for 10 min in RNase buffer with RNase (20µg/ml) at 37°C and one more time in RNase buffer without RNase. Then slides were washed for 2-3 h at 60°C in washing buffer II under gentle agitation, for 10 min at 60°C in 2xSSC solution and for 10 min at 60°C in 0.5xSSC solution. After this procedure the slides were dehydrated in an ascending series of ethanol, and air-dried. The slides were exposed to BIOMAX^{TM} MR X-ray films (Kodak) overnight and then dipped into NTB-3 nuclear track emulsion (Kodak) and exposed for 10-14 days (depending on intensity of signal visualised by the film). The slides were developed in developing solution (D19 Kodak), fixed (2% acetic acid for 1 min and 47.5% sodium tiosulfate for 5 min) and stained by Giemsa solution. After this the slides were mounted with EUKITT® and coverslips.

3. Cell culture

3.1 CHO cell culture

CHO cells were cultured in CHO-medium at 37°C, 5 % CO_2 and 90 % relative humidity in 54 cm^2 dish (Nunc) with 8 ml medium or in six-well plates (d = 35 mm; area = 9,69 cm^2) with 3 ml medium. Cells were passaged when they were confluent (usually after 3-4 days). Medium was removed and cells were detached by incubation with 4 ml trypsin solution (Gibco) for 5 min at 37°C. Cells were centrifuged (200xg, 5 min, RT) and the pellet was resuspended in 10

ml fresh medium. Cells were split 1:5 for maintenance or seeded in six-well plates for transfection (300 µl per well).

3.1.1 Stable transfection of CHO-cells

(Lipofectamine Plus manual, Life technologies)

For transfection of CHO cells, the Lipofectamine Plus kit (Life Technologies) was used. One day before transfection, 2 x 10^5 cells were seeded per 100 mm dish. When cell density had reached 60-70% (usually after 18-24 h) the cells were washed with CHO-medium without FCS and antibiotics and transfected with 8 µg total DNA (containing the neomycin resistance gene) per 100 mm dish. Transfection was performed as described in the manufactures protocol. On the next day the medium was exchanged against selection medium containing geneticin (G418 Gibco *f.c.* 0.8 mg/ml). The cells were grown during 2-3 weeks in selection medium that was changed every 2-3 days, until single clones appeared. Single clones were then isolated and placed into the 96-wells plate. Clones were cultured as described above, frozen and stored at -80°C. Clones were analysed for matn2 expression by Western blot and immunostaining, and the clone, which had the highest level of expression, was used for protein production.

3.1.2 Cell culture of stably transfected CHO cells

Clones of stably transfected CHO cells were cultured as described above. For the production of matrilin-2-Histag protein, the cells were slowly adapted to medium without serum, then seeded in 150 cm^2 dish and 8 ml medium without serum was added. Cells were allowed to express and secrete matrilin-2-HisMyc into medium for 24-36 h and then the medium, containing the matn2 protein was collected. After addition of fresh medium this procedure was repeated 5-6 times per dish.

3.2 S16 cell culture

S16 cells were cultured in S16-medium at 37°C, 5 % CO_2 and 90 % relative humidity in 54 cm^2 dish (Nunc) with 8 ml medium or in 24-well plates (area = 2 cm^2) with 1 ml medium. Cells were passaged when they were confluent (usually after 3-4 days). Medium was removed and cells were detached by incubation with 4 ml trypsin solution (Gibco) for 5 min at 37°C. Cells were centrifuged (200xg, 5 min, RT) and the pellet was resuspended in 8 ml fresh medium. Cells were routinely split 1:5 for maintenance.

3.2.1 Agarose drop migration assay

Agarose drop migration assay was performed as described by Milner and co-workers (1997). In this assay, S16 cells were resuspended in Sato medium containing 10% FCS in high confluence ($1x10^7$ per ml) and pre-warmed to 37°C. After this, 1/3 volume of 1% pre-warmed low melting agarose was added into cells to make final concentration 0.33% and mixed. Then the mixture of agarose and cell solution was plated as small drops onto tissue culture plastic (24 well dish), which was then placed at 4°C for 15 min to allow the agarose to solidify. Following this, the cooled drop was covered with Sato medium containing the chosen ECM molecule. Cells migrated for 24-48 h and migration was observed using a phase contrast microscope.

3.2.2 Adhesion assay

Adhesion assay was performed using the protocol described (McGarvey et al., 1984; Milner, 1997; Chernousov et al., 2001). S16 Schwann cell suspension ($1x10^5$ per ml) in Sato medium was added to the wells of a 24-well tissue culture dish, which was coated with the different ECM substrates before the assay. Then cells were incubated for 30 min at 37°C. The adhesion assay was stopped by addition of DMEM and washing off loosely attached cells. The attached cells were then fixed, and adhesion was quantified by counting all attached cells under a phase contrast microscope.

3.3 Dorsal root ganglion (DRG) culture

3.3.1 Preparation

DRG cultures were prepared from mouse embryos (E12-E18). In brief the culture was performed as follows:

1) Dissection

Mice were decapitated and the spinal cord with the attached DRG removed. The spinal cord with the DRG was transferred into a petri dish containing DRG medium. Next, the DRG were plucked away like grapes carefully using fine forceps and then put into tubes with DRG medium.

2) Removal of cell debris and plating of DRG explants

After centrifugation (800 x g, 5 min, 4^0C) and resuspension in DRG medium containing GFs, cellular debris was removed and DRG were plated in a density of approximately 1 embryo/2cm^2.

3.3.2 Axon outgrowth by DRG explants

DRG, prepared as described above, were placed on dishes coated with different ECM substrates. The ganglia were incubated in DRG medium with nerve growth factor for 24h. To analyse axon outgrowth an immunostaining with anti-neurofilament antibodies was performed.

3.3.3 Cell migration from DRG explants

DRG (E12) were placed on the dishes coated with different ECM substrates. Growth factors (NGF, NT3) promoting axon outgrowth were added into the medium. After 48h in culture the migration of cells along growing axons was monitored by immunostaining with sox10 (ESC marker) and DAPI (nuclear localization) was performed. Cells were counted using immunofluorescence microscopy. To eliminate the effect of axon growth on cellular migration, axon growth was inhibited by removal of growth factors (NGF, NT3) from the medium. Heregulin was added to the medium to promote Schwann cell survival and

differentiation. The migration of Schwann cells out of the ganglia was allowed for 48h. Analysis was performed by immunostaining employing anti-sox10 antibodies.

3.3.4 Dissociation of DRGs

DRG were dissociated as described (Svennigsen et al., 2003). In brief, the procedure was performed as follows: DRG, prepared as described above, were put into L15 medium (Gibco) containing 0.05% trypsin and incubated for 15-30 min at 37°C (incubation time depended on the embryonic stage). After this, a blocking solution (10% FCS in L15) was added to inhibit trypsin activity. The suspension was precipitated by a short centrifugation (100 x g) and washed twice by L15 medium. After addition of 2 ml L15 medium with 0.01% DNase the DRG were titrated using a fire-polished Pasteur pipette to dissociate the still loosely attached tissue. Singularised cells were washed twice with L15 medium and resuspended in Neuronal medium containing GFs. Then the cells were plated in a final density of 1000 cells/mm^2.

3.3.5 Stripe assay

Stripe assay was performed as described (Vielmetter et al., 1990). Silicon matrix was obtained from the MPI in Tübingen, Germany. This matrix has parallel channels, 50 µm width, which are separated by bars of 40 µm width. On both ends the parallel channels merge into an inlet and outlet channel, respectively. This matrix was placed onto a glass surface e.g. a coverslip. The first protein solution was filled into the open channel and the protein was allowed to bind to the glass (incubation periods of 2 h at 37°C). Unbound protein was removed by 3 washes with PBS. Then, a blocking solution containing fluorescein-conjugated BSA (1% in PBS, Molecular Probes) was injected into the channels and incubated for 2 h at 37°C. Next, the channels were rinsed three times with injected PBS, after which the matrix was removed. The coverslips could additionally be coated with a second protein. DRG explants and dissociated cells were grown on these coverslips for 24 h as described above. For analysis the samples were fixed and subjected to an immunostaining procedure with NF antibodies.

3.4 Preparation of dissociated hippocampal cultures

For preparation of dissociated hippocampal cultures, mice of postnatal day 1 – 4 were used. Hippocampi were prepared by Galina Dityateva. Preparations were performed as described (Dityatev et al., 2000).

In brief, the procedure was performed as follows:

1) Dissection

Mice were decapitated and brains removed from the skull. Brains were cut along the midline and hippocampi were prepared and split into 1mm thick pieces.

2) Digestion

Hippocampi were washed twice with dissection solution and treated with trypsin and DNAseI for 5 min at RT. Digestion solution was removed and hippocampi were washed twice. The reaction was stopped by adding trypsin inhibitor.

3) Dissociation

Hippocampi were resuspended in a dissection solution containing DNAseI. Titration with Pasteur pipettes having successively smaller diameters were used to dissociate hippocampi to a homogeneous suspension.

4) Removal of cell debris and plating of cells

By subsequent centrifugation (80 x g, 15 min, 4^0C) and resuspension in dissection buffer, cellular debris was removed. Cells were counted in a Neubauer cell chamber and plated to provide a density of 250 cells/mm^2 (for neurite outgrowth) or 1.000 cells/mm^2 (for synaptogenesis assay).

3.5 Morphometric analysis

For morphometric analysis of neurite outgrowth and cellular migration the inverted microscope Axioplan 2 imaging and Axiovision release 2 SP1 imaging system were used for image acquisition of stained cells and operator-controlled tracing of neurites. Statistical evaluation was performed using the paired t-test applied to compare the averaged values derived from three independent experiments.

4. Immunofluorescence

4.1 Immunocytochemical analysis of cell cultures

Cells were seeded on coverslips and allowed to grow for appropriate time. After removal of the medium the cells were carefully washed once with PBS and then fixed with cold methanol for 5 min at RT. After this, the cells were washed twice with PBT (0.1% Triton) for 5 min and blocked in PBT (0.01% Triton) containing 2% BSA or 5% goat serum for 1 h at RT. Solutions with primary antibodies (PBS with 0.01% Triton and 1% goat serum) were applied onto the samples and incubated overnight at 4°C. After the washing steps (three times with PBT) solutions containing the appropriate secondary antibodies were added and incubated in the dark for 1 h at RT. Samples were washed twice with PBT for 5 min at RT, once with PBS and DAPI (1÷1000, to visualise the nuclei) for 5 min at RT and a last time with water. Finally, the coverslips were mounted on objectives with Aqua Poly-Mount medium (Polysciences Inc) and could be stored in the dark at 4°C prior to analysis.

4.2 Immunocytochemical analysis of the tissue sections

4.2.1 Preparation of tissue sections

Embryos of different developmental stages, as well as brains from three weeks old mice were fixed with 4% PFA in PBS overnight (the latter were perfused before the isolation of the brains). After fixation the tissues were washed twice with PBS and cryoprotected by incubation with 30% sucrose in PBS overnight. Tissues were frozen in Tissue Tek® and stored at –80°C. Tissues were cut on a cryostat (Leica…) in 10–15 µm sections and collected on adhesion micro slides (Marienfeld). The air-dried slides could be stored in sealed boxes at –80°C.

4.2.2 Immunofluorescence staining of mouse tissue

The slides were warmed up to RT and fixed with 4% PFA in PBS for 5 min at RT or with cold ethanol for 20 min at –20°C. After this, the slides were washed three times with PBT

(0.1% Triton) for 10 min and blocked in PBT (0.01% Triton) containing 2% BSA or 5% goat serum for 1 h at RT. Solutions with primary antibodies (PBS with 0.01% Triton and 1% goat serum) were applied onto slides, covered with pieces of parafilm and incubated 1 – 3 days in chambers containing water (to avoid evaporation of liquid) at 4°C. After the washing steps (three times with PBT) solutions containing appropriate secondary antibodies were added and the slides were incubated in the dark for 1 h at RT. Sections were washed twice with PBT for 5 min at RT, once with PBS and DAPI (1÷1000, to see nuclei localization) for 5 min at RT and last time with water. Finally slides were mounted with Aqua Poly-Mount medium (Polysciences Inc) and covered by coverslips. The slides could be stored in the dark at 4°C prior to analysis.

5. Animals and surgery

For cell culture experiments, surgery and expression study we used C57Bl/6 mice as well as ErbB3 and Matn2 deficient mice that were on a mixed background (129/ C57Bl/6). For surgical experiments, animals were anaesthetised with anaesthetision solution (25 µl per g). The sciatic nerves were transected in the wright thigh. These animals were killed 5 (n=3), 7 (n=3) and 14 (n=4) days postoperation (dpo). The femoral nerves were crushed with forceps in the left thigh. These animals were killed 3 dpo (n=3).
Deeply anaesthetised animals were killed by perfusion through the left ventricle of the heart with fixative (4% paraformaldehyde in 0.1M PBS was used).

6. Computer analysis

6.1 Sequence analysis

Computer based sequence analysis and alignments of DNA sequences and protein sequences was performed using the Lasergene-programe (DNASTAR, *Inc*., www.dnastar.com). The following databases were used: Medline-, BLASTN- and BLASTP-Server of NCBI (National Center for Biotechnology Information, www.ncbi.nlm.nih.gov).

6.2 Statistical analysis

All values are reported as mean ± SEM (standard error of mean). Student's test and non-parametric U-test were used to assess statistical significance using Sigma Plot 5.0. Differences between >3 groups were tested for significance using nonparametric Kruskal-Wallis one way analysis of variance.

V. Results

V.1 Matrilin-2 (matn2) as a component of the peripheral nervous system

1.1 Matn2 is differentially expressed in erbB3 mutant and wt mouse embryos

It has been shown previously that matn2 has a wide tissue distribution. Expression of matn2 in mouse cartilage and bones has been studied quite well, whereas expression in the nervous system and embryonic tissues was not described in great detail yet. In the present study I investigated the expression pattern of matn2 by in situ hybridization. I found it in different regions of the brain, such as the choroid plexus and trigeminal ganglion V, as well as the genital ridge, cartilage, bone, skin, and in sheets of inner organs. In the PNS expression of matn2 was observed in dorsal root ganglia (DRG) and along peripheral nerves. To investigate the expression pattern of matn2 in the PNS in more detail, erbB3 mutant mice were used, that had already served to identify matn2 as being expressed in the PNS (Michaela Miehe, Dissertation, Universität Hamburg 2003). These mice have several abnormalities in the PNS. One characteristic feature of these mutant embryos is the lack of Schwann cell precursors along peripheral nerves (Riethmacher et. al., 1997; Britsch et. al., 1998). The expression of matn2 was not found in DRG and along peripheral nerves in erbB3 mutant embryos (Fig. 1C-F) using in situ hybridization. In addition, this result was confirmed by real time (RT) PCR of cDNA prepared from DRG of wt and mutant embryos (Fig. 1A). It's known that Schwann cells start to migrate from DRG on embryonic day E10- E12. Thus, the localisation of the transcripts in wt embryos as well as the absence in erbB3 mutant mice argues for a Schwann cell specific expression of matn2. The signal observed in the lower part of DRG of wt embryos most likely is derived from Schwann cells, which are ready to migrate. To corroborate this assumption, the expression of matn2 was analysed in different cell types by RT-PCR, Northern Blot and immunofluorescence analysis. Matn2 was found to be expressed by at least two Schwann cell types: primary embryonic Schwann cells (prepared from DRG of mouse embryos) and S16 cells (an immortalized Schwann cell line from rat sciatic nerve), whereas the precursor of embryonic Schwann cells, neural crest stem cells, do not express matn2 (Fig. 1B).

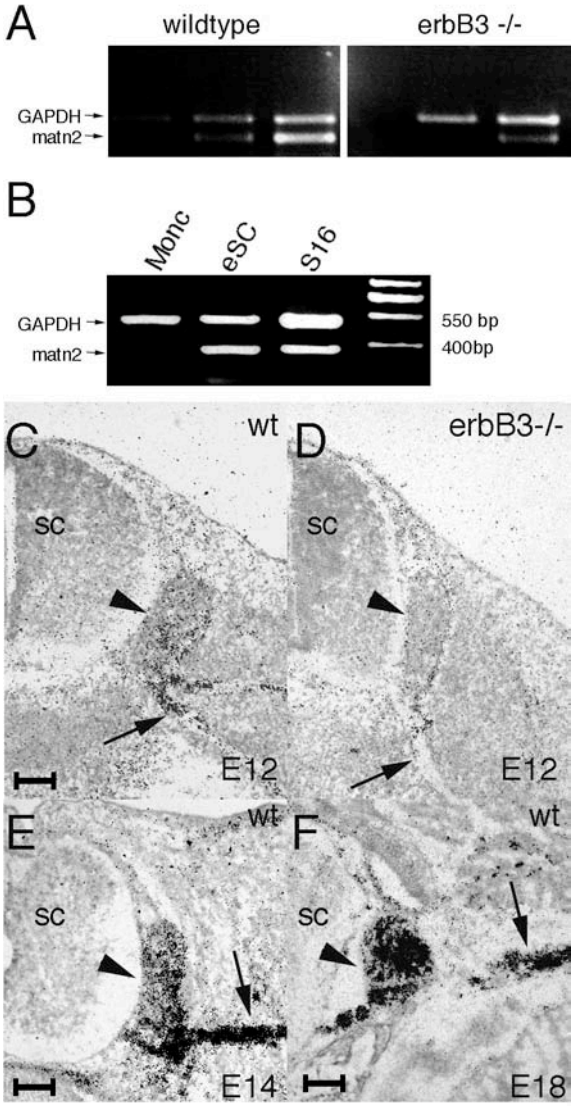


Fig. 1 Expression of matn2 in wt and erbB3 -/- embryos and in different cell types. (**A**) Semi-quantitative PCR analysis of cDNA prepared from DRG of wt and erbB3 mutant. GAPDH (upper band) was used as an internal control. At comparable GAPDH levels, much higher levels of matn2 specific amplicons were present in wt compared to mutant. (**B**) Analysis of matn2 expression by RT-PCR. GAPDH (upper band) was used as internal control. Matn2 (lower band) is expressed in a rat Schwann cell line (S16) and in mouse primary embryonic Schwann cells (eSC) while no expression of matn2 in neural crest stem cells (Monc), the precursors of Schwann cells, could be found. (**C-F**) S^{35} in situ hybridisation with a matn2 specific probe on *cross* cryosections of mouse embryos (E12-18). Arrowheads indicate the expression of matn2 in DRG of wt mice (**C,E,F**) and absence of this expression in mutants (**D**). Arrows point towards peripheral nerve projections. Note the absence of signals in the mutant embryo **(D)**. At later stages of embryonic development matn2 expression becomes stronger in the Schwann cell lineage **(E,F)**.

1.2 Matn2 promotes Schwann cells (S16) adhesion

Given the expression of matn2 in Schwann cells I next wanted to identify potential effects of the protein on this cell type. Therefore I investigated the adhesion of Schwann cells to different ECM substrates, namely laminin, fibronectin and matn2. First, it was necessary to determine the adhesion time, during which cells adhere to a given surface. This time is specific for each cell type. For instance, primary Schwann cells need 3 h to adhere to a surface (Chernousov at al., 2001). I wanted to use S16 cells and found experimentally that for these cells 30 min is the ideal time in an adhesion assay. Another important step was the pre-

incubation of the cell suspension before plating to restore cytoskeleton molecules and receptors, which are damaged during the trypsination procedure. After adhesion the adherent cells were stained with toluidine blue and using a phase microscope the numbers were determined. As shown in Fig. 2A, matn2 promotes Schwann cell adhesion, although to a lesser extent than laminin or fibronectin.

1.3 Matn2 promotes Schwann cells (S16) migration

Next the ability of matn2 to promote Schwann cell migration was investigated and compared with other ECM substrates that are present in peripheral nerves, such as laminin or fibronectin. For this purpose, the Varani migration assay (Varani et al., 1978) was used. This assay measures the migration of cells from a high-density population of cells contained in an agarose drop (for details see Materials and Methods). Interestingly, Schwann cells began to migrate out of a drop within 2 h after plating, and continued to migrate radially for a number of days, producing a uniform "corona" of cells around the drop. Migration was quantified daily by measuring the distance between the leading edge of the corona and the edge of the agarose drop. As shown in Fig. 2B-F, matn2 promoted Schwann cell migration stronger than laminin or fibronectin. Fig. 2F shows the effects of different ECM molecules (all at 10 µg/ml coating concentration) to promote Schwann cell migration over a period of 3 days.

1.4 Matn2 promotes axon outgrowth of dorsal root ganglion neurons

In order to investigate whether matn2 could influence neurite outgrowth of sensory neurons from DRG, the length of axons was measured after 24 h in culture. DRG explants were cultured on different ECM substrates: matn2, laminin or a mixture of laminin and matn2 in serum-free medium containing growth factors. As shown in Fig. 3A, neurite length was significantly increased on a matn2-coated surface compared to a poly-D-ornithine (PDO) coated surface. Interestingly, matn2 was almost as competent in neurite outgrowth promotion as laminin, a known and potent stimulator of axon growth.

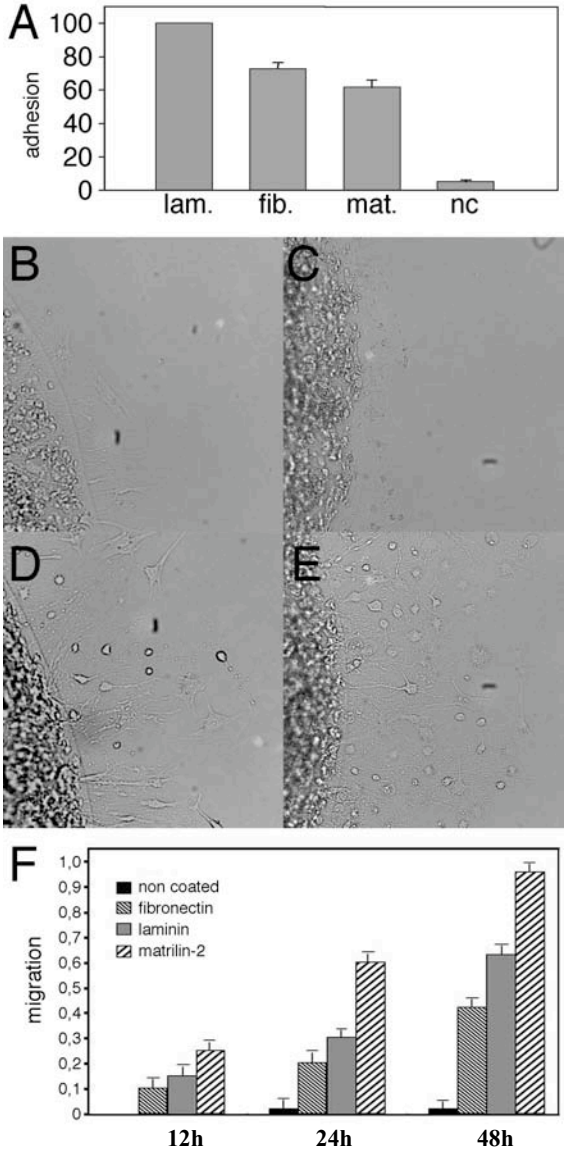


Fig. 2 Comparison of Schwann cell (S16) adhesion to different ECM substrates and S16 migration on those substrates. (**A**) Schwann cell suspension was added for 30 min to the wells of a 24-well tissue culture dish, which has been coated by different ECM substrates before the assay. After washing, cells were fixed and the adhesion was quantified by counting all attached cells with a phase contrast microscope. Results are expressed as a percentage of the number of cells adhering to laminin, that was normalized to 100% (n=3 experiments). (**B-E**) Cell migration was observed using a phase contrast microscope. Note that after 2 days, cells on the matn2 substrate **(E)** migrated further and appeared more dispersed than cells on others substrates non-coated **(B)**, fibronectin **(C)**, or laminin **(D)**. (**F**) Distances (mm) of Schwann cell migration on different ECM substrates: fibronectin, laminin, matn2 and non-coated. All ECM molecules promoted Schwann cells migration over the non-coated control. Four independent experiments were done. The values shown are the mean ± SD. lam., laminin; fib., fibronectin; mat., matn2; nc, non-coated; h, hours.

1.5 Matn2 promotes cell migration from DRG

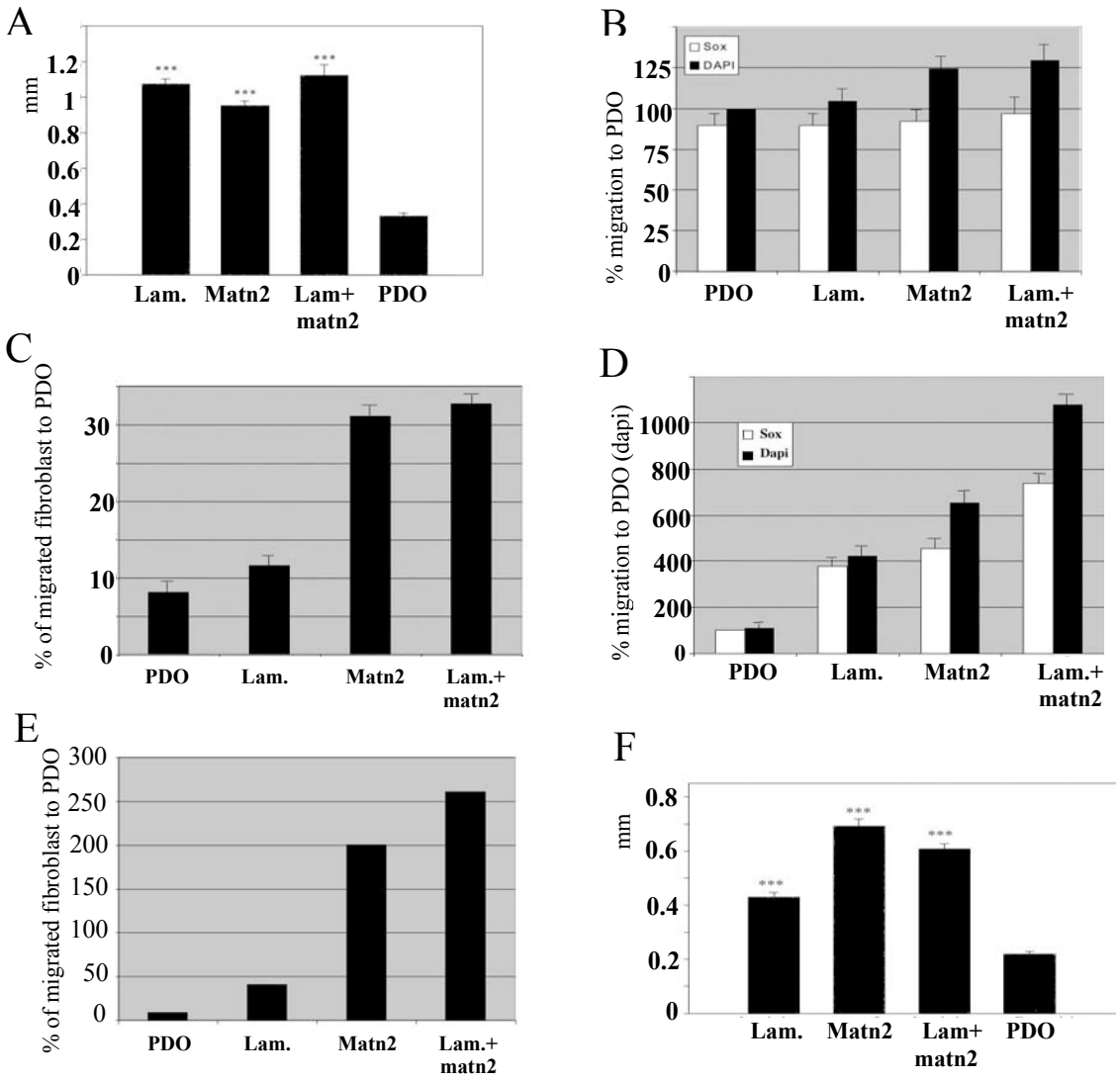
Schwann cells migrate along outgrowing neurites during development, and the effect of axons on this migration behaviour is significant. Migration of Schwann cells depends on axon-Schwann cells interactions. But additionally secreted factors, like neuregulin, strongly promote Schwann cell migration.

The ability of matn2 to promote embryonic Schwann cell migration from DRG was first analysed without inhibition of axon outgrowth. In these experiments, DRGs prepared from

mouse embryos (E12) were placed on dishes coated with matn2, laminin or a mixture of matn2 and laminin. The ganglia were incubated in serum free medium supplemented with neurotrophic factors. After allowing the cells to migrate along growing axons for 48 h, the cultures were fixed and stained by sox10 antibodies (specific for glial cells) and DAPI (to see all migrated cells). Using immunofluorescence microscopy the number of cells was determined. Under these conditions there was no difference in Schwann cell (sox10 positive) migration between the different ECM substrates tested. However, the total number of cells that had migrated was increased when matn2 alone and matn2+laminin was used in comparison with laminin alone or PDO (Fig. 3B). We assumed that all non-glial (sox10 negative) cells in our assay are fibroblasts. The increase in the total number of migrating cells on matn2 was determined by subtraction of sox10 from DAPI positive cells. As shown in Fig. 3C significantly more fibroblasts had migrated (≈30% from total number cells migrated on PDO) when matn2 was used for coating in comparison with laminin when less fibroblasts migrated (≈10% from total number cells migrated on PDO).

As mentioned before axons have a strong influence on the migration behaviour of Schwann cells and thus might make it impossible to detect effects deriving from the different ECM molecules used in our assay. We therefore wanted to eliminate these axonal effects. We did this by culturing DRG under conditions that inhibit axon outgrowth, as described earlier (Chernousov et al., 2001). In short, neurotrophic factors were removed from the medium and thus neurites could not grow. To keep the embryonic Schwann cells alive that are dependent on axonal signals nrg had to be included in the medium. Schwann cells were visualised by fluorescence microscopy after staining with sox10 specific antibodies (Fig. 3G) and their migration away from DRG on different ECM substrates was determined (Fig. 3D). Under these conditions the total number of cells that had migrated was increased, when matn2 or matn2+laminin were used in comparison to other substrates. Moreover, matn2 also strongly promoted the migration of Schwann cells (sox10 positive). Thus, while in experiments without inhibition of axon growth only the number of migrating fibroblasts was elevated when matn2 was used in experiments with inhibition of axon growth also the amount of migrating Schwann cells was increased. One possible explanation for this observation is that the migration of Schwann cells depends much more on axonal signals than the migration of fibroblasts. Consistently the numbers of migrating fibroblasts was comparable in both experiments (Fig. 3E). Additionally, the distances of cellular migration were determined and the biggest distance of migration could be observed when matn2 alone was used as coating reagent (Fig. 3F). Statistical analysis has shown that migration on matn2 was significantly

increased (***p <0.0001), as well as migration on laminin+matn2 (**p<0,0014) compared to laminin alone.



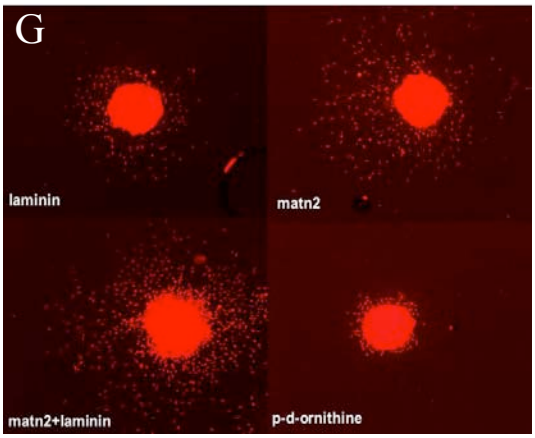


Fig. 3 Effects of different ECM substrates on axon outgrowth and cellular migration in cultures of DRG.

(A) Axon outgrowth from DRG cultures. The values shown are the mean ± SD. Statistical analysis demonstrated significant increase (***p<0.0001) of axonal growth on all ECM substrates compared to PDO. Promotion of axon growth by matn2 is comparable to the promotion by laminin. **(B,C)** Migration of DRG cells without inhibition of axon growth. Cells were identified and visualised using immunofluorescence microscopy and sox10 specific antibodies. **(B)** Migration of cells in wt cultures grown on PDO was normalized to 100% and compared to the other observed migration. **(C)** Percentages of sox10 negative cells (fibroblasts) to number of cells migrated on PDO (100%). A significant increase in non-

Schwann cell migration was observed from PDO to matn2. **(D-G)** Cellular migration in DRG cultures with inhibition of axon growth as visualised by fluorescence microscopy **(G)**. The number of cells that had migrated on PDO was normalized to 100% and compared to the other values **(D)**. Note the strong promotion of Schwann cell migration on laminin, matn2 and lam+matn2. As before when axonal growth was not inhibited an increase in non-Schwann cell migration from PDO to matn2 could be observed **(E)**. Thus, these experiments demonstrated that matn2 not only promotes migration of fibroblasts but, additionally, strongly promotes Schwann cell migration. The distances of migration were quantified by measuring on microphotographs the distance from the edge of a ganglion to the leading edge of migrating Schwann cells, i.e., the cohort of cells that had migrated the greatest distance from the ganglion **(F)**. Statistical analysis showed significant increase (***$p<0.0001$) of migration on all ECM substrates compared to the migration on PDO. Note that migration on matn2 alone as well as on lam+matn2 was significantly increased compared to laminin alone (***$p<0.0001$ and **$p<0.0014$, respectively). lam., laminin; PDO, poly-D-ornithine. The values shown are the mean ± SD of measurements made on four ganglia for five separate cultures.

1.6 Axon outgrowth is decreased in matn2 knock out DRG co-culture

In order to substantiate the biochemical data, where matn2 was used as coating reagent, in axon outgrowth assay with embryonic DRG and showed a significant increase in axonal growth, I next employed matn2 mutant embryos. DRG were isolated from wt and matn2 mutant embryos (E12) and cultured as described before in dishes coated with PDO or laminin. The ganglia were incubated in serum-free medium with neurotrophic factors for 72h. The time of culture was increased in these experiments to allow the deposition of endogenous matn2 and thus to detect potential differences between axonal growth in wt and mutant cultures. Finally an immunostaining with anti-neurofilament antibodies (to visualize the neurites) and counterstaining with DAPI (to localize nuclei) was performed. A significant decrease of axonal length was observed in mutant cultures compared to wt cultures (Fig. 4A). This difference was not expected since matn2 mutant mice show no obvious abnormalities in the PNS during embryonic development and in the adult (see Introduction).

1.7 Cell migration is decreased in matn2 knock out DRG co-culture

In parallel to the axon outgrowth assay, the migration of cells from mutant and wt DRG was analysed. Migration was allowed for 72 h along growing axons, and the identity of migrating cells was determined by staining with anti-Sox10 antibodies and DAPI. As shown in Fig. 4B, the number of migrating cells in mutant DRG cultures was decreased in comparison with wt

cultures on both applied substrates, PDO and laminin. Importantly, when laminin was used a decrease in fibroblast and Schwann cell migration was detected whereas on PDO only the number of migrating Schwann cells rather than fibroblasts (Fig. 4C) was reduced. Additionally, there was not a big difference in distances of Schwann cells migration (data not shown), as it was observed when matn2 was used as coating substrate.

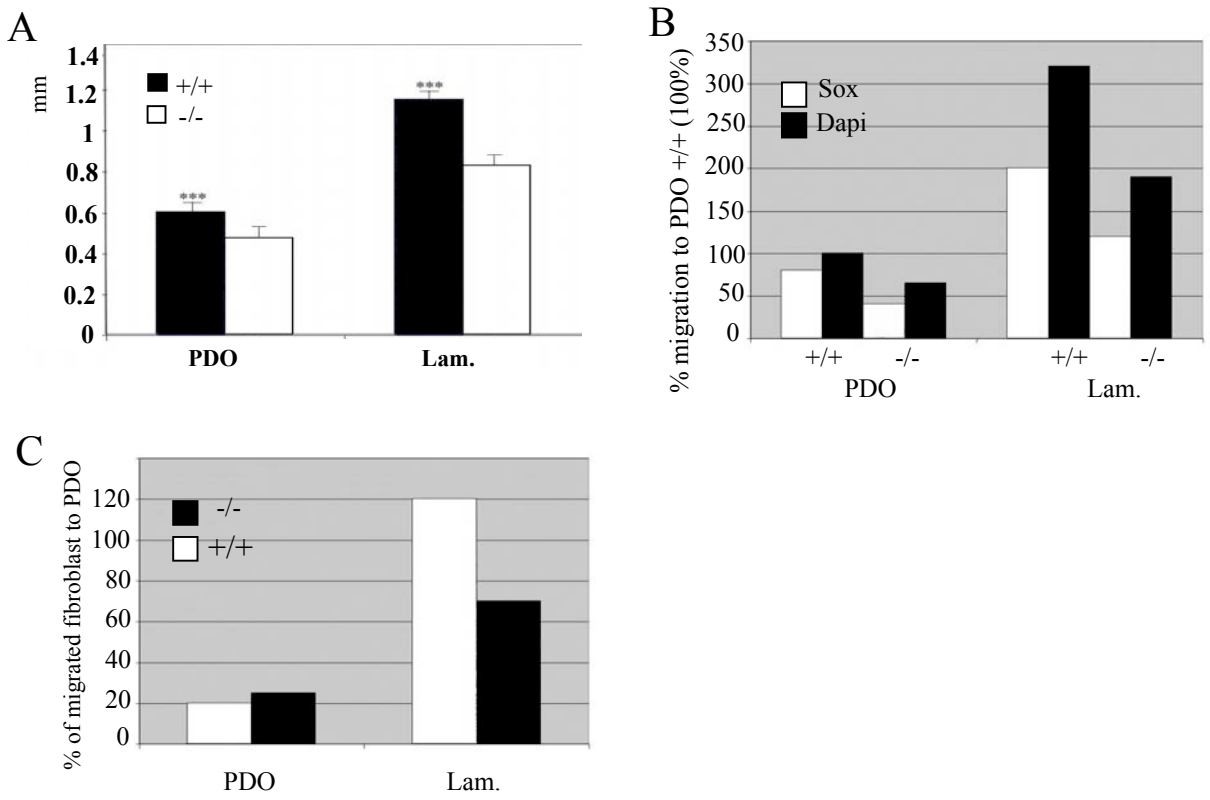


Fig. 4 Axon outgrowth and cells migration are decreased from matn2 -/- DRG. (A) Axon outgrowth from DRG sensory neurons. The results are presented as % in relation to the length of axons from wt DRG growing on PDO. Statistical analysis showed significant reduction (***$p<0.0001$) of axonal growth in mutant DRG cultures both on PDO and on laminin. **(B)** Cellular migration in DRG cultures from wt and matn2 deficient DRG. Migration of cells in wt cultures grown on PDO was normalized to 100% and compared to the other observed migration. The number of cells migrating in mutant DRG cultures was strongly decreased in comparison to the wt control in both cases, on PDO and on laminin. **(C)** Migration of fibroblasts from matrn2 deficient DRG. The results are presented as % of sox10 negative cells (fibroblasts) to the total number of migrated cells on PDO (100%). Note that the number of migrated fibroblasts (sox10 negative) on laminin was strongly decreased in mutant cultures compared to wt cultures, whereas on PDO migration was comparable. Lam., laminin; PDO, poly-D-ornitine. The values shown are the mean ± SD of measurements made on four ganglia for four separate cultures.

1.8 Matn4 is not upregulated in the PNS of matn2 knock out mice

Although I could observe clear influences of matrilin2 on cells of the PNS in vitro, no obvious phenotype had been seen in the matn-2 homozygous mutant mice. The lack of a phenotype in the developing PNS could be due to redundant functions of different matrilins. Since matrilin-4 (matn4) shows a similar expression pattern as matn2, it is a good candidate for compensation. As was previously shown by Northern and Western blot analyses the expression levels of matn4 mRNA and protein in different tissues (brain, eye and lung) of mutant mice were comparable to those of control animals (Mates et al., 2004). Since matrilin-4 (matn4) is also expressed in the PNS, I compared the expression levels of matn4 in DRG and Schwann cells of mouse embryos using immunofluorescence, in situ hybridisation and semi-quantitative PCR assays. Immunostaining of DRG cultures with polyclonal matn4 antibody revealed a very small upregulation of matn4 in matn2-/- mice (Fig. 5A). However, no variations in expression levels could be detected between different genotypes by semi-quantitative PCR (Fig. 5B) and S-35 in-situ hybridisation, and thus led to the conclusion that matn4 as in other tissues examined (Mates et al., 2004) is not upregulated in the PNS tissues of matn2 mutant embryos during development.

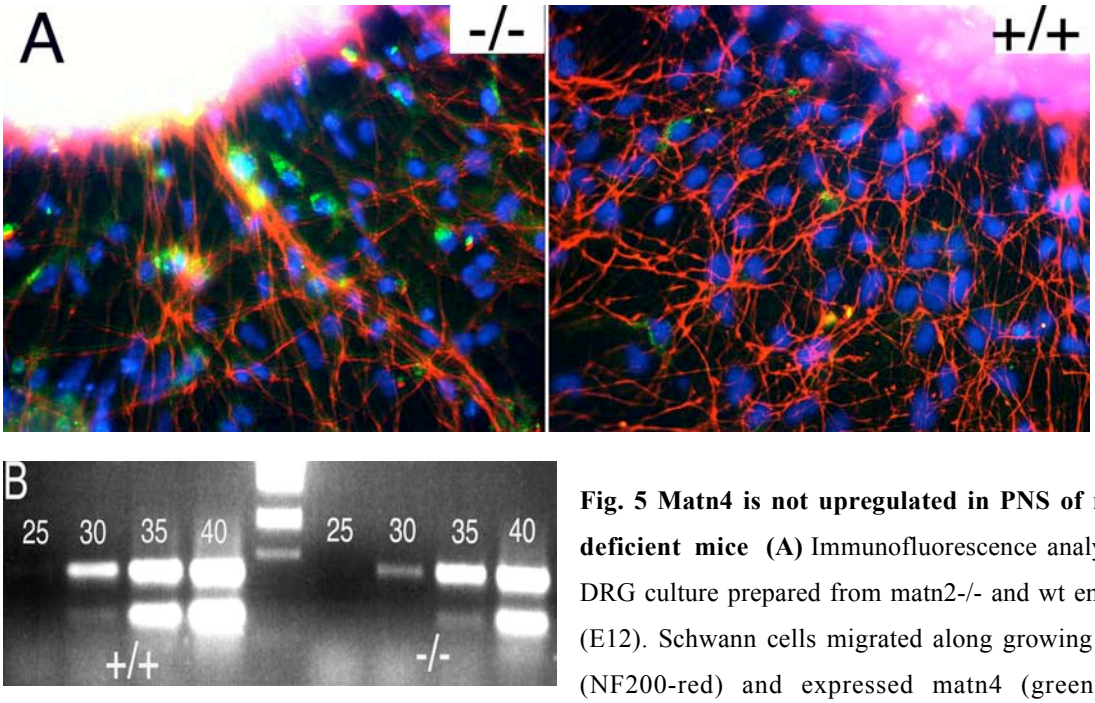


Fig. 5 Matn4 is not upregulated in PNS of matn2 deficient mice (A) Immunofluorescence analysis of DRG culture prepared from matn2-/- and wt embryos (E12). Schwann cells migrated along growing axons (NF200-red) and expressed matn4 (green) n=3 experiments. Note that only a small portion of migrating cells expressed detectable amounts of matn4 and that the intensity of the signals and the amount of expressing cells seemed to be elevated in matn2 mutants. **(B)** Semi-quantitative PCR on cDNA prepared from DRG of wt and matn2 mutant embryos. GAPDH (upper band) was used as an internal control. At comparable GAPDH levels (35 / 40 cycles) similar levels of matn4 specific

amplicons were present in wt compared to mutant (n=3 experiments) and as the in-situ-hybridisation experiments (data not shown) did not reveal any upregulation of matn4 in matn2 mutant tissue.

1.9 Matn2 is a preferred substrate for DRG sensory neuron axonal growth

The response of mouse DRG sensory neuron axons to matn2 coated surface was investigated in a classical stripe assay. The ability of matn2 to promote axonal growth was compared to different ECM molecules, such as laminin (which has been shown to be an excellent substrate for DRG axons), fibronectin and poly-l-lysine (PLL). This experiment was done in the presence of NGF to support neuronal survival and to elicit axon outgrowth. 2% Alexa488 conjugated BSA was used to block unspecific binding sites and simultaneously to allow visualisation of the stripes at the end of the experiment. As was shown before (Vielmetter et al., 1990) DRG sensory neuron axons prefer to grow on laminin in comparison to PLL. In my experiments this preference was confirmed (Fig. 6A) also demonstrating that the method successfully works in my hands. As a negative control the blocking solution (BSA) was used to coat the stripes, and surprisingly, axon preferred to grow on BSA compared to pure PLL (Fig. 6B).

When DRG axons had the choice to grow between matn2 and PLL, they always preferred matn2 (Fig.6C), although this preference was not as strong as between laminin and PLL. When DRG sensory neuron axons were confronted with alternating stripes of laminin and matn2 or fibronectin and matn2, axons did not show any preference between these different substrates (Fig.6D,E). When exposed to alternating stripes of laminin alone and a mixture of laminin and matn2, DRG sensory neuron axons preferred the mixture of matn2 and laminin over laminin alone (Fig. 6F). The observed preference was not an effect of BSA (Fig. 6G). Taken together, these results showed on the one hand that matn2 alone could promote axonal growth of sensory neurons and, on the other hand, that the mixture of laminin and matn2 was even more preferred than the universally accepted axonal growth promoter laminin alone.

1.10 Matn2 does not promote branching of DRG neurons

When plated on uniform, growth-permissive substrates like laminin or matn2, dissociated adult DRG neurons started to elaborate processes after being only a few hours in vitro. During 48 h, the neuritic tree kept growing and got more and more complex. The ability of young DRG axons (P3) to branch was compared on the following ECM substrates: matn2, laminin

and matn2+laminin for 12 h in culture. At this time point the neuritic tree was already complex, however still allowing a quantitative analysis (Fig. 7 A,B,C). Previous studies had shown that laminin is rather stimulating neurite extension than neurite branching. In the present study, I found that the effect of matn2 on branching is similar to that of laminin. On a matn2 (7c) coated surface DRG axons were shorter than on laminin (7A) (see above), but the number of branching points was comparable to laminin (maybe slightly higher). Interestingly, when a mixture of matn2 and laminin (7C) was used a tendency of increased axon lengths and numbers of branching points became apparent, although the effects were not highly significant (Fig. 7A,B,C,D). Mean numbers of branching point per longest neurite were quantified, and as shown on Fig. 7D, these means are not significantly different between different ECM substrates.

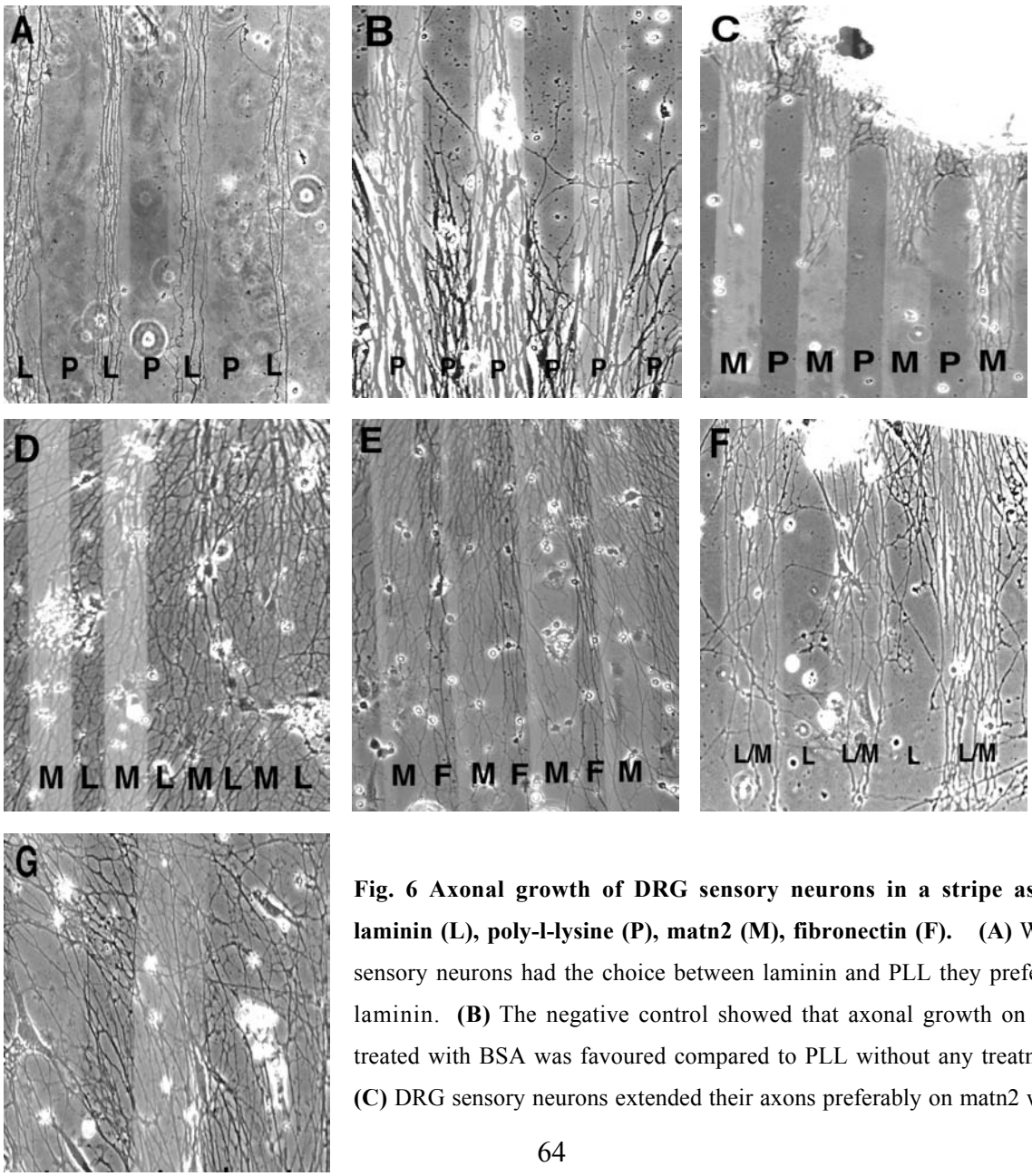


Fig. 6 Axonal growth of DRG sensory neurons in a stripe assay: laminin (L), poly-l-lysine (P), matn2 (M), fibronectin (F). (A) When sensory neurons had the choice between laminin and PLL they preferred laminin. **(B)** The negative control showed that axonal growth on PLL treated with BSA was favoured compared to PLL without any treatment. **(C)** DRG sensory neurons extended their axons preferably on matn2 when

offered the choice between matn2 and PLL. When stripes of matn2 and laminin **(D)** or matn2 and fibronectin **(E)**, were offered neurites did not show any preference. **(F)** When neurites had the choice between laminin and a mixture of laminin and matn2, they preferred the mixture. This effect was matn2 specific and could not be elicited by BSA **(G)**. N=3 separate experiments for each substrate combination. P., poly-l-lysine; M., matrilin; F., fibronectin; L., laminin; L/M., laminin+matrilin.

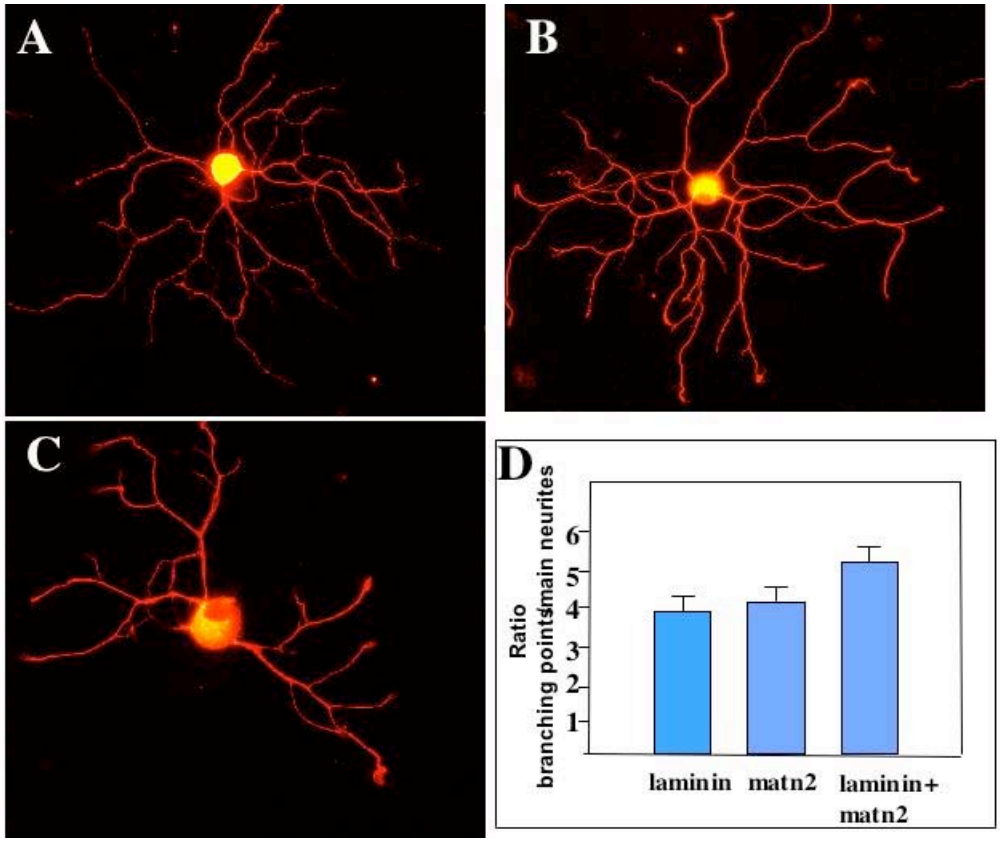


Fig. 7 Neurite branching on different ECM substrates. Morphology of neurites of dissociated DRG neurons (P3), which were grown for 12 h on laminin **(A),** a mixture of laminin and matn2 **(B)** or matn2 **(C),** visualised by tubulin immunostaining. **(D)** Quantitative analysis of the mean number of branching points per longest neurite (n=3 experiments).

1.11 Histological analysis of peripheral nerves in matn2 knock out mice

As was mentioned before, matn2 deficient mice show no obvious abnormalities during embryonic development or the adult (Mates et al., 2004). However, peripheral nerves of matn2-/- mice were not thoroughly investigated in this study. Therefore, in the present study, peripheral nerve morphology of matn2 mutant animals was analysed to rule out the possibility

that temporal defects might have been overlooked. Histological analysis of homozygous matn2-/- embryos on E12.5 demonstrated that peripheral nerves appear normal and show no delay or degeneration. Moreover, the number of Schwann-cell precursors present in the nerves of the matn2 mutants did not differ from the wildtype situation. Thus, peripheral nerves of matn2-/- embryos are normal in their histological appearance and are not delayed in their development (Fig. 8A,B).

Additionally, histological analysis of semithin sections from sciatic nerves of adult matn2-/- animals did not show any abnormalities in myelination. Peripheral axons were normally myelinated in mutant mice, and the number of myelinated fibers in mutant mice was similar in comparison with the control (Fig. 8C,D).

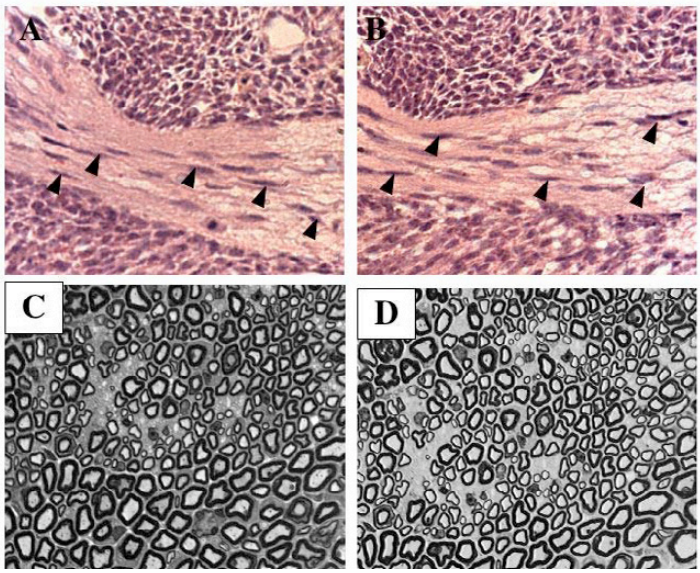


Fig. 8 Histological analysis of peripheral nerves of matn2-/- mice. Peripheral nerves of mutant **(A)** and wt embryos **(B)** at E12.5 were stained with hematoxylin / eosin. Arrows point towards Schwann-cell precursors in both mutant and wt. Histological analysis of sciatic nerve semithin sections from mutant **(C)** and control **(D)** animals show normal myelination. (n=2 experiments)

1.12 Peripheral nerve injury causes an upregulation of matn2

Expression of matn2 in the perineurium of mouse peripheral and spinal nerves had been superficially described in previous studies (Piecha et al., 1999). My experiments demonstrated significant effects of matn2 on axon outgrowth and Schwann cell migration *in-vitro*, however matn2 mutant animals show no obvious phenotypes. In order to investigate a potential function of matn2 in peripheral nerve regeneration I performed additional experiments. First matn2 immunoreactivity was investigated in the transected sciatic nerve after 5-14 days post operation (dpo) and the crushed femoral nerve (examined at 3 dpo) of adult mice. Immunohistochemical analysis revealed a strong upregulation of matn2 in damaged nerves in both cases (Fig. 9A). In addition, matn2 protein was detected in damaged nerves (7 dpo) by

Western blot analysis (Fig. 9B). The upregulation of matn2 in the distal part was slightly stronger than in the proximal part. Expression of matn2 was observed in the perineurium of lesioned and intact sciatic nerve as well as in endoneurium of damaged nerves (Fig. 9A). Interestingly, the perineurium of the femoral nerve was not immunoreactive for matn2 and remained negative even after lesion (Fig. 9A).

It is known that drastic changes occur in peripheral nerves after transection: The distal stump undergoes Wallerian degeneration, and in the proximal stump the breakdown of myelin sheaths for up to several nodes of Ranvier is initiated. These processes are accompanied by Schwann cell proliferation, macrophage invasion and downregulation of myelin-related genes. Expression of matn2 was investigated in the distal and proximal stumps of lesioned sciatic nerves during this period.

Adult mouse sciatic nerve showed neurofilament (NF)-positive axons near the injury site. Many more axons were identified by positive NF immunoreactivity in the proximal stump in comparison with distal stump (Fig. 10A,B). The injury site was strongly immunoreactive for matn2 in both the proximal and the distal stump. The observed immunoreactivity resembled in shape and localisation the typical spindle-shaped Schwann cells, which normally intimately enwrap regenerating axons (Fig. 10A,B). In order to clarify which celltypes were expressing matn2 and where exactly it is localised we performed some colabelling experiments. Schwann cells were identified in the proximal and distal stumps by immunoreactivity for p75 (low affinity NGF-receptor, a marker for Schwann cells), which is known to be upregulated in Schwann cells of the distal stump following injury of sciatic nerves (Taniuchi et al., 1986). Colocalisation of p75 and matn2 (Fig. 10C,D) confirmed the expression of matn2 by Schwann cells. It is very likely that matn2 is mainly deposited into the injured nerve by Schwann cells. The endoneurial (basal lamina) tubes were immunoreactive for nidogen-1 (which is a marker for the basement membrane) and matn2 (Fig. 10E,F), demonstrating that matn2 is also deposited into the basement membrane. In addition, immunoreactivity for MBP (myelin basic protein) showed that matn2 is not localised in myelin sheaths (Fig. 10G,H). Thus, it is likely that matn2 together with laminin and collagen IV is a structural component of basal lamina tubes but is not present in myelin sheaths.

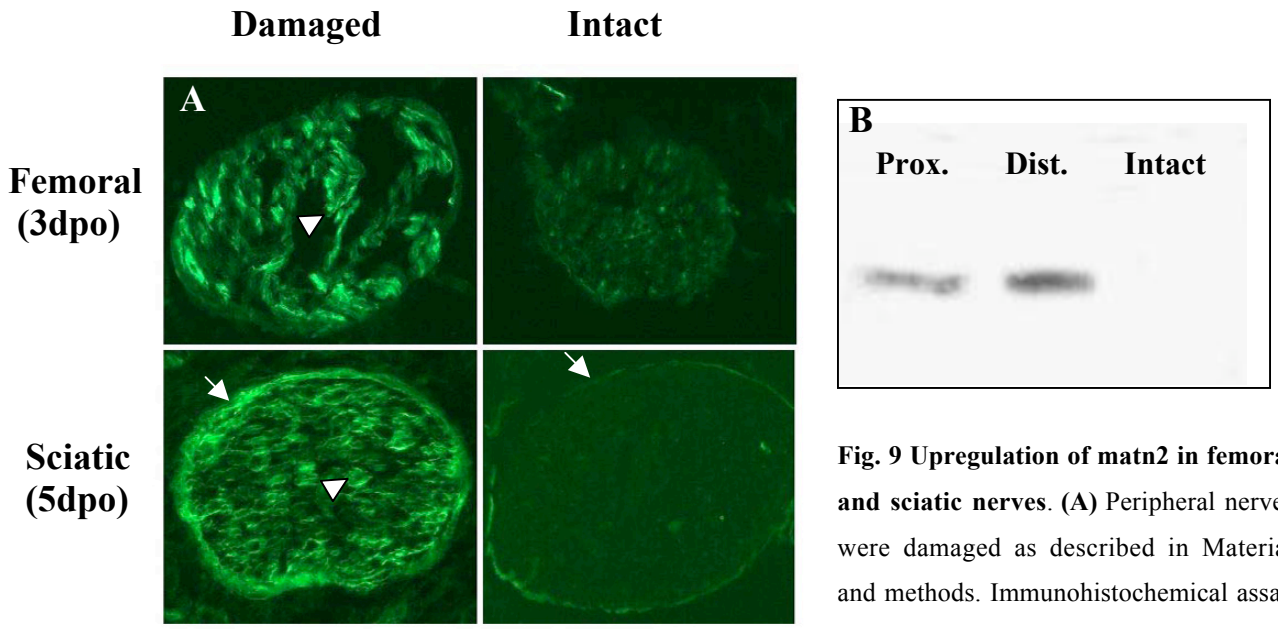
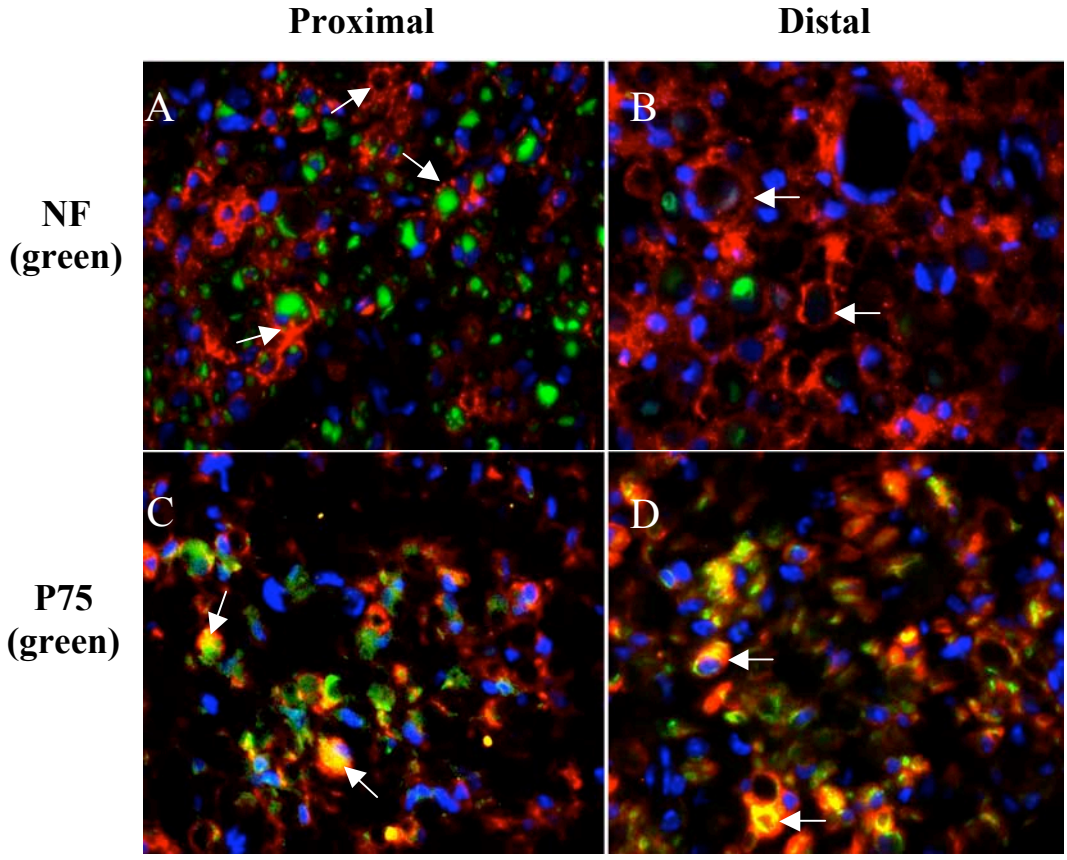


Fig. 9 Upregulation of matn2 in femoral and sciatic nerves. **(A)** Peripheral nerves were damaged as described in Material and methods. Immunohistochemical assay was performed close to the site of lesion after 3 days post operation (dpo) for femoral nerve, and 5 dpo for sciatic nerve. Arrows show localisation of matn2 in the perineurium of sciatic nerve, arrowhead shows localisation of matn2 in the endoneurium (n=4 experiments). **(B)** Upregulation of matn2 in damaged sciatic nerve (7 dpo) analysed by Western blotting. Intact nerve, as well as proximal and distal stumps of injured nerve were isolated and homogenised. Equal amounts of homogenates were subjected to SDS-PAGE. After transfer to nitrocellulose membrane and incubation with antibodies against matn2 the visualisation was achieved with the ECL kit (n=2).



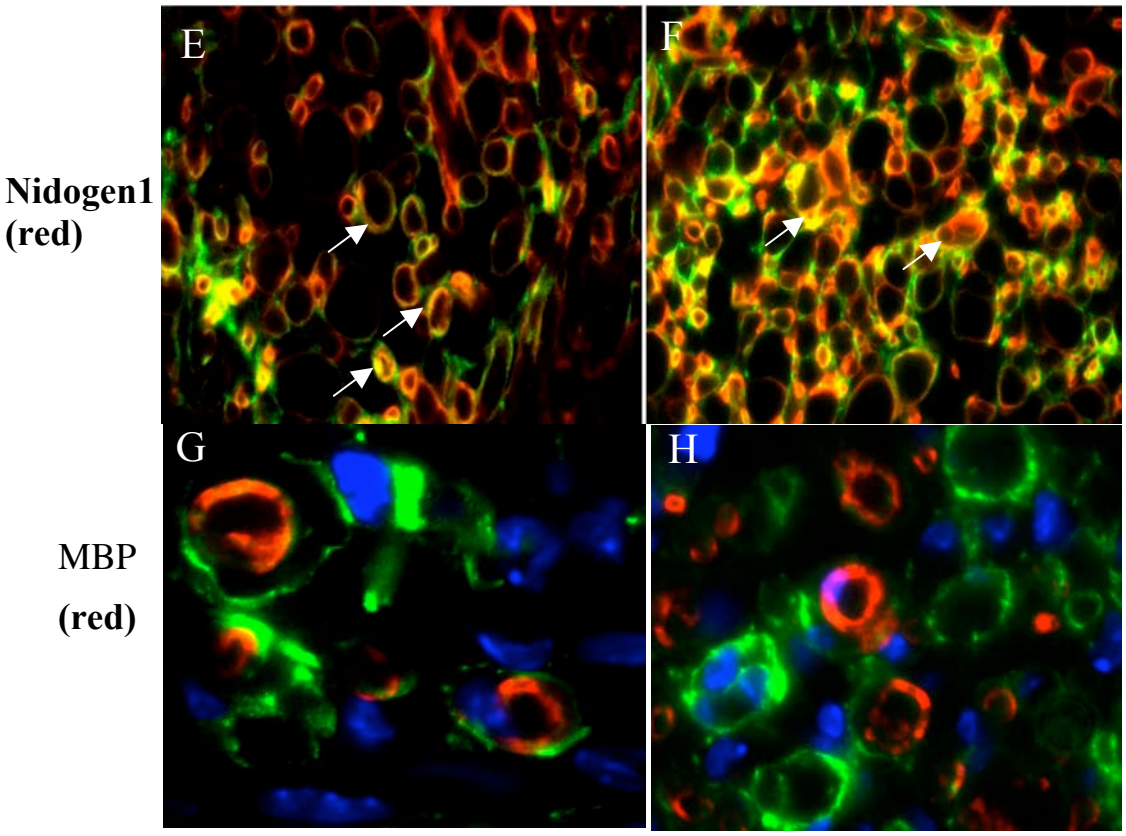


Fig. 10 Immunohistochemicial analysis of matn2 expression in adult mice after sciatic nerve injury 7dpo. **(A)** In the proximal stump NF-positive axons (green) are wrapped by basal lamina tubes, which are positive for matn2 (red, arrows). **(B)** In the distal stump number of NF positive axons was dramatically decreased due to Wallerian degeneration, but matn2 was still present in the endoneurial tubes. **(C)** Co-localisation of matn2 and the Schwann cell marker p75 (green) in the proximal and distal **(D)** stumps of injured nerve (arrows). Note that p75 was localized on the cell surface, as it is a membrane-bound receptor whereas matn2 (red) was wider distributed in the extracellular environment. **(E)** In the proximal and **(F)** distal stumps of injured sciatic nerve, matn2 (green) showed colocalisation with the basement membrane marker nidogen 1 (red, arrows). However matn2 (green) could not be detected in myelin sheaths of proximal **(G)** and distal **(H)** stumps of injured nerve, as visualised by co-immunostaining with myelin basic protein (MBP, red) antibodies. All stainings were performed on four independent animals after perfusion.

1.13 Matn4 is upregulated following injury of the peripheral nerves

Similar to matn2, the expression of matn4 was investigated in the transected sciatic nerve (examined 5 -14 dpo) and in the crushed femoral nerve (examined 3 dpo) of adult mice. Strong upregulation of matn4 could be observed in the endoneurium of damaged nerves (Fig.

11A-C), whereas no immunoreactivity for matn4 was found in neither the perineurium of the damaged nerve nor in intact nerves (Fig. 11D). Interestingly, expression of matn4 in the endoneurium was observed not only in the tubes of the endoneurium, but also in structures probably corresponding to injured axons (Fig. 11A-C). To further characterise the expression of matn4 in injured nerves I again performed a detailed immunohistochemical analysis of injured sciatic nerves (7 dpo and 14 dpo) employing colabelling of matn4 and NF. Surprisingly some of the NF-positive axons, that were present in the proximal stump of the sciatic nerve one week after transection, were also immunoreactive for matn4 (Fig. 12A,C), whereas in the distal stump (7 dpo) close to the lesion point no axonal expression of matn4 was observed (Fig. 12B). Again only few axons were still positive for NF in the distal stump (7 dpo) far from the lesion site, and a subfraction of those were immunoreactive for matn4 (Fig. 12D). Two weeks after transection, when the regenerative machinery had almost restored the proximal part of the injured nerve and newly generated axons had already penetrated into the distal stump, surviving axons (present in the proximal stumps) and newly generated thin axons (present in both, proximal and distal stumps) were no longer immunoreactive for matn4. Closer inspection further revealed that matn4 in the proximal stump was predominantly associated with the basal lamina, which wrapped innervated axons, whereas in the distal stump matn4 was more often localised in fragments of degenerated axons (Fig. 12I,K). Since the nerve was crushed, axonal expression of matn4 after injury is likely to occur independently of the neuronal cell body either via translation of axonal matn4 mRNA or via endocytosis of matn4 from the extracellular space. Therefore I monitored the expression of matn4 in the somata of DRG and spinal cord neurons 7 do. Consistently, immunoreactivity of matn4 was only detectable within the white matter of the spinal cord, where it most likely stems from astrocytes or oligodendrocytes. In the grey matter, where neuronal bodies are located, no expression of matn4 was found (Fig. 13A,B). Within the DRG only weak immunoreactivity was detectable and again seemed to be residing in glial cells rather than neurons. Regarding this expression pattern and the fact that there was also no difference between the lesioned (Fig. 13A,C) and the control (Fig. 13B,D) side I could conclude that no upregulation of matn4 occured in spinal cord and DRG neurons after lesion 7 dpo. This finding does not rule out that matn4 mRNA is present and translated in some axons after lesion nor that matn4 protein is translocated to damaged axons.

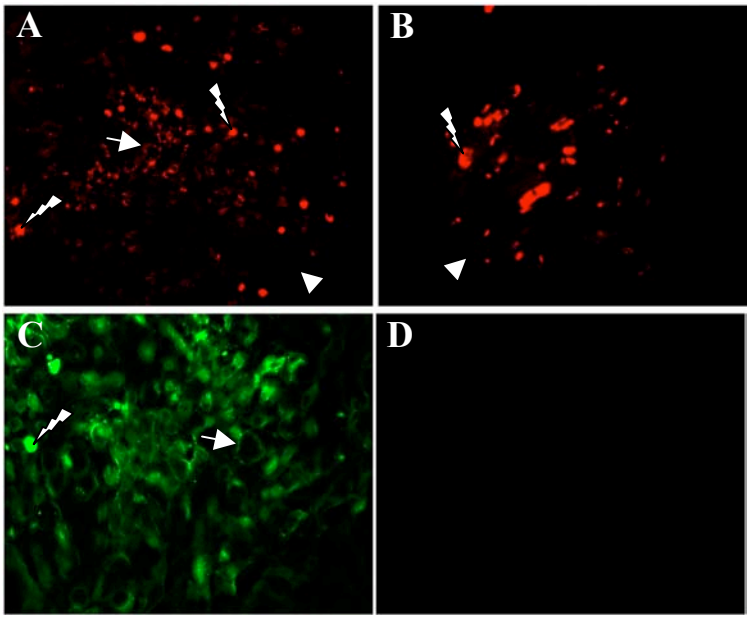


Fig.11 Upregulation of matn4 in femoral and sciatic nerves. (A,C) Sciatic and femoral nerves were damaged as described in Material and Methods. Immunohistochemical analysis was performed close to the site of lesion 7 dpo. Arrows show localisation of matn4 in endoneurium tube of the sciatic nerve, bolts show axonal localisation of matn2, arrowhead show absence of matn4 in perineurium. **(B)** Femoral nerve 3 dpo. Matn4 was associated with endoneurium of the femoral nerve and not with perineurium. **(D)** Intact sciatic nerve 5 dpo. No expression of matn4 was found. Immunostaining was performed on three independently perfused animals.

The expression of matn4 in the endoneurium of transected sciatic nerves was further characterised. Schwann cells, which were identified by their p75 expression, were also positive for matn4 (Fig. 14A,B) demonstrating that matn4 as matn2 expression is closely associated with Schwann cells. Immunoreactivity of matn4 was also found to be associated with the basal lamina as visualised by co-immunostaining with nidogen 1 in both proximal and distal stumps (Fig. 14C,D), although the signal in the basal lamina was clearly weaker than in axons. Evidently, matn4 is not a component of myelin sheaths since the area, which was immunoreactive for matn4 was not overlapping with MBP positive myelin sheaths in the proximal stump and in the distal stump, where axons partially degenerated and signal concentrated on periphery of axonal fascicles (Fig. 14E,F).

So in summary, matn4 and matn2 showed substantial overlap regarding their upregulation upon nerve injury and regarding their expression domains, however, matn4 expression could addionally be detected within axons.

Proximal **Distal**

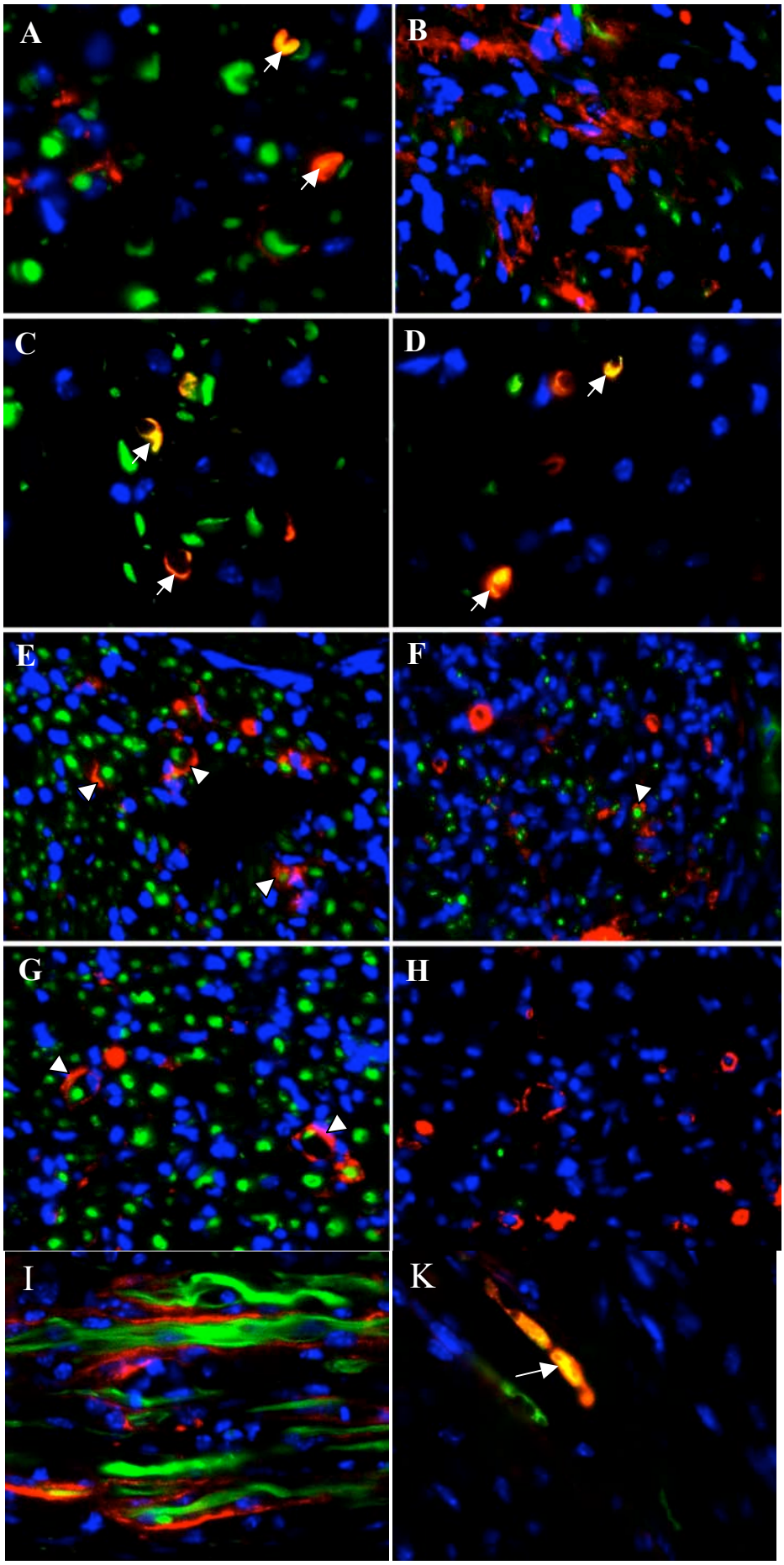


Fig. 12 Immunohistochemistry for matn4 (red) and neurofilament (green) of adult mice 7 dpo (A-D) and 14 dpo (E-H). (A) The proximal stump close to the lesion point. Some axons that survived were NF-positive and in addition positive for matn4 (arrows). **(B)** The distal stump close to the lesion site. The number of NF-positive axons was dramatically decreased due to Wallerian degeneration, and no co-localisation of matn4 and NF was found. **(C)** The proximal stump far from the lesion site. Some of the axons that survived were immunoreactive for NF and matn4 (arrows). **(D)** The distal stump far from the site of lesion. Here some axons that had survived could be detected that were immunoreactive for matn4 and NF (arrows). The proximal stump 14 dpo close **(E)** and far **(G)** from the lesion site. No newly generated axons were immunoreactive for matn4, but the endoneurial tubes were immonoreactive for matn4 and wrapped around NF-positive axons (arrowhead). The distal stump 14 dpo. Newly generated thin axons in the proximal stump **(F)** grew beyond the site of lesion but did not penetrate far into the distal stump yet **(H)**. No matn4 and NF co-localisation was observed. **(I)** Immunoreactivity of matn4 was found to be associated with the basal lamina, which wrapped axon bundles in the proximal stump 7 dpo. **(K)** In the distal stump immunoreactivity for matn4 could only be found associated with fragments of degenerated axons (arrow). All stainings were performed on four independently perfused animals.

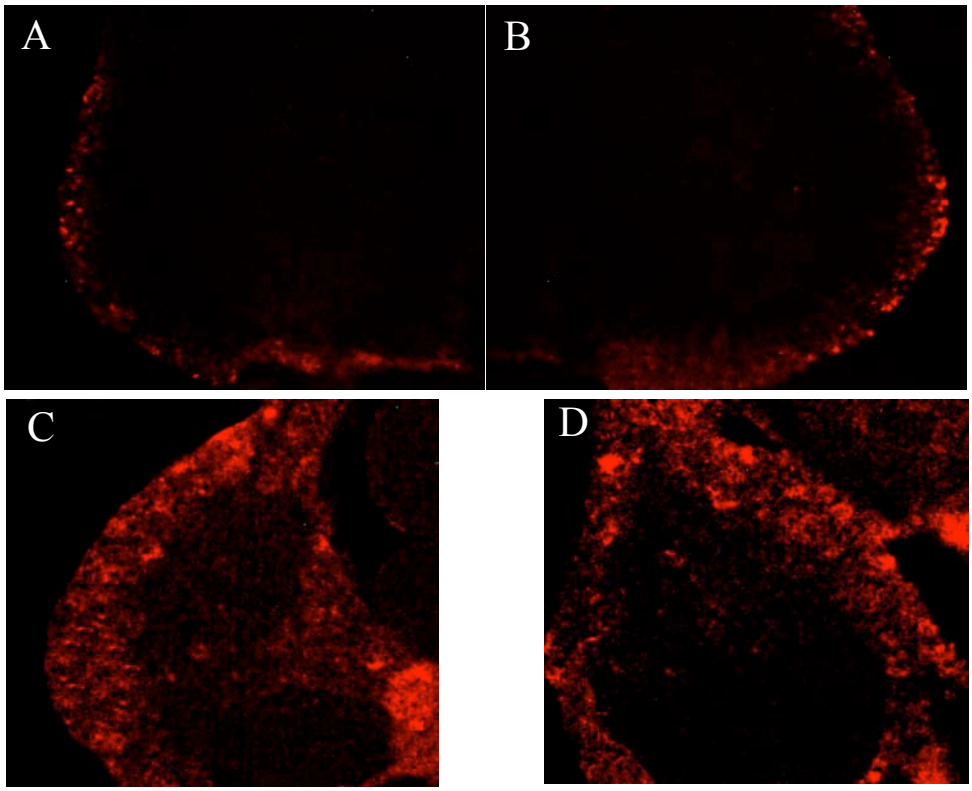


Fig. 13 Immunohistichemistry for matn4 in the spinal cord (A,B) and DRG (C,D) at 7 dpo. Panels **A,C** demonstrate damaged side, **B,D**-intact side. Matn4 immunoreactivity is present on glial cells of spinal cord and presumably in glial cells of DRG. No matn4 upregulation in damaged side was found. Immunostaining was performed on two independently perfused animals.

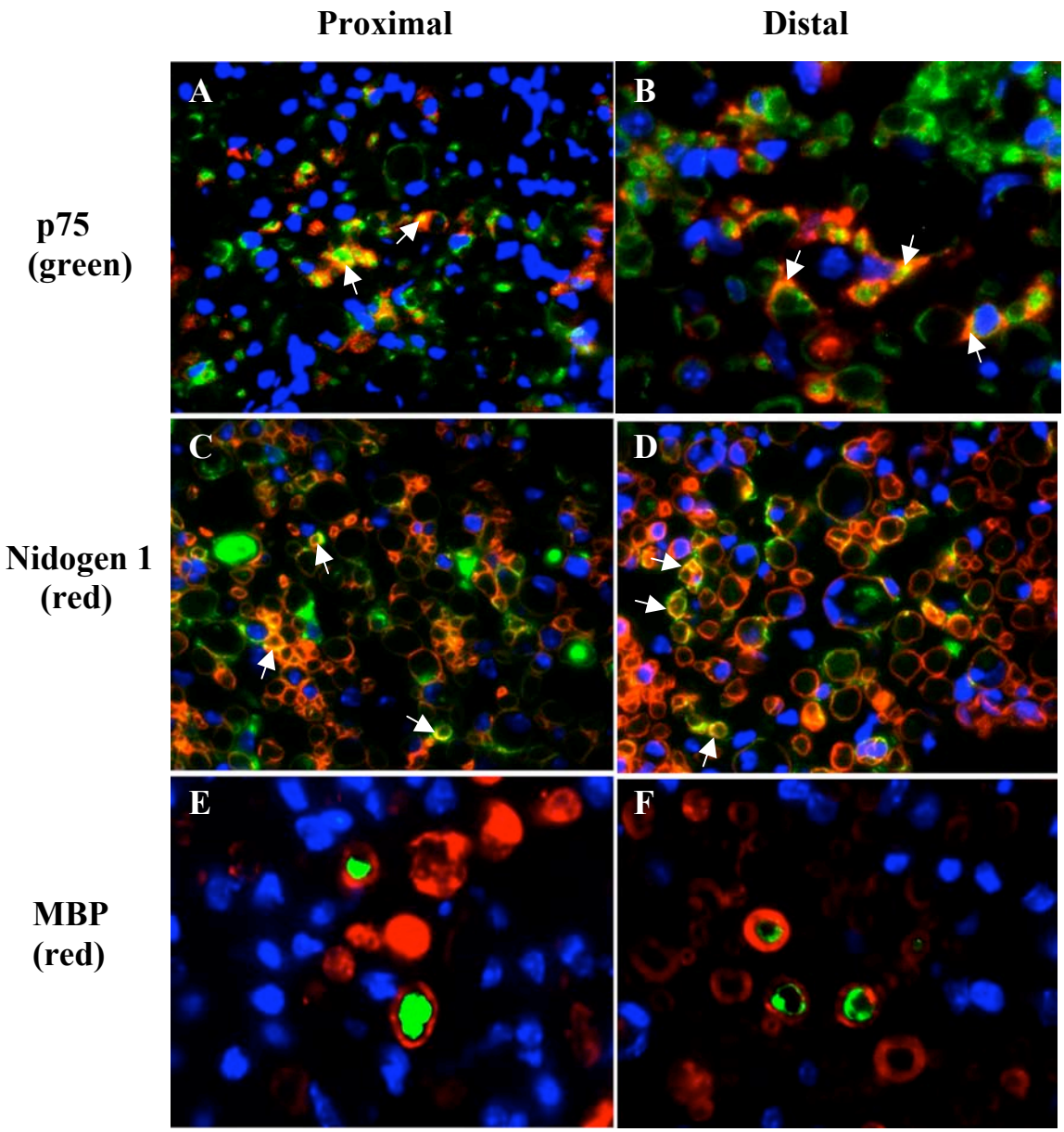


Fig. 14 Immunohistochemistry for matn4 and p75 (A,B), nidogen 1 (C,D) and MBP (E,F) after nerve transection of adult mice 7 dpo. (A) Co-localisation of matn4 (red) and the Schwann cell marker p75 (green) in proximal and distal **(B)** stumps of injured nerve (arrows). Note that p75 was localized on the cell surface, as it is a membrane-bound receptor whereas matn4 (red) was wider distributed in the extracellular environment. **(C)** Proximal and **(D)** distal stumps of injured sciatic nerve. Some of the matn4 immunoreactivity (green) co-localised with the basement membrane marker nidogen 1 (red, arrows). Matn4 (green) was not localised within the myelin sheaths of proximal **(E)** and distal **(F)** stumps of injured nerve, as visualised by co-immunostaining with antibodies against MBP. All immunostainings were performed on four independently perfused animals.

V.2 Matn2 as a component of central nervous system

2.1 Expression of matn2 in the mouse hippocampus

Expression of matn2 in the mouse CNS has not been investigated before. I therefore decided to analyse the distribution of matn2 in the adult mouse brain by in-situ hybridization and immunohistochemistry. Expression of matn2 was observed in different regions of the brain, such as the choroids plexus, cortex, olfactory bulb, thalamus and the hippocampus. A detailed analysis of the expression pattern in the hippocampus revealed high levels of matn2 expression in CA1, CA2 pyramidal cell layers as well as in subicullum, while in the dentate gyrus and CA3 region much lower expression levels of matn2 were observed (Fig. 14A). The specificity of the antibody was demonstrated by the comparison of the matn2 positive layers of pyramidal cells in CA1 and CA2 (Fig. 14B-F), and the absence of these signals in the matn2 deficient animals (Fig 14G,H). In addition, some perineuronal nets producing interneurons (visualised by Wisteria floribunda agglutinin (WFA) staining) were strongly immunoreactive for matn2 (Fig. 14B-F). Surprisingly, some WFA positive cells in matn2 deficient brains appeared also positive for matn2 (Fig. 14G,H), although the levels of expression were lower than in wt brains. This unexpected signal might be due to a crossreactivity of the matn2 specific antibodies to a not yet identified matn2-like molecule, which is present in perineuronal nets. However, there is almost no co-localisation of matn2 and the marker for perineuronal nets, suggesting that matn2 is not an abundant component of these perineuronal nets. In the CA3 layer (Fig. 14I,K), the dentate gyrus (not shown) and the subicullum (Fig. 14L,M) of wt animals matn2 specific signals where detected, whereas these signals were absent in the corresponding areas of matn2 mutant brains (Fig. 14N,O).

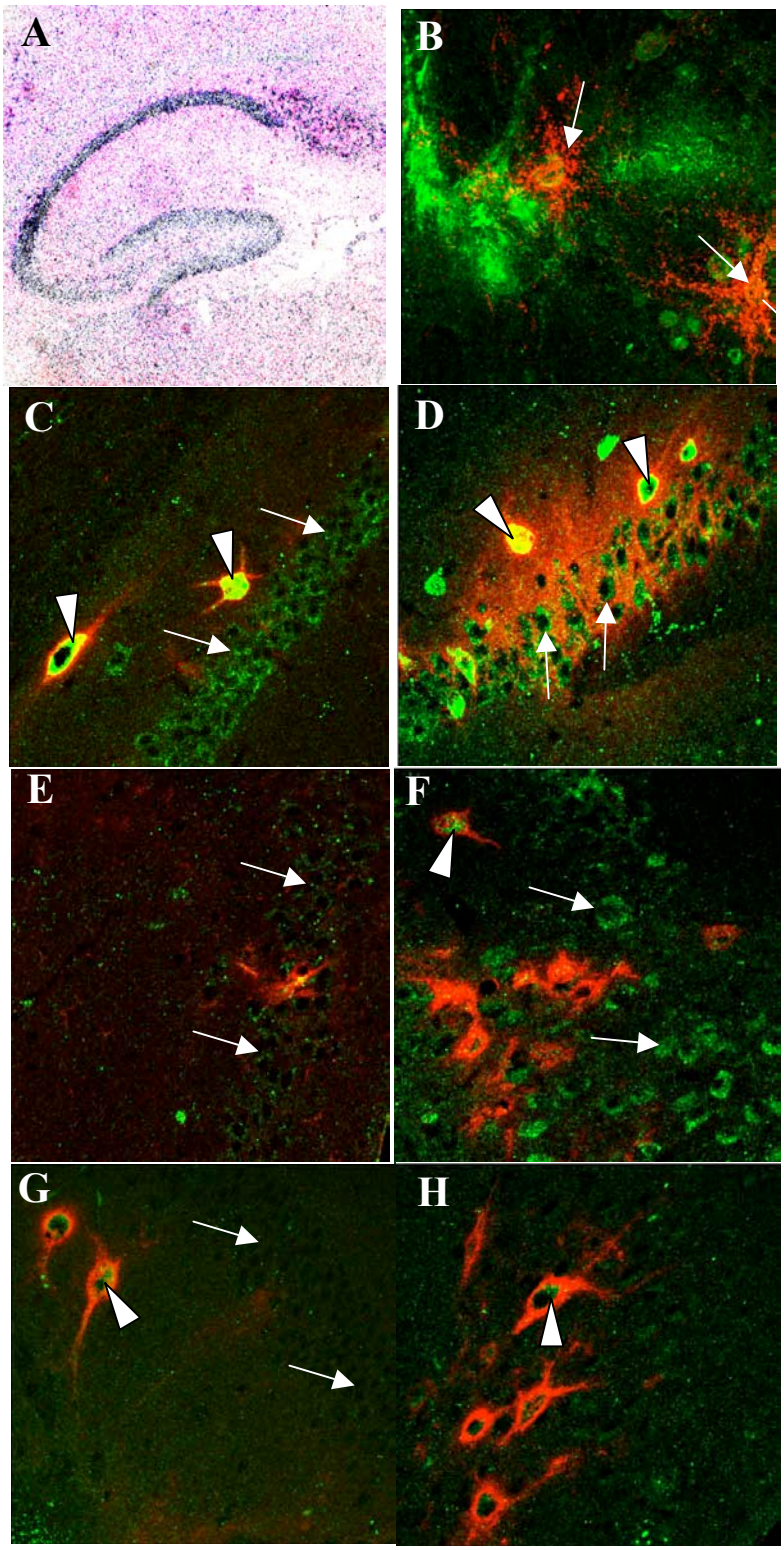


Fig. 15 Distribution of matn2 in mouse hippocampus. (A) S^{35}-in situ hybridization with matn2 specific probe showed strong localisation of matn2 mRNA in pyramidal cell layers of hippocampus and in the subicullum (n=3 experiments). **(B-H)** Co-immunostaining with polyclonal antibody against matn2 (green) and WFA (red). **(B)** Expression matn2 in culture of hippocampal neurons, matn2 was not localised in nets surrounded neurons (arrows). Expression of matn2 (arrows) in CA1 **(C)**, CA2 **(D)** and CA3 **(E)** pyramidal cell layer, as well as in the subicullum **(F)** of wt mice, whereas in CA1 **(G)** and subicullum **(H)** of matn2-deficient brains no matn2 specific signals were observed. Co-immunostaining of different regions of hippocampus with a marker for perineuronal

nets WFA (red) did not reveal substantial amounts of matn2 in these nets, although some net producing cells were matn2 positive (arrowheads), as well as in control matn2 deficient brain **(G,H).** All immunostainings were performed on three independently perfused animals.

2.2 Distribution of different populations of interneurons is normal in matn2 mutant mice

Since matn2 is widely expressed in all hippocampal layers, it was tempting to speculate that absence of this ECM component in matn2 mutants might have an effect on axonal projections and or the distribution of different types of interneurons, especially in CA1 and CA2 pyramidal cell layers. To investigate this I performed immunofluorescence assays using different markers for interneurons such as calbindin (CAB) and parvalbumin (PV). The distribution of immunoreactive somata as well as the staining intensity of different neuronal compartments were examined and compared in the wt and matn2 deficient mouse hippocampus.

The distribution of PV-containing interneurons was analysed in CA1 and CA2 pyramidal layers of the hippocampus of wt and matn2-deficient animals. No obvious differences in these regions regarding the distribution of somata, dendrites, and axon terminals could be detected using PV-specific antisera. PV-positive cell body localized in the stratum pyramidale, and dendrites of PV-positive cells span all layers and largely oriented in radial directions (Fig. 16A,B).

Additionally, a CaBP-immunostaining was performed. It has been found previously, that the granule cells of the dentate gyrus, superficial cells of the CA1 region and axons of granule cells, the so called mossy fibers, are CaBP-immunoreactive. Again a similar distribution of CaBP-positive neurons was observed in sections of the dentate gyrus from matn2-mutant and wt animals. CaBP-immunoreactive neurons were located in the stratum granulosum and the hilus (where immunoreactive cells were rare) and less frequently in the stratium moleculare (Fig. 16C,D). Although matn2 expression in these regions of the adult brain is very prominent, I was unable to detect any obvious abnormalities in theses analyses.

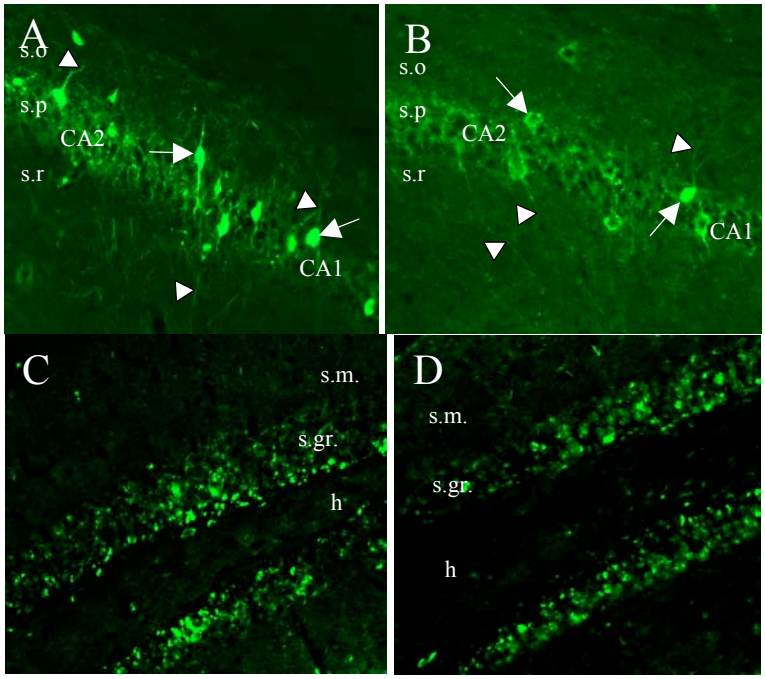


Fig. 16 Distribution of PV-immunoractive and CaBP-immunoreactive neurons in the hippocampus of wt and matn2 deficient mice. Both types of neurons have similar distribution in wt **(A,C)** and mutant mice **(B,D)**. **(A,B)** Similar distribution of PV-positive interneurons (arrows) in CA1 and CA2 pyramidal cell layers of matn2 deficient **(B)** and wt **(A)** brain. Arrowheads show dendritic projections that are largely extending in radial directions. **(C,D)** Similar distribution of CaBP-containining neurons in dentate gurus of wt **(C)** and mutant brain **(D)**. p., stratum pyramidalle; s.r., stratum radiatum; s.o., stratum oriens; h., hilus; s.m., stratum moleculare; s.gr. stratum granulosum. All immunostainings were performed on four independently perfused animals.

2.3 Matn2 promotes axon outgrowth of hippocampal neurons, but does not stimulate neuritogenesis

As I have demonstrated that matn2 stimulates axon outgrowth of DRG sensory neurons I wanted to test whether matn2 exerts similar effects on the growth of hippocampal neurons. Therfore, the neuritelengths in cultures of hippocampal neurons that had been plated for 24 h on different coating substrates (matn2, laminin, PLL or a mixture of laminin and matn2) was determined. The neuritelength was significantly increased when the neurons were grown on matn2 compared to PLL. However, when compared to neurons grown on laminin they were significantly shorter (Fig. 17A). Interestingly, the neuritelength on matn2 and laminin together was comparable to laminin alone. In parallel, the number of neurites per neuron was calculated. Compared to PLL there was no effect of matn2 on neuritogenesis, although compared to laminin, a known suppressor of neuritogenesis in cultures of postnatal neurons older than P10, an approximately twofold increase could be observed (Fig. 17B). Interestingly, when a mixture of matn2 and laminin was used an increase in the numbers of neurites was observed compared to laminin alone, although the length of neurites was similar for these two substrates (Fig. 17A,B).

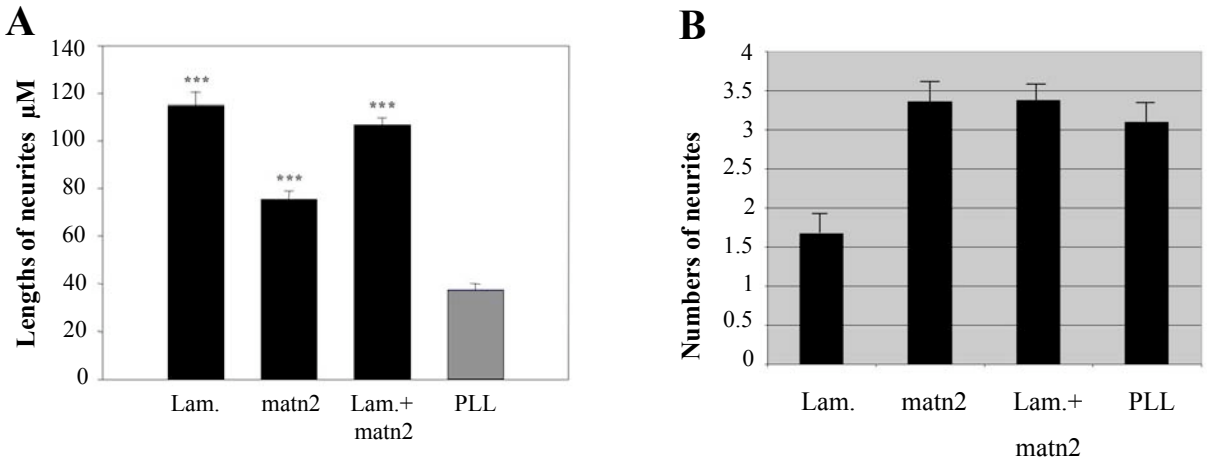


Fig. 17 Effects of different ECM substrates on neurite outgrowth and neuritogenesis of hippocampal neurons. (A) Neurite outgrowth assay. Statistical analysis showed significant increase (***$p<0.0001$) of neurite outgrowth on all ECM substrates compared to PLL. **(B)** Numbers of neurites was increased on matn2 compared to laminin (Lam.), but did not differ from PLL (n=3 experiments).

VI. Discussion

The aim of the present dissertation was to analyse the expression pattern of the ECM protein matn2 in the nervous system and to characterize its biochemical properties especially in the context of PNS and CNS development and regeneration. In the first part I focussed my investigation upon the distribution and functions of matn2 in PNS. Using different in vitro approaches as well as matn2 deficient animal model, I could provide strong evidence for potential roles of matn2 during PNS development and regeneration. I demonstrated that matn2 promotes cell adhesion, the migration of different types of Schwann cells and axon outgrowth of DRG sensory neurons. As severe abnormalities in matn2 deficient mice could not be identified (Mates et al., 2004), our biochemical data, the expression pattern and the strong upregulation of matn2 and matn4 after peripheral nerve transection suggest that matn2 might serve important functions during peripheral nerve regeneration.
In a second set of experiments, I have characterised the expression pattern of matn2 in the murine CNS. Matn2 specific transcripts and protein were found widely distributed in the hippocampus. With the help of coimmunolabeling I could show that it is not a component of perineuronal nets and its absence does not cause obvious changes in different neuronal populations of the hippocampus. However, like on peripheral sensory neurons matn2 protein showed a stimulation of neurite outgrowth of hippocampal neurons. These biochemical data point towards a potential role of matn2 during development of CNS although mutant mice do not show obvious abnomalies (Mates et al., 2004).

1 Distribution and functions of matrilins in the PNS

1.1 Expression of matn2 in the PNS

As was described above, Schwann cells are the main producer of ECM in the PNS. Therefore, erbB3 deficient mice, which lack these cells along axonal projections, provide a good system to screen for genes expressed and potentially be of functional importance in theses cells. A DNA-microarray analysis revealed that matn2 is a differentially expressed gene in wt DRG cultures when compared to erbB3-/- cultures (E12.5) *(Michaela Miehe, Dissertation, Universität Hamburg*). This result prompted us to investigate possible roles of matn2 in developing peripheral glia. First the data of the DNA-microarray analysis had to be confirmed

by different approaches, such as S^{35} in situ hybridization and semi-quantitative PCR. In erbB3 mutant embryos the expression of matn2 was absent in DRG and along peripheral nerves, whereas this pattern was present in wt embryos of the same developmental stage. Additionally, a semi-quantitative PCR revealed upregulation of matn2 in wt versus erbB3 mutant cultures. These findings argued for a Schwann cells specific expression of matn2 in the PNS. Supplementary confirmation of this idea was obtained when the expression of matn2 was investigated in different cell types by RT-PCR. Interestingly, matn2 was found to be expressed by both non-myelinating, immature (embryonic Schwann cells) and mature, myelinating Schwann cells (S16, rat Schwann cell line), whereas neural crest stem cells (NCSCs), which are the precursors of Schwann cells showed no expression. NCSCs, that migrate early in development, express different groups of ECM molecules and receptors and during their migration encounter different ECM molecules, providing important guidance cues (Perris and Perissinotto, 2000). My analysis has provided no evidence that matn2 is important for this developmental process as it is not expressed in NCSCs nor along their migratory routes. However, the expression and strong upregulation in migratory Schwann cells during later stages of development (E12-E18) point towards a potential biological role associated with Schwann cell differentiation or migration.

1.2 Functions of matn2 in the PNS

The development and later in the adult the functionality of the PNS is strongly influenced by the ECM that is largely produced by Schwann cells. Different ECM molecules are implicated in axon growth, cell migration, axon branching and myelination (Chernousov and Carey, 2000). As matn2 is expressed in the PNS and can serve as an adaptor protein in the ECM it seems to be a good candidate for mediating some of these functions or to modulate the activity of other involved ECM molecules. Using purified recombinant matn2 protein, as a substrate for growing DRG cultures, we revealed that matn2 promotes growth of sensory axons, cellular migration and adhesion.

1.2.1 Matn2 promotes cell migration and adhesion

Normally ECM molecules do influence the adhesion of the cells, when they are present on the surface (Milner et al., 1997; Chernousov et al., 2001). Using a simple adhesion assay we

demonstrated, that matn2, when applied to a surface as a coating substrate, promotes Schwann cell adhesion. Additionally, using different assays, I could show that matn2 does not only promote adhesion but is also very efficiently promoting cellular migration. In the agarose drop assay I investigated the migration behaviour of the rat Schwann cell line S16 on different ECM surfaces. Interestingly matn2 promoted the migration of these cells more efficiently than the established migration promoters laminin and fibronectin. In consistence with this result I found that cellular migration from DRG explant cultures (with and without inhibition of axon growth) was enhanced on a matn2 coated surface. Again matn2 was the most permissive substrate for Schwann cell migration when compared to laminin or fibronectin. Thus, matn2 can similarly influence the behaviour of different Schwann cell types, as the embryonic Schwann cells in the DRG cultures and the S16 cells in the agarose drop assay represent non-myelinating and myelinating Schwann cells, respectively. In addition a 20% increase in fibroblast migration was observed, when matn2 was used as a coating substrate in comparison with laminin, demonstrating that matn2 can also affect other celltypes apart from Schwann cells. Interestingly, this stimulation of fibroblast migration did not depend on axon extension, because similar numbers of migrating fibroblast were observed in experiments with or without axon growth. In contrast, Schwann cell migration showed strong differences in experiments without compared to experiments with inhibition of axon growth. When Schwann cells were allowed to migrate along growing axons, only small differences in the number of migrating cells between different coating substrates could be detected. On the other hand Schwann cell migration varied strongly on different surfaces when axon extension was inhibited and in these experiments matn2 was the strongest promoter. One possible explanation for this difference could reside in the fact that axons themselves provide trophic factors and guidance cues, such as neuregulin that is secreted or presented by axons (Meyer et al., 1997). Thus, axons can strongly influence proliferation and migration of Schwann cells and thereby mask the effects exerted by the coated material. It is also conceivable that direct Schwann cell-axon-interaction not only competes with but also actively interferes with Schwann cell-ECM interactions.

Interestingly, the mixture of matn2 and laminin had the strongest permissive effect for Schwann cell migration, whereas the distances of Schwann cell migration were bigger on matn2 alone. In a previous study interactions between matn2 and laminin have been described (Piecha et al., 2002). These interactions could block some active binding sites on matn2 and or laminin that are important for migration. As migration is always a balance between adhesion and disengagement to allow movement it is also possible that the mixture of laminin

and matn2 is important for the onset of the migration process while matn2 alone might favour long distance migration. These biochemical data clearly prove the potential of matn2 to promote and influence Schwann cell migration *in-vitro* and suggested that this molecule might also be involved during the corresponding *in-vivo* processes.

1.2.2 Matn2 promotes axon growth but does not affect branching

Our *in-vitro* findings demonstrated that matn2 is a permissive substrate for axonal growth of DRG sensory neurons. Matn2, when used as a uniform coating substrate, exerted stronger stimulation of axonal growth than PDO but weaker stimulation than laminin. When DRG axons were confronted with stripes of PLL and matn2 in a classical stripe assay, they preferred to grow on matn2. Given the choice between matn2 and laminin or between matn2 and fibronectin I could not detect any preference. This is somewhat surprising, since laminin has been shown to be the most permissive substrate for axonal growth (Vielmetter et al., 1990; Nguyen-Ba-Charvet et al., 2001; Moreau-Fauvarque et al., 2003). Additionally, using this assay I found that DRG sensory neuron axons clearly prefer to grow on stripes containing a mixture of laminin and matn2 compared to stripes coated with laminin alone. These data obtained in the stripe assay are in good agreement with the data I obtained in the simple axon outgrowth assay, where similarly the mixture of laminin and matn2 seemed most permissive for axonal growth.

In search of additional functions of matn2 I investigated its influence on branching of DRG sensory neuron axons. The mean of branching points per main neurite grown on matn2 was similar to those grown on laminin or grown on mixture of these two proteins. The results demonstrated that like laminin, that was known to promote axonal extension but in cultures of young neurons only moderately enhance branching, matn2 too, does not intensely stimulate branching of DRG neurons.

1.2.3 Cell migration and axon growth are decreased in matn2 deficient mice

Since matn2 deficient mice had been generated (Mates et al., 2004) we intended to confirm our *in-vitro* findings using DRG cultures prepared from matn2 mutant mice. In these experiments the time of culture was prolonged to 72 h in comparison with the experiments where matn2 had been used as a coating substrate. The ratio for this modification was to allow the accumulation of endogenous matn2, secreted from Schwann cell precursors. Thus, in these

experiments we did not want to investigate the effects of exogenous matn2, applied to the surface prior to the culture, but to analyse effects due to the absence of matn2 during the process of axon growth and Schwann cell migration. It is noteworthy that the matn2 deficient animals are phenotypically normal (Mates et al., 2004), however using our DRG culture assay we found a clear decrease in axon outgrowth and cellular migration in matn2-/- DRG compared to wt cultures. The experiments were performed on two different surfaces: on PDO and on laminin. Axonal length was significantly decreased on both surfaces, which confirms our previous data and allows us to conclude that matn2 has an impact on axonal growth *in-vitro*. The cell migration assay using DRG cultures was performed without the inhibition of axon growth, and I could observe a strong decrease in Schwann cell migration on both surfaces used in this assay. It seems likely that the observed decrease in cellular migration is not only due to the reduced axonal growth as the effect on migration is stronger than the effect on axon growth. In support for this interpretation in experiments using recombinant matn2 without the inhibition of axonal growth no effect on Schwann cell migration could be detected. Thus, if the decrease in migration would be solely due to the axonal defect the decrease should be similar to the observed reduction in axonal length. So the observed differences in this assay must be attributed to endogenous expression of matn2 by Schwann cells. These results suggest that the endogenous matn2 secreted by the Schwann cells migrating in our cultures either deposited in the ECM or membrane associated somehow positively influence the process of migration.

The exact mechanisms by which matn2 affects neurite outgrowth and Schwann cell migration are not known. There are several possible target proteins mediating these matn2 dependent effects. The integrins, transmembrane glycoproteins that mediate interactions between cytoplasm and the extracellular environment, constitute one potential candidate family. Two different classes of integrines are expressed in the PNS: five β_1 integrins, $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_1\beta_3$, $\alpha_6\beta_1$ and $\alpha_150\beta_1$; and two α_v integrins, $\alpha_v\beta_3$ and $\alpha_v\beta_8$ (Milner et al., 1997). In previous studies, several ECM ligands for integrins such as collagens, laminin, fibronectin, tenascins and vitronectin were revealed (Previtali et al., 2001). Some of them such as laminin or fibronectin affect axonal velocity and direct axon growth (Kuhn et al., 1995; Luckenbill-Edds et al., 1995). Laminin can interact with $\alpha_1\beta_1$ and $\alpha_1\beta_3$ expressed on the growth cones and mediates neurite outgrowth in cultured mouse DRG neurons (Tomaselli et al., 1993; DeFreitas et al., 1995). Additionally, the influence of ECM-integrin-interactions on Schwann cell migration was investigated in several studies, showing that laminin and fibronectin promote Schwann cell migration. The migration on laminin was shown to be dependent on β_1-

integrin, whereas the migration on fibronectin was α_v-integrin dependent (Milner et al., 1997). Among the integrins expressed in Schwann cells, $\alpha_v\beta_3$ and $\alpha_v\beta_1$ seem to be the most promising candidates to interact with matn2, as the WFA subunit (which is also present in matrilins) is a well known ligand for these integrins (Previtali et al., 2001). Several groups, including our own, tried to confirm direct matn2 integrin interactions using different approaches. However, so far no clear interactions have been found (data unpublished), making it unlikely that the integrins are direct receptors for matrilins.

This immunoglobulin superfamily includes a lot of members, among them L1, Ng-CAM, NILE, N-CAM and N-cadherin all of which are expressed in the PNS. Many of these molecules are involved in adhesion between axon and axon, axon and Schwann cell, and thereby regulate axonal growth, adhesion and fasciculation. For example, *in-vitro* experiments that use antibodies against L1 and N-CAM provide strong evidence for such a role. L1 antibodies inhibit at least 80% of neurite outgrowth on a Schwann cell surface in co-cultures of Schwann cells and DRG (Bixby et al., 1988; Kleitman et al., 1988). Some ECM molecules, such as tenascins or CS-PGs can modulate homophilic and heterophilic binding between different CAM family members, thereby influencing neurite formation, extension and guidance during neuronal development (Salmivirta et al., 1991; Milev et al., 1994; Norenberg et al., 1995; Milev et al., 1997; Milev et al., 1998; Volkmer et al., 1998). Since CAMs are mostly responsible for cell-cell interactions and not for cell-ECM contacts, it seems not likely that matn2 might serve as a ligand for CAMs. Nevertheless this possibility should not be excluded and potential interaction should be in the focus of further investigations.

The last class of molecules that has been suggested to function as ECM receptors are cell surface proteoglycans. Several proteoglycans have been identified on the surface of Schwann cells, such as glypican-1, syndecan-3, dystroglycan. Glypican-1 isolated from Schwann cells binds to laminin *in-vitro* and is localized in the outer Schwann cell membrane that contacts the laminin-rich basal lamina (Carey et al., 1990; Carey and Stahl, 1990). Syndecan-3 does not bind laminin, but therfore binds to p200, the Schwann cell specific collagen subunit (Chernousov and Carey, 1993; Chernousov et al., 1996). α-Dystroglycan has been identified in peripheral nerves and serves as a receptor for agrin and laminin-2 (Yamada et al., 1994b; Yamada et al., 1996). Another membrane associated protein NG2 has been described to be expressed by fibroblast present in peripheral nerves (Zhang et al., 2001). In another publication, NG2 was reported to be localized on immature Schwann cells and in a subpopulation of non-myelinating Schwann cells in mature peripheral nerves, and thus closely resembles the matn2 localisation. So NG2 might be a potential interactor for matn2 in

mediating fibroblasts and immature Schwann cell migration (Schneider et al., 2001). Additionally, NG2 and neurocan-C are present on surfaces of neurons and on glial cells in the CNS, where they are important for PNN formation and in mediating ECM-cell contacts (Butt et al., 1999; Oohira et al., 2000). Two further observations could support the theory that matrilins may mediate their functions through interactions with cell surface proteglycans. First, each matrilin has several glycosylation sites, which may be targets for GAG chains of glycoproteins. Second, it has been shown recently, that matn1 and biglycan or decorin connect collagen VI microfibrils to both collagen II and aggrecan (Wiberg et al., 2003). Thus, matn1 (and probably other matns) forms a linkage between collagen VI microfibrils and other macromolecular components in the ECM of cartilage. All of these molecules: byglycan, decorin and aggrecan are also present in the nervous system, especially in the CNS (see introduction). Thus, even if direct interactions between matn2 and cell surface molecules do not exist, matn2 could mediate its functions by stabilisation of the ECM in the CNS through interaction with different types of proteoglycans, and in the PNS through interaction with laminin and fibronectin, the two main components of the basal lamina and of collagen fibrils, respectively.

1.3 Histological analysis of peripheral nerves in matn2 knock out mice

Although matn2 knock out mice turned out to be phenotypically normal during embryonic development and in the adult (Mates et al., 2004), our in vitro findings, prompted us to carefully investigate the PNS of these mice. We expected to find some subtle abnormalities that might have escaped the general analysis. For instance, the number of non-myelinating Schwann cells, migrating along growing axons during development might be transiently decreased in mutant embryos and later be compensated by prolonged proliferation. Additionally, there could be subtle defects in myelin formation or fasciculation in adult animals. However, unfortunately these hypothesises could not be confirmed. Peripheral nerves appeared normal during all stages of development that we investigated, and never any signs of degeneration could be observed. Moreover the number of Schwann-cell precursors present in the nerves of mutant embryos did not differ from wt numbers at any stage. Additionally, a close examination of the myelin ultrastructure revealed no abnormalities. However, this is just another example where clear-cut *in-vitro* results failed to be confirmed by the *in-vivo* model. Sometimes it is easier to see an effect by *in-vitro* experiments when cells are lacking their normal “complex” extracellular environment thereby masking effects

that would occur upon addition or deletion of a specific protein (Jones and Jones, 2000a). So under *in-vitro* conditions subtle differences may be detected because they are "amplified" or not compensated by the complex *in-vivo* environment. Also it is conceivable that the secretion of matn2 during *in-vitro* culture is enhanced compared to the wildtype situation, leading to an exaggerated difference between matn2 knock out and wt cells in these assays.

1.4 Matrilins are upregulated following injury of the peripheral nerves

Schwann cells play important roles during peripheral nerve regeneration. They promote regeneration by increasing the synthesis of cell adhesion molecules (CAMs), such as N-CAM, Ng-CAM/L1, N-cadherin, and L2/HNK-1, by elaborating the basement membrane that contains many extracellular matrix proteins, such as laminin, fibronectin, and tenascin, and by producing many neurotrophic factors and their receptors (Liuzzi and Tedeschi, 1991; Fu and Gordon, 1997; Dezawa, 2000; Dezawa and Adachi-Usami, 2000; Dezawa, 2002). An ECM molecule, like matn2, that is capable to influence neurite outgrowth and Schwann cell migration *in-vitro* could also be important for peripheral nerve regeneration. Therefore, we wanted to address this and investigated the expression pattern following nerve injury.

1.4.1 Matn2 expression in normal and injured peripheral nerves

In this study we described the expression of matn2 by non-myelinating and myelinating Schwann cells. Expression of matn2 could be detected in the endoneurium and perineurium of intact adult sciatic nerves, but only in the endoneurium of intact adult femoral nerve. A very significant finding in the present study is that following different forms of peripheral nerve injury, a strong upregulation of matn2 was observed in the proximal and distal stump. Interestingly the upregulation was higher in the distal stump into which axons regenerate. Probably, the upregulation of matn2 is associated with Schwann cell gliosis, which occurs at the tip of the proximal stump and throughout the distal stump during the first two postoperative weeks (Bunge et al., 1987; Clemence et al., 1989). Newly generated Schwann cells have a different phenotype compared to the mature Schwann cells present in undamaged adult nerves, and resemble younger non-myelinating cells. For instance, myelin genes are down regulated in these cells (Trapp et al., 1988; LeBlanc and Poduslo, 1990), whereas neurotrophic factors and ECM molecules (laminin, fibronectin, collagens) which are

important for fasciculation, axonal prolongation and Schwann cell migration are strongly upregulated in damaged nerves (Bunge, 1983; Daniloff et al., 1986; Bixby et al., 1988; Brodkey et al., 1993; Martini, 1994). In this context the upregulation of matn2 in damaged nerves argues for an involvement in these processes. In order to localize the upregulation of matn2 on a cellular level I performed coimmunolabelling experiments, which showed colocalization of matn2 with the Schwann cell marker p75 and the basal lamina tube marker nidogen1. Schwann cells are the main producers of the basal lamina tubes, which support axon growth and Schwann cell migration (Bunge et al., 1986; Bunge et al., 1989; Bunge et al., 1990). Laminin and collagen IV are the main components of the basal lamina tubes and also known binding partners of matn2 (Piecha et al., 2002). Schwann cells form basal lamina-covered bands of Büngner to direct regenerating axons following nerve injury (Thomas, 1964; Bunge and Wood, 1987). Matn2 expression in the basal lamina may facilitate formation of these bands and the growth of axons along them, and additionally, might help to confine the regenerating axons within the basal lamina tubes, which would be advantageous for axonal guidance to the specific targets.

1.4.2 Matn4 expression in normal and injured peripheral nerves

Another member of the matrilin family, matn4, shows a similar tissue distribution as matn2 (Wagener et al., 1998). However, no detailed expression analysis for matn4 regarding the PNS had been performed. Our findings showed, that matn4 is not present in intact peripheral nerves, but is strongly upregulated following nerve injury. Matn4 expression was detected in the endoneurium (but not in the perineurium) of damaged nerves. Matn4 immunoreactivity was associated with Schwann cells and basal lamina tubes, and thus bears a strong resemblance to the localisation of matn2, also regarding the upregulation kinetics upon nerve injury. But, in addition to this overlapping expression we found another expression domain in degenerated axons, where exclusively matn4 was found. A strong upregulation of matn4 was observed several days after transection in proximal and distal stumps. Two weeks after transection a small decrease of matn2 expression level was observed, especially in the proximal stump. At this stage, macrophages have already removed most of the cellular debris and axonal fragments from the proximal stump. Interestingly no matn4 immunoreactivity was observed in newly generated axons or axons that survived the lesion present in the proximal stump. In contrast, matn4 immunoreactivity was still detectable in axonal fragments in the

distal stump two weeks after lesion. Like in the proximal stump newly generated axons, which already started to extend into the distal stump, were negative for matn4. These observations suggest that matn4 immunoreactivity is only associated with damaged axons or axonal fragments that appear after injury and not with newly generated axons or undamaged axons. This interesting axonal expression pattern of matn4 is atypical for ECM proteins, which usually do not show axonal localisation. In order to analyse this axonal expression in more detail I decided to monitor matn4 expression in neuronal bodies located in the segments affected by the injury. However, no immunoreactivity for matn4 was detected in the grey matter of the spinal cord, where neuronal somata are located. Immunoreactivity was only associated with the white matter of the spinal cord, where it most likely is expressed by oligodendrocytes or possibly by astrocytes. Within the DRG matn4 expression could be ascribed to the glial compartment and again was not found in neuronal cell bodies. Additionally, no upregulation of matn4 was detectable in the spinal cord and DRG 7 dpo when damaged and intact sides were compared, showing that the upregulation resides in the peripheral compartment of the nerves. Thus, axonal upregulation of matn4 occurs independently of the cell body and can only be explained by either presence and translation of matn4 mRNA within damaged axons, or via endocytosis of matn4 from the extracellular space. So the mechanism for this axonal localisation and the presence of matn4 in fragmented axons after peripheral nerve transection requires further investigation.

The biochemical properties of matn4 regarding influences on axonal outgrowth or Schwann cell migration have not been investigated yet. However, the structural similarity to matn2, the potential to heterodimerize with matn2 and identical binding partners as matn2 suggest that matn4 as matn2 is a permissive substrate for axon outgrowth and Schwann cell migration. In combination with the very similar expression pattern and the upregulation following nerve injury it seems likely that matn2 and matn4 either are both required or have redundant functions.

2 Distribution and functions of matrilins in CNS

2.1 Expression and functions of matn2 in adult murine brain

Using different approaches we were able to show a localisation of matn2 in the mouse hippocampus, where it is expressed in pyramidal cell layers, the subicullum and to a lesser

extent also in the dentate gyrus. Matn2 was associated with all cells in the pyramidal cell layers. Colabelling experiments with parvalbumin, which is associated with PNNs, revealed that matn2 is not an abundant component of these PNNs. According to the influences of matn2 on axonal growth and cellular migration revealed in our experiments with sensory neurons and Schwann cells we hypothesised that similarly matn2 functions might be associated with axon growth, neuronal or glial cell migration, the modulation of axonal targeting in the hippocampus or synaptogenesis.

A neurite outgrowth assay with hippocampal neurons revealed a strong stimulation of neurite outgrowth on matn2. I additionally investigated neuritogenesis in these hippocampal cultures. I found that the number of neurites on matn2 was comparable to PLL, however when compared to laminin an approximately twofold increase in neurite number on matn2 was observed. Interestingly, when a combination of matn2 and laminin was used for coating the observed neurite length was comparable to the length observed on laminin alone while the number of neurites was similar to the number of neurites found on matn2 alone. It appears that matn2 and laminin share mechanisms for the stimulation of neurite outgrowth of hippocampal neurons. Hence the mixture of matn2 and laminin promoted neurite outgrowth stronger than matn2 alone and similar to laminin alone but no additive effect on the stimulation could be observed. Regarding neuritogenesis in hippocampal cultures, it seems that laminin does suppress neuritogenesis, whereas matn2 shows a weak stimulation and when used in combination with laminin could overcome the inhibitory action of laminin. These experiments clearly demonstrated that matn2 is a permissive substrate for neurite outgrowth and moderately enhances neuritogenesis.

2.2 Matn2 has no influence on the distribution of different populations of interneurons in the hippocampus

In the present study, we analysed the distribution of distinct types of interneurons, identified by the expression of neurochemical markers, in hippocampi of matn2 deficient mice.

We focussed on the distribution of PV-containing perisomatic inhibitory neurons and CaBP-containing dendritic inhibitory cells. When we compared these cells in brains from matn2 deficient and wt animals, we observed a similar distribution and also the morphological features were not aberrant. Thus, matn2 has no influence on the distribution or survival of this types of neurons in mouse hippocampus.

So in agreement with the general description of the matn2 mutant by Mates et al. (Mates et al., 2004) my more detailed analysis of the CNS in matn2 deficient animals did not reveal any obvious abnormalities. This does not rule out the possibility that some differences exist in the CNS of these animals or that they are somehow impaired. Like in other tissues one also has to take a potential functional redundancy of the different matrilins into consideration. Upregulation of other matn family members in the different matn mutant mice has been investigated but was never observed. So no compensatory upregulation mechanism in mice exists for this protein family. However, one cannot rule out the possibility that existing levels are sufficient for compensation and thus there is no requirement for upregulation. Maybe generation of double or triple mutants will shed more light into the functions of this family of ECM molecules.

VII. Summary

The present work describes the expression pattern of the ECM protein matn2 in the peripheral and central nervous system and also reveals some functions. To study these functions matn2 was expressed heterogeneously and used as a purified protein in different *in-vitro* assays to investigate its effects on processes like Schwann cell migration, axon outgrowth, adhesion, axon branching or neuritogenesis. Additionally in this study two different knock out mouse models were used: erbB3 deficient mice, that initially helped to reveal the expression of matn2 in the PNS and matn2 deficient mice that were on the one hand used to confirm the biochemical in-vitro observations in an in-vivo model and to carefully investigate these animals in order to unravel phenotypes in the PNS or CNS.

Matn2 was identified by a DNA microarray approach as a differently expressed gene comparing erbB3 mutant and wt embryos. In this study the down regulation of matn2 in erbB3 mutant embryos, as revealed by the microarray, was confirmed using different approaches, such as S^{35} *in-situ* hybridization and semi-quantitative PCR.

The expression of matn2 in the PNS was further characterized by S^{35} *in-situ* hybridization and immunohistochemical methods. Matn2 expression was found in DRG as well as in the endoneurium and perineurium of peripheral nerves. A close inspection of the expression on a cellular level revealed that matn2 protein was associated with immature Schwann cells in the developing peripheral nerve, with myelinating Schwann cells of adult mouse and also in embryonic primary Schwann cells as well as in a rat Schwann cell line (S16).

The effects of recombinant matn2 on migration and adhesion of S16 cells was investigated by the agarose drop migration assay and an adhesion assay, respectively. Matn2 strongly stimulated migration of S16 Schwann cells, as well as the adhesion of these cells in comparison with other ECM proteins tested in parallel experiments.

To investigate functions of matn2 in the nervous system, we used several *in-vitro* approaches. We found that purified matn2 promotes migration of Schwann cells and fibroblasts from dorsal root ganglia (DRG) explant cultures and increases neurite outgrowth of DRG sensory neurons. Furthermore, DRG axons preferred to grow on matn2 when they were challenged

with different ECM substrates in a classical stripe assay. A branching assay revealed that matn2 does not promote branching of DRG neurons. Additionally, in DRG cultures prepared from matn2 deficient mice axonal length and the number of migrating cells were decreased in comparison to cultures from wild-type mice.

All of these findings suggested that matn2 has some functions during PNS development and led us to investigate whether it might also be important during peripheral nerve regeneration. I could show that matn2 is strongly upregulated in crashed femoral and transected sciatic nerves. In the damaged nerves matn2 immunoreactivity was associated with non-myelinating Schwann cells and basal lamina tubes, both of which are very important for peripheral nerve regeneration. Interestingly another member of the matrilins family, namely matn4, was also upregulated following peripheral nerve injury. Although this protein is not expressed in intact nerves, it shows similar to matn2 an upregulation in Schwann cells and in basal lamina tubes after injury. In addition, I was able to detect matn4-specific immunoreactivity within degenerating or fragmented axons after injury.

In the CNS, my analysis revealed expression of matn2 in several regions of the mouse brain such as the hippocampus and the olfactory bulb. Matn2 immunoreactivity was not associated with perineuronal nets and seemed evenly distributed between all cells in pyramidal cell layers. Immunohistological analysis of CNS tissue from matn2 deficient animals revealed no obvious abnormalities regarding distribution of different interneuron populations or axonal projections. Similar to the observations with peripheral sensory neurons I found that matn2 promotes neurite outgrowth of cultured hippocampal neurons. Interestingly, when a combination of matn2 and laminin was used, we observed an increase in the number of neurites compared to laminin alone, although the length of neurites was similar on these two surfaces. Thus, on hippocampal neurons matn2 has also a positive effect on neuritogenesis in contrast to DRG sensory neurons where no such effect was detectable.

This work is the first study, where distribution and function of the ECM protein matn2 in the nervous system of mice has been investigated. Although matn2 deficient mice show normal embryonic development and are apparently normal in the adult, the functional features of matn2 and its strong upregulation following peripheral nerve injury suggest important roles of this protein during regenerative processes of the nervous system, that are currently under investigation.

VII. Zusammenfassung

In der vorliegenden Arbeit wird das Expressionsmuster des ECM-Moleküls Matn2 im peripheren und zentralen Nervenssystem beschrieben sowie einige Funktionen aufgezeigt. Um diese Funktionen zu untersuchen, wurde Matn2 heterolog exprimiert und als gereinigtes Protein in verschiedenen *in-vitro*-Versuchen eingesetzt. Dabei wurden Prozesse wie die Wanderung von Schwannzellen, Axonwachstum, Adhäsion, axonales Verzweigen und Neuritogenese untersucht. Außerdem wurden in dieser Arbeit zwei verschieden Mausmodelle eingesetzt: Zum einen ErbB3 defiziente Mäuse, die zur Identifizierung der Expression von Matn2 im PNS eingesetzt wurden und zum anderen Matn2 defiziente Mäuse. Diese wurden sorgfältig auf mögliche Defekte im PNS und ZNS untersucht und dienten des weiteren zur Überprüfung und Bestätigung der in den *in-vitro* gewonnenen Daten.

Matn2 wurde in einem DNA-Mikroarray-Ansatz als ein Gen identifiziert, das beim Vergleich von ErbB3-defizienten und Wildtyp Embryonen im PNS vom unmutierten Embryonen hoch, beziehungsweise in mutanten herunterreguliert wird. In dieser Arbeit wurde dieser Mikroarray Befund zunächst mithilfe verschiedener Methoden, wie der S^{35} *in-situ*-Hybridisierung und der semiquantitativen PCR-Analyse bestätigt.

Die Expression von Matn2 im PNS wurde nachfolgend genauer mittels S^{35} *in-situ*-Hybridisierung und immunhistochemischen Methoden charakterisiert. Matn2-spezifische Expression wurde sowohl in den Spinalganglien (DRG) als auch im Endo- sowie Perineurium peripherer Nerven detektiert. Eine genaue Charakterisierung der Proteinexpression auf zellulärer Ebene ergab, dass Matn2 in der Maus mit unreifen Schwannzellen im sich entwickelnden Nerv und mit myelinisierenden Schwannzellen assoziert ist. Außerdem konnte ich zeigen, dass Matn2 sowohl in primären embryonalen Schwannzellen als auch in einer Schwannzelllinie der Ratte (S16), die myelinisierenden Schwannzellen entspricht, exprimiert wird.

Mithilfe eines „Agarosetropfen-Migrationsassays“ sowie eines Adhäsionversuchs konnte ich zeigen, dass rekombinantes Matn2 stark die Migration der S16-Zellen sowie deren Adhäsion fördert. Die Matn2-spezifische Migrationsförderung war im direkten Vergleich mit anderen ECM-Molekülen deutlich stärker.

Um die möglichen Funktionen des Matn2 im PNS weiter zu untersuchen habe ich verschiedene *in-vitro*-Ansätze verfolgt. In Kulturen von embryonalen Spinalganglien konnte gereinigtes Matn2 sowohl die Wanderung von Schwannzellen als auch die Wanderung von

Fibroblasten fördern. Auch zeigte sich in diesen Experimenten eine positive Wirkung von Matn2 auf das axonale Wachstum sensorischer Neurone. In einem sogenannten „Stripe-Assay“ zeigte sich, dass eine mit Matn2 beschichtete Oberfläche zu den bevorzugten Oberflächen gehört. Eine Matn2 abhängige Stimulation der axonalen Verzweigung konnte ich nicht beobachten. Zusätzlich konnte ich mithilfe von Spinalganglienkulturen, die aus matn2 defizienten Embryonen gewonnen wurden, zeigen, dass sowohl die Länge der Axone als auch die Anzahl wandernder Zellen im direkten Vergleich mit Wildtyp-Kulturen deutlich reduziert ist.

Alle diese Befunde deuteten darauf hin, dass Matn2 einige Funktionen während der Entwicklung des PNS übernehmen könnte und uns interessierte daher, ob Matn2 möglicherweise auch bei der Regeneration eine Rolle spielen könnte. Ich konnte zeigen, dass Matn2 nach Quetschung des Nervus femoralis sowie nach Durchtrennung des Nervus Ischiadicus stark hochreguliert. In geschädigten Nerven war Matn2, mittels immunologischer Methoden nachgewiesen, sowohl in nicht-myelinisierenden Schwannzellen als auch in der Basallamina detektierbar. Interessanterweise war ein weiteres Mitglied der Matrilinfamilie, nämlich Matn4, ebenfalls nach einer Nervverletzung hochreguliert. Obwohl dieses Protein normalerweise nicht in intakten Nerven vorliegt, zeigte es nach der Schädigung eine Expression in Schwannzellen und der Basallamina. Zusätzlich konnte Matn4 in degenerierenden sowie in fragmentierten Axonen nachgewiesen werden.

Im ZNS der Maus fand sich Matn2 vor allem im Hippocampus und im Bulbus Olfactorius. Mittels immunhstochemischer Methoden konnte ich zeigen, dass Matn2 kein wesentlicher Bestandteil der „Perineuronalen Netzwerke“ (PNN) ist, sonder eher gleichmäßig verteilt in den pyramidalen Zellschichten vorliegt. In den Matn2 defizienten Mäusen haben ich mithilfe von Markergenen verschiedene Populationen von Interneuronen im Hippocampus untersucht, konnte jedoch keine auffälligen Veränderungen in den Mutanten feststellen. Ähnlich wie schon in den Experimenten mit peripheren sensorischen Neuronen, konnte Matn2 auch das Auswachsen der Neuriten in Kulturen hippocampaler Neurone fördern. In diesen Untersuchungen zeigte sich zudem, dass Matn2 alleine und in Kombination mit Laminin eine Erhöhung der Neuritenanzahl bewirkte, während deren Länge auf allen verwendeten Substraten im Vergleich mit PLL vergleichbar erhöht war. Im Gegensatz zu den Experimenten mit peripheren, sensorischen Neuronen hatte Matn2 auf hippocampale Neurone auch einen stimulierenden Einfluss auf die Neuritogenese.

In dieser Arbeit wird zum ersten Mal detailliert die Verteilung und Funktion des ECM Proteins Matn2 im Nervensystem von Mäusen untersucht. Obwohl Matn2 defiziente Mäuse

sich normal entwickeln und auch im Erwachsenenalter unauffällig sind, deuten sowohl die funktionellen Eigenschaften als auch die starke Hochregulation infolge einer Verletzung des Nervs auf eine wichtige Rolle bei regenerativen Prozessen im Nervensystem hin. Diese werden derzeit von mir untersucht.

VIII. APPENDIX

1.2 ABBREVIATIONS

µ	micro (10^{-6})
× g	g-force
°C	grad celsius
aa	amino acid
A	adenine
Acc.	accession number
Amp	ampicillin
APS	ammoniumperoxodisulfate
ATP	adenosine triphosphate
bp	base pairs
BSA	bovine serum albumine
C	Cytosine
cDNA	complementary deoxyribonucleic acid
CHO	Chinese Hamster Ovarian
Da	dalton
dATP	2'-desoxyadenosinetriphosphate
dCTP	2'-desoxycytidinetriphosphate
DEPC	diethylpyrocarbonate
dGTP	2'-desoxyguanosinetriphosphate
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNase	desoxyribonuclease
dNTP	2'-desoxyribonucleotide-5'-triphosphate
DTT	dithiothreitol
E. coli	escherichia coli
EDTA	ethylendiamintetraacetic acid
FCS	fetal calf serum
g	gram
G	guanosine

h	hour
HEPES	2-(4-(2-Hydroxyethyl)-piperzino)-ethansulfonic acid
kb	kilo base pairs
l	litre
LB	Luria Bertani
m	milli (10^{-3})
MEM	minimal essential medium
min	minute
MOPS	(4-(N-morpholino)-propan)-sulfonic acid
n	nano (10^{-9}), number
PAGE	polyacrylamide gel electrophoresis
PBS	phosphat-buffered saline
PCR	polymerase chain reaction
PEG	polyethylenglycol
PFA	Para-formaldehyde
PMSF	phenylmethylsulfonylfluoride
rpm	rounds per minute
p	statistical significance
RNA	ribonucleic acid
RNase	ribonuclease
RT	room temperature
s	second
SDS	sodium dodecyl sulfate
T	thymine
TE	tris-EDTA
TEMED	N,N,N',N'-tetraethylenamine
Tris	tris(-hydroxymethyl)-aminomethane
U	unit (emzymatic)
V	volt
v/v	volume per volume
Vol.	volume
w/v	weight per volume
ZMNH	Zentrum für Molekulare Neurobiologie Hamburg

Amino acids were abbreviated using the one letter code

2. Oligonucleotides

Nr.	Primer	Sequence (5`-3`)	remarks
1	GAPDH-f	ACCACAGTCCATGCCATCAC	
2	GAPDH-rev	TCCACCACCCTGTTGCTGTA	
3	matn2-f-SQPCR	TGCAAGAGATGCACTGAAGG	
4	matn2-rev-SQPCR	ATTTCCTTGGCTGAGCTGAA	
5	matn4-f-SQPCR	CTGATGGCAAGAGCTGTGAC	
6	matn4-rev-SQPCR	ATGTACTCCACGGCCAAAAC	
7	matn2-f-Kpn	CCACGCAGGTACCCAGAGTA	Kpn 1 site
8	matn2-rev-Not	**TCA**GCGGCCGCTCTGTATTTT	Not1 site, **Stop** codon
9	T7	TAATACGACTCACTATAGGG	For sequencing
10	BGH	TAGAAGGCACAGTCGAGG	For sequencing
11	M1	ACCATGACAGGGCTTGCCAT	For sequencing
12	M2	TGCTGGGTTCCTTTGTCTGC	For sequencing
13	M3	CACAGAGGAGTCCTTTGTCT	For sequencing
14	M4	TGAGGATGGAAAGCACTGCA	For sequencing
15	M5	AGAACTACAGGAGATTGCCT	For sequencing
16	Ex2/rev	CAGTGCAGTCTAAAAATACAT	For genotyping
17	Ex2/in	ATCAGGGACACCCAGAAAT	For genotyping
18	Ires3	TACGCTTGAGGAGAGCCATT	For genotyping
19	neo 2L	CGAATTCGCCAATGACAAGACGCTGG	For genotyping
20	Intron A1	GGGTGTCTGAGTCTTTGAAGCTGGAG	For genotyping
21	Exon A2	ACCTGTAATCTCCCGACTGTCCTGAA	For genotyping
22	matn2-f	CGCAAGAGATGCACTGAAGG	In situ
23	matn2-rev	CATGCTGCTGAGTCCTGTCT	In situ
24	matn4-rev	TCACTTTCGGCTAGCCAGCT	In situ

3. Accessionnumbers

Protein	**Organism**	**Accession N^{o}**

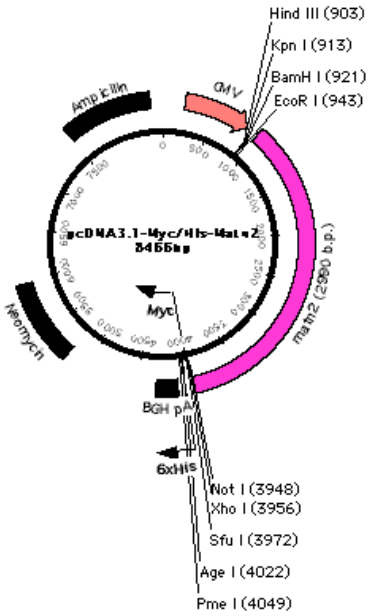
Matrilin-2	mouse	AAH05429
Matrilin-4	mouse	NP 038620.1

4 Plasmid constructs

4.1 pcDNA-Matn2-MycHis$_6$ (N)

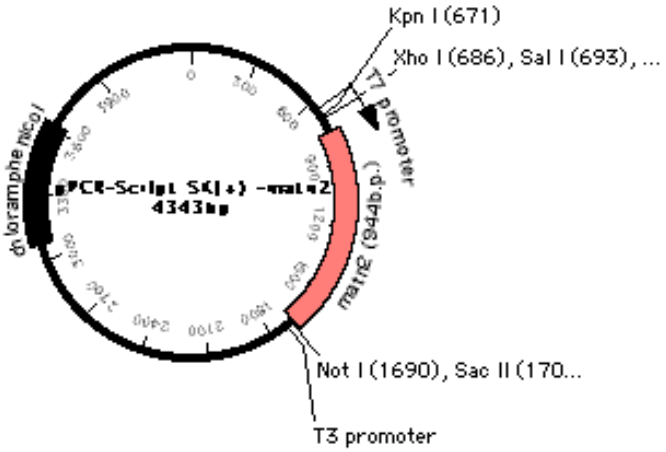
The matn2 cDNA was received from the "Deutsches Ressourcenzentrum für Genomforschung GmbH" (RZPD) in a Sport6 vector as the clone (I.M.A.G.E. G$_p$998F188539Q3). In this clone the SalI restriction site corresponded to the 5'end and the NotI restriction site corresponded to the 3'end.

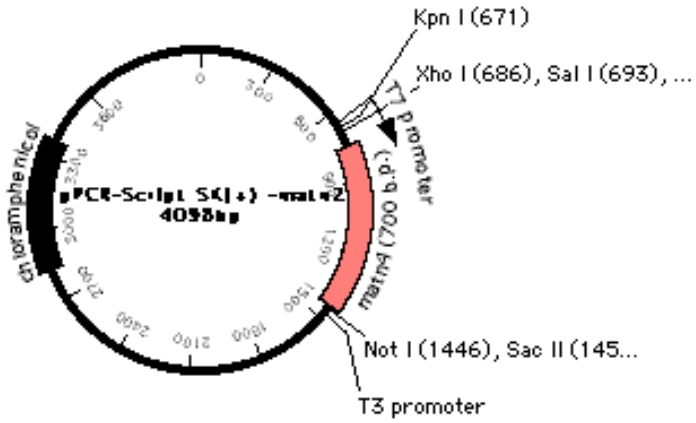
By polymerase chain reaction using the following primers the stop codon was deleted: 5´-CCACGCAGGTACCCAGAGTA-3´(containing KpnI restriction site) and 5´-TCAGCGGCCGCTCTGTATTTT-3`(containing NotI restriction site in front of the stop codon). After amplification and digestion with KpnI and NotI the PCR product (592 bp) was cloned into matn2-Sport6 vector previously cleaved with the same enzymes. A fragment corresponding to the full-length matn2 precursor could be retrieved from this vector. After a restriction with SalI, the ends were filled in, resulting in blunt end formation. Next a restriction with NotI was carried out and the resulting fragment was then inserted into pcDNA3.1-Myc/His$_6$ (C) cleaved previously with EcoRV (blunt) and NotI.



4.2 Cloning of matn2 and matn4 fragments into pPCR-Script SK(+) vector

PCR-Script™ Cam Cloning Kit was used for cloning the PCR fragments of matn2 and matn4. Briefly, the ends of purified PCR products (which were generated with Pfu DNA polymerase) were Treated as described in the manufactures protocol. After this the PCR products were inserted into pPCR-Script™ CamSK(+) cloning vector with a ratio insert-to-vector of 40-to-1.





5 Publications and poster presentations

5.1 poster presentations:

Dmitriy Malin[1], Attila Aszódi[2], Raimund Wagener[3] and Dieter Riethmacher[1]
Matrilin-2, a new extracellular matrix component of the Peripheral Nervous System.
Meeting of the federation of European neuroscience societies (FENS), Lisboa,
11th-15th July 2004

D. Malin[1], A. Dityatev[1,2], G. Dityateva[1], A. Aszódi[3], R. Wagener[4] and D. Riethmacher[1]
Distribution and functions of the extracellular matrix protein matrilin-2 in the nervous system of mice. 6th Meeting of the German Neuroscience Society, 30th Göttingen Neurobiology conference, Göttingen, 17th –20th February 2005

5.2 publications:

D. Malin[1], A. Aszódi[2], R. Wagener[3] and D. Riethmacher[1]
Distribution and functions of extracellular matrix protein matrilin-2 in the peripheral nervous system of mice, submitted

CURRICULUM VITAE

Name: Dmitriy Malin

Date of birth: 11 April, 1975

Place of birth: Russia

Nationality: russian

Citizenship: Russia

Education:

Moscow Academy of Fine Chemical Technology, Dept. of Biotechnology and Fine

Organic Synthesis.

Experience:

1996-1999 Work in the Zelinskiy Institute of Organic Chemistry, Russian Academy of Scienses, Laboratory of Carbohydrate Chemistry, 47 Leninskiy pr. 117913 Moscow, Russian Federation. „Synthesis of (T-labelled)dolichilphosphat-a,D-mannopyranose“.

1999-2000 Work in the Center " Bioengineering" Russian Academy of Sciences, Laboratory of synthesis of oligonucleotides, 7/1 60-years October pr., 117 334 Moscow, Russian Federation. Main topic of present research: „Transport systems for efficient delivery of oligonucleotides into the cells“.

2001 - present Ph.D. Student, Zentrum for Molekulare Neurobiologie UKE, University Hamburg, AG Riethmacher. Main topic of present research: „Matrilins: possible role in Nervous system“.

Supervisors:

Dr. Dieter Riethmacher
ZMNH, Falkenried 94
20251 Hamburg
Phone: 49-40-42803-5354
Fax: 49-40-42803-5359
E-mail: drieth@zmnh.uni-hamburg.de

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