Die Reorganisation der Fascia dentata der Ratte nach entorhinaler Denervierung: eine regulatorische Rolle für das Chondroitinsulfat Proteoglykan NG2

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'What is this,' said the Leopard, 'that is so 'sclusively dark,

and yet so full of little pieces of light?'

Rudyard Kipling, How the leopard got his spots

Abstract

Lesion of the rat entorhinal cortex denervates the outer molecular layer of the fascia dentata followed by layer-specific axonal sprouting of uninjured fibers in the denervated zone. One of the candidate molecules regulating the laminar-specific sprouting response in the outer molecular layer is the transmembrane chondroitin sulfate proteoglycan NG2. NG2 is found in glial scars and has been suggested to impede axonal regeneration following injury of the spinal cord.

The present study adressed the question whether NG2 could also regulate axonal growth in denervated areas of the brain. Therefore, (1) changes in NG2 mRNA and NG2 protein levels, (2) the cellular and the extracellular localisation of the molecule, (3) the identity of NG2 expressing cells, and (4) the generation of NG2-positive cells were studied in the rat fascia dentata before and following entorhinal deafferentation. Laser microdissection was employed to selectively harvest the denervated molecular layer and combined with quantitative reverse transcription-PCR to measure changes in NG2 mRNA amount (6h, 12h, 2d, 4d, 7d post lesion). The study revealed increases of NG2 mRNA at day 2 (2.5-fold) and day 4 (2-fold) post lesion. Immunocytochemistry was used to detect changes in NG2 protein distribution (1d, 4d, 7d, 10d, 14d, 30d, 6 months post lesion). NG2 staining was increased in the denervated outer molecular layer at 1 day post lesion, reached a maximum at 10 days post lesion, and returned to control levels within 6 month. Interestingly, the accumulation of NG2 protein was strongly restricted to the denervated outer molecular layer forming a border to the unaffected inner molecular layer. Using electron microscopy, NG2-immunoprecipitate was localized not only on glial surfaces and in the extracellular matrix but also in the vicinity of neuronal profiles indicating that NG2 is secreted following denervation. Double-labelings of NG2-immunopositive cells with markers for astrocytes, microglia/macrophages, and oligodendrocytes suggested that NG2-cells are a distinct glial subpopulation before and after entorhinal deafferentation. Bromodeoxyuridine-labeling revealed that some of the NG2-positive cells are postlesional generated.

Taken together, the data revealed a layer-specific upregulation of NG2 in the denervated outer molecular layer of the fascia dentata that coincides with the sprouting response of

uninjured fibers. This suggests that NG2 could regulate lesion-induced axonal growth in denervated areas of the brain.

Introduction

The central nervous system (CNS) of an adult organism is characterized by a large number of interconnected neurons that are insulated by glial cells. For many years the adult brain was believed to be a stable organ in the sense of a finished end product of the developmental process. It was considered "mature" and believed to be incapable of regeneration. Ramon y Cajal postulated in 1928: “The functional specialization of the brain imposed upon the neurones two great lacunae: proliferative inability and irreversibility of intraproto-plasmatic differentiation. It is for this reason that, once the development has ended, the fonts of growth and regeneration of the axons and dendrites dried up irrevocably. In adult centres the nerve paths are something fixed, ended, immutable. Everything may die, nothing may regenerate. Pathologists consider it an unimpeachable dogma that there is no regeneration of the central paths, and therefore that there is no restoration of the normal physiology of the interrupted conductors in the spinal cord. A vast series of anatomico-pathological experiments in animals, and an enormous number of clinical cases that have been methodically followed by autopsy, serve as a foundation for this doctrine, which is universally accepted today.” (Ramon y Cajal, 1928).

To date, it is widely accepted in neuroscience that the neuronal circuitry of the CNS is continuously reshaped in the adult brain. It is this plasticity of the nervous system that allows an organism to adapt to changing environments and to react appropriately to internal as well as external stimuli. However, focal injury of the CNS as well as neurodegenerative diseases damage the adult brain and result in severely impaired neuronal function. Although injured CNS axons are, indeed, incapable of regeneration in the adult brain, a lesion-induced form of neuronal plasticity exists that may compensate for the loss of neurons following injury: “axonal sprouting”. This form of central nervous plasticity, where uninjured axons sprout new axon collaterals that reinnervate denervated areas, was first observed in the spinal cord (Liu and Chambers, 1958), and in the brain in the septum (Raisman, 1969). By now this phenomenon has been documented for several species (Cotman et al., 1981), brain regions (Raisman and Field, 1973; Rubel et al., 1981; Chen and Hillman, 1982; Wells and Tripp, 1987; Schwegler, 1995), and is now regarded as

a naturally occurring reorganization response of the brain to different kinds of lesions (Steward, 1994).

It is of fundamental interest to understand the molecular and cellular basis of this lesion-induced central nervous system plasticity. Understanding this basis may lead to the development of novel therapeutic strategies that may help in the treatment of acute brain trauma or neurodegenerative diseases.

Reorganisation processes after central nervous system injury

A focal injury of the adult mammalian brain leads to a number of temporally ordered reactive responses in neurons and glial cells in the affected areas. The affected brain areas are (1) the directly damaged regions, (2) the regions that are in the close vininity of the injury, (3) the axotomised regions in which retrograde degeneration takes place, and (4) the denervated regions lacking input (Fig. 1). The temporally ordered cellular responses triggered by the lesion are (a) neuronal cell death, (b) sequential activation of microglial cells and astrocytes, (c) transneuronal degeneration processes, and (d) lesion-induced sprouting of uninjured neurons in distal target areas of the axotomized neurons (Raisman, 1969; Steward, 1989; Steward et al., 1993; Kelley and Steward, 1997; Hirsch and Bahr, 1999).

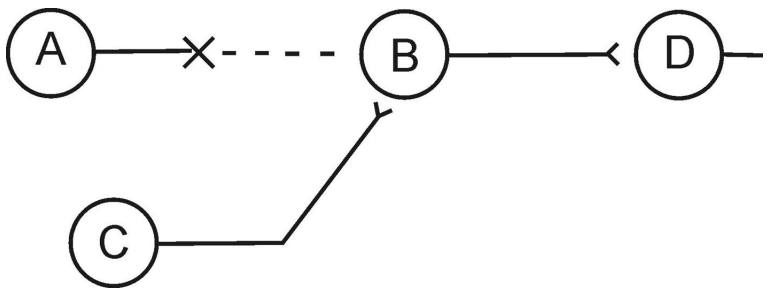

Figure 1 *Schematic illustration: Effects of a CNS lesion on the neuronal network*

A lesion of the CNS affects the neuronal network: Cell A is axotomized (the cross indicates the directly damaged region). Terminals of cell A degenerate (dashed line), and cell B loses its input from cell A. Cell C is not directly damaged by the lesion, but terminates in the vicinity of

degenerating terminals. Cell D is a target neuron of cell B and may be affected transneuronally if cell B dies or atrophies (Deller and Frotscher, 1997).

The context of the present work is the regulation of the lesion-induced sprouting of uninjured neurons in a target area lacking input. A candidate molecule, the chondroitinsulfate proteoglycan (CSPG) NG2, was investigated in a time course analysis in the denervated outer molecular layer (OML) of the rat dentate gyrus, in order to ascertain wether NG2 may contribute to the regulation of the lesion-induced sprouting response in injured adult rat brain.

Axonal sprouting after entorhinal cortex lesion

The phenomenon of lesion-induced sprouting can be well studied using the entorhinal cortex lesion (ECL) model (Lynch et al., 1972; Del Turco et al., 2003). In this lesion model the fibers of the *Tractus perforans* are transected electrolytically. The transected fibers originated from stellate neurons of layer II in the entorhinal cortex (EC) and terminate mainly in the ipsi- and contralateral outer molecular layer (OML) of the dentate gyrus, where they contact distal dendrites of the granule cells from the granule cell layer (Steward and Scoville, 1976; Deller et al., 1996b). Additionally, fibers from the EC terminate in *stratum lacunosum-moleculare* of hippocampal subfields CA1-CA3. The transection of the *Tractus perforans* leads to several degeneration processes and results in the denervation of the OML. The denervation is followed by collateral sprouting of un-injured fibers that terminate also in the denervated layer, e.g. fibers from the contralateral EC and from the medial septum, thereby reinnervating the denervated OML (Lynch et al., 1976). Remarkably, the collateral sprouting of the un-injured fibers is strictly layer-specific (Frotscher et al., 1997). Only those fibers reinnervate the OML that terminate in the denervated zone already before lesioning. Fibers from the adjacent inner molecular layer (IML) do not invade the denervated zone (Fig. 2). Therefore, the laminar organization of the dentate gyrus is maintained following lesion of the entorhinal cortex (Deller et al., 2001).

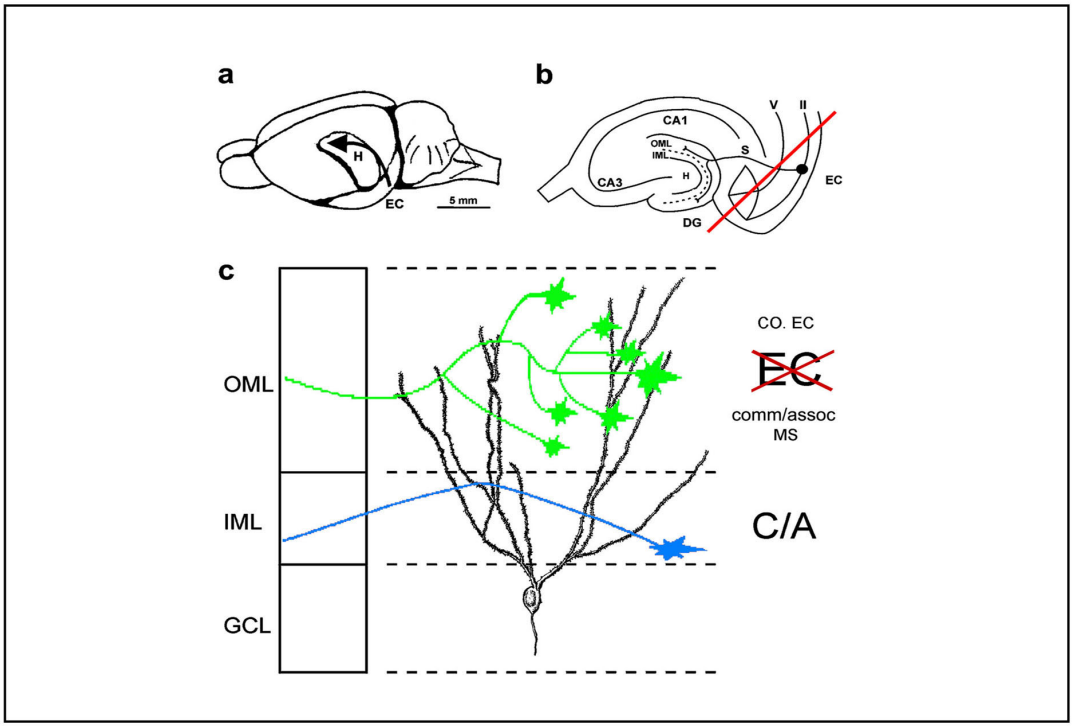

Figure 2 *Schematic illustration: The entorhinal cortex lesion model (Deller, 1998)*

a. Topography of the entorhino-hippocampal projection. Fibers of the EC project to the hippocampus (H) in a topographically ordered, lamina-specific manner.

b. Horizontal section of the hippocampal formation. Neurons located in layer II of the EC form the perforant pathway projecting to the OML of the DG. Additionally, fibers from the EC project to stratum lacunosum-moleculare of CA1-CA3. The red line indicates the site where the perforant pathway is transected.

c. Lamina-specific termination and sprouting of afferent fibers in the DG. A typical granule cell is illustrated. On the right hand side the major afferent fiber systems are indicated. The size of the lettering indicates the relative contribution of these fibers to the innervation of the DG. Following ECL, the entorhinal input is lost. The remaining fiber systems sprout and reinnervate the DG in a layer-specific fashion. The green fiber indicates sprouting afferents that reinnervate the OML following denervation. Fibers that terminate in the non-denervated adjacent IML (blue fiber) do not invade the denervated zone.

CA1, CA3: hippocampal subfields, DG: dentate gyrus, EC: entorhinal cortex, ECL: entorhinal cortex lesion, GCL: granule cell layer; H: Hippocampus, IML: inner molecular layer, OML: outer molecular layer

ECL is a well established lesion model to investigate the regulation of sprouting mechanisms in injured brain (Lynch et al., 1972; Cotman et al., 1973; Matthews et al., 1976; Steward, 1976). Two of its advantages are:

1.) The lengthy distance between the lesion site and the denervated OML. This allows an investigator to discriminate between lesion-induced neurodegenerative processes (e.g. local damage and glial scar formation at the entorhinal cortex) and lesion-induced reactive processes occuring in the denervated area (OML). The lesion-induced reactive processes are the initial degeneration of entorhinal fibers, followed by a local reaction of glial cells and sprouting of surviving fibers that contribute to the reinnervation of the denervated dentate gyrus.

2.) The layer-specific termination of the *Tractus perforans* in the OML. This layer-specific termination of entorhinal fibers allows an investigator to differentiate between sprouting fibers terminating within the denervated OML and those fibers originating from non-denervated adjacent layers, e.g the IML (Deller et al., 1995a; Deller et al., 1996a).

The phenomenon of layer-specific collateral sprouting in the dentate gyrus following ECL led to the question which cells and molecules could be involved or even regulate the reinnervation process (Steward, 1995; Savaskan and Nitsch, 2001; Deller et al., 2001). As fas as the molecular regulation of the sprouting response is concerned, it has been hypothesized that at least two groups of molecules play a major role: (a) molecules that are intrinsic to the sprouting cell, the so-called “intrinsic” molecules, and (b) molecules of the environment of the sprouting cell, the so-called “extrinsic” molecules (Fawcett, 1997; Frotscher et al., 1997; Deller et al., 1997; Deller et al., 1999).

Molecular regulation of sprouting

Intrinsic molecular cues are expressed by growing neurons themselves (e.g. during development) and determine their growth capacity (Caroni, 1997; Benowitz and Routtenberg, 1997). Molecules that may regulate the growth response of sprouting fibers following ECL are e.g. the growth associated protein GAP43, or CAP23 (Benowitz et al., 1990; Masliah et al., 1991; Fawcett, 1992).

Extrinsic molecular cues reside in the microenvironment of growing axons (Rauch, 1997). Extrinsic molecules are found in the vicinity and in the target area of growing axons where they are important for neuronal pattern formation (Faissner, 1993; Rauch and Schafer, 2003). These molecules may guide growing axons not only to the correct target region but also to the correct target cell (Rauch, 1997; Margolis and Margolis, 1997). They regulate the degree of axonal branching, the number of synapses that are formed and they may play a role in synaptic stabilization (Faissner et al., 1994; Faissner and Steindler, 1995; Rauch, 2004). Extrinsic molecules that may be involved in the regulation of lesion-induced changes in the dentate gyrus are cell adhesion molecules (e.g. N-CAM), diffusible molecules (e.g. growth factors and cytokines), and extracellular matrix (ECM) molecules (e.g. tenascin-C, Neurocan). All of these molecules can be produced either by nerve or by glial cells within the target region (Fig. 3). The various regulatory molecules are subdivided into those stimulating axonal growth and those inhibiting axonal growth (Goodman, 1996; Tessier-Lavigne and Goodman, 1996; Bovolenta and Fernaud-Espinosa, 2000). Within the group of ECM molecules that might regulate lesion-induced sprouting after ECL, some members of the family of chondroitin sulfate proteoglycans (CSPGs) were found (Haas et al., 1999; Thon et al., 2000; Deller et al., 2001; Savaskan and Nitsch, 2001).

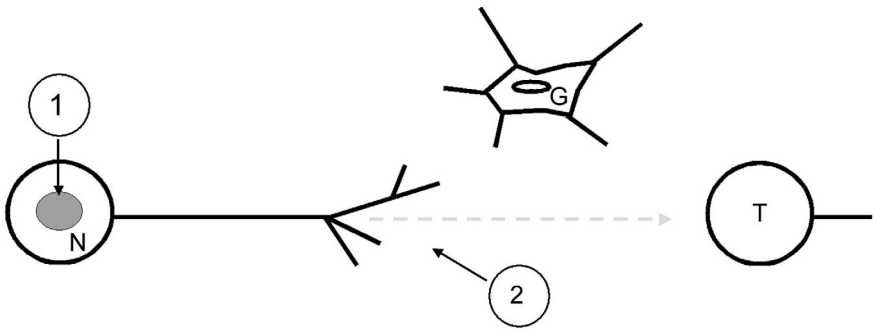

Figure 3 *Schematic illustration: Molecular regulation of sprouting. Modified from (Deller and Frotscher, 1997)*

1. Intrinsic molecules determine the growth capacity of uninjured nerve cells (N) sprouting and reinnervating the target cell (T).

2. The extrinsic molecules reside in the vicinity of sprouting axons. They may be involved in the regulation of sprouting axons and can be produced by glial cells (G).

Chondroitinsulfate proteoglycans (CSPGs)

Molecules of the CSPG family are functionally versatile and are known to be involved in a large number of biological processes during development of the zentral nervous system (Miller et al., 1995; Margolis and Margolis, 1997) as well as in the adult brain (Rauch et al., 1991; Snow and Letourneau, 1992; Grumet et al., 1996). Although some CSPGs have growth promoting effects on growing axons (Faissner et al., 1994; Anderson et al., 1998) most CSPG species inhibit axonal growth. Since CSPGs were enriched at lesion sites following brain injury, it has widely been assumed that they contribute to the regeneration failure of axons within the CNS (Fawcett and Asher, 1999; Asher et al., 2001; Morgenstern et al., 2002; Rhodes et al., 2003).

A member of the CSPG family: NG2

A prominent member of the CSPG family is NG2 (Fig. 4). This CSPG was first described as a cell surface molecule expressed by immature neural cells by Wilson and coworkers (Wilson et al., 1981). The full-length NG2 is a transmembrane CSPG integral membrane protein consisting of three domains: a large extracellular region with three glycosaminoglycan side chains, a single transmembrane domain, and a short cytoplasmatic tail (Nishiyama et al., 1991; Levine and Nishiyama, 1996; Stallcup, 2002). NG2, which is not specific to nervous tissue, is present on the surface of multipotent glial precursor cells during brain development (Levine and Stallcup, 1987; Levine et al., 1993). Furthermore, NG2 is expressed by a subset of glia in the mature brain (Levine and Nishiyama, 1996), and expressed by glia and macrophages in the vicinity of an injury (Levine, 1994; Jones et al., 2002; Jones et al., 2003). NG2 binds to type V and VI collagen and to other ligands, such as the platelet-derived growth factor-alpha receptor (Tillet et al., 1997; Levine and Nishiyama, 1996; Sandvig et al., 2004).

Different cell lines express different forms of the NG2 core protein. Many of the cell lines express the full length 300kDa core protein, lacking the cytoplasmatic domain but contain almost the entire ectodomain and release a 290kDa form into the medium. There also exist cell lines that express a truncated 275 kDa core protein form, lacking the cytoplasmatic domain and at least 64 amino acids of the ectodomain (Nishiyama et al., 1995).

In vitro studies have documented a considerable axon growth-inhibitory potential of the full-length NG2 proteoglycan and of the core protein of NG2, that is obtained by enzymatic removal of the chondroitin sulfate side chains. The chondroitinase treatment indicated that the inhibitory potential of NG2 was mediated by the core protein rather than through the glycosaminoglycan chains (Dou and Levine, 1994; Fidler et al., 1999; Ughrin et al., 2003). Additionally, several *in vivo* studies showed an enrichment of NG2 in the glial scar following CNS injury (Fawcett and Asher, 1999; Chen et al., 2002a; Camand et al., 2004; Rezajooi et al., 2004). Although a glial scar does not form in the denervated OML following ECL, all glial cells in the OML strongly react to denervation. Micro- and astroglia glial cells phagocytose degenerating axons and their terminals (e.g., Rose et al., 1976; Gall et al., 1979; Bechmann and Nitsch, 1997; Deller et al., 1999; Jensen et al., 1999; 2000; Bechmann and Nitsch, 2000; Drojdahl et al., 2004). Reactive astrocytes are believed to regulate the axonal sprouting response by altering the composition of the extracellular matrix (e.g., Deller et al., 1997; 2000; 2001; Haas et al., 1999; Thon et al., 2000) and produce most of the CSPGs in the denervated OML (Savaskan et al., 2000b; Deller et al., 2001; Thon et al., 2000).

The definite cell type producing NG2 in the nervous system is still under debate (Bu et al., 2001; Jones et al., 2002; Chekenya et al., 2002; Greenwood and Butt, 2003; Peters and Sethares, 2004a; Belachew et al., 2003; Butt et al., 2002) and even less is known about the cell type producing NG2 in the denervated OML after ECL.

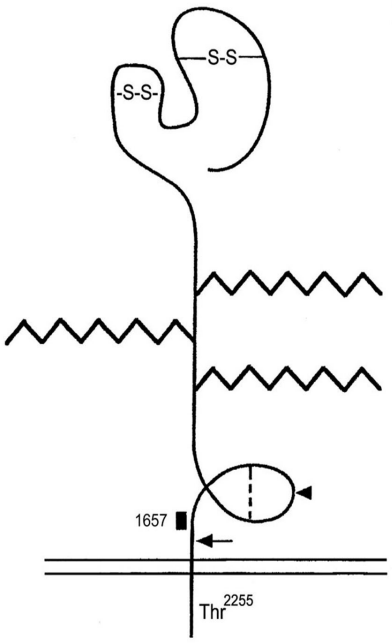

Figure 4 *Schematic illustration: Drawing of NG2 (Levine and Nishiyama, 1996)*

"The double lines represent the plasma membrane, the jagged lines the chondroitin sulfate glycosaminoglycan chains, -S-S- represents disulfide bonds, the arrowhead denotes the site of proteolytic cleavage that generates the 275 kDa fragment of the core protein, and the arrow denotes a cleavage site that generates the 290kDa fragment. The position of threonine ^{2255} is indicated and the dotted line represents the sites of potential intramolecular interactions that retain the association of the 275 kDa fragment with the cytoplasmic domain of the core protein. The position of the 1657 peptide is indicated with a solid block."

Concerning the regulation of layer-specific sprouting in the denervated OML, the glia-derived CSPG NG2 is of interest for several reasons:

1.) NG2 strongly inhibits axonal growth. NG2 has been implicated in the regulation of axonal growth and axonal regeneration following injury of the spinal cord (Fidler et al., 1999; Levine et al., 2001; Jones et al., 2002). In the spinal cord it is thought to inhibit axonal regeneration across the glial scar. This regulatory role of NG2 under conditions of CNS injury suggests that NG2 could also regulate axonal growth processes in denervated areas of the brain.
Therefore, the working hypothesis is that NG2 is also involved in the regulation of the layer-specific sprouting response of uninjured fibers in the denervated OML.

2.) Processing of NG2 could change the composition of the ECM in the denervated OML. *In vitro* and at the glial scar NG2 can be cleaved by metalloproteases at its membrane domain (Nishiyama et al., 1995; Larsen et al., 2003). Whether or not processing of NG2 occurs in denervated brain areas is unknown. Since cleaved NG2 could chance the composition of the ECM it may play a regulatory role for the sprouting response of growing axons following ECL.

3.) The identity of NG2 producing cells in denervated areas of the brain is unknown. If NG2 is involved in layer-specific reorganisation processes following ECL it may be interesting to know by which cell type this CSPG is produced.

Aims of the study

The experiments reported here were designed to gain insight into the molecular regulation of the layer-specific collateral sprouting response following ECL. To study wether the CSPG NG2 could be involved in the reorganisation processes in the rat fascia dentata, changes in NG2 were analyzed following ECL and correlated with the sprouting response. In detail, the following questions were investigated using the indicated methods:

1.) To study wether NG2 is expressed in denervated areas of the brain, the ECL model was used.

2.) To elucidate the time course of changes in amount of NG2 mRNA expression of the denervated OML, lasermicrodissection (LMD) was combined with subsequent quantitative reverse transcription PCR (qPCR).

3.) To elucidate the time course of NG2 protein changes, immunohistochemical methods were used.

Both, amount of NG2 mRNA and protein in the denervated fascia dentata were investigated in a time course analysis and correlated with the postlesional sprouting response.

4.) To verify wether NG2 is secreted into the ECM of the denervated OML, electron microscopy was used.

5.) To identify the NG2-expressing cell type, double-labelings of NG2-positive cells with markers for astrocytes, microglia/macrophages, and mature oligodendrocytes were performed before and after entorhinal deafferentation.

6.) To determine wether or not some NG2-positive cells were generated post lesion, double-labeling of NG2-positive cells with 5´-bromo-2´-deoxyuridine (BrdU) for generated cells was performed after ECL.

Materials and Methods

Animals, surgical procedures, and tissue preparation

Animals

A total of seventy adult male Sprague Dawley rats (250-350g; Charles River Wiga, Sulzfeld, Germany) housed under standard laboratory conditions were used in this study. For light microscopy, control rats (n=4), and EC-lesioned rats surviving for 1 day (d), 4d, 7d, 10d, 14d, 30d, and 6 months post lesion were employed (n=4 for each time point). For electron microscopy, EC-lesioned rats surviving for 10d (n=2) were used. For LMD and qPCR, control rats (n=4), and EC-lesioned rats surviving for 6 hours (h), 12h, 2d, 4d, and 7d post lesion were used (n=4 for each time point). For BrdU-labeling, EC-lesioned rats surviving for 10d (n=2), and control rats (n=2) were used.

Entorhinal cortex lesion

All surgical procedures were performed under deep pentobarbital-sodium anaesthesia (50mg/kg body weight Narcoren, Merial GmbH, Hallbergmoos, Germany) in agreement with the German law on the use of laboratory animals. For lesioning of the entorhinal cortex, the head of the animal was horizontally aligned in a stereotaxic frame (Kopf Instruments, Tunjunga, USA). Using sterile procedures, the skin was retracted and a hole was drilled above the lesion site without disruption of the underlying dura using a handheld dental drill (Proxxon Micromot 50, Hickory NC, USA; drill heat: Dremel 107, Leinenfelden-Echterdingen, Germany). A standard electrocoagulator (Erbe, Tübingen, Germany) was used to perform a complete unilateral electrolytic lesion (3 single pulses, 2.0μA, 1s each). One cut was placed in the frontal plane and one cut was placed in the sagittal plane between the entorhinal area and the hippocampus. The two electrolytic cuts result in a complete destruction of the ipsilateral afferents from the entorhinal cortex to the fascia dentata (Deller et al., 1995a; Deller et al., 1996a; Deller et al., 1996c). The following coordinates measured from the interaural line were used: frontal cut: AP +1, L +3 to +7, V

down to the base of the skull; sagittal cut: AP +1 to +4, L +6.7, V down to the base of the skull (Paxinos and Watson, 1982). Finally, the head wound was closed and the animal was carefully returned to cage. For postoperative analgesia metamizol-sodium analgetica was added to the drinking water (0.1% Novaminsulfon, Ratiopharm Merckle GmbH, Blaubeuren, Germany).

Tissue preparation

Rats were deeply anesthetized with an overdose of pentobarbital-sodium anaesthesia (300mg/kg body weight, Merckle) and for immunohistochemistry transcardially perfused with 0.9% NaCl followed by 4% paraformaldehyde in 0,1M phosphate buffered saline (PBS, pH: 7.4). Brains were removed and post-fixed for 24h in the same fixative. For electron microscopy 0.1% glutaraldehyde was added. Serial frontal or horizontal sections (50µm) were cut with a vibratome (VT 1000S, Leica, Bensheim, Germany).

For LMD and qPCR, rats were anaesthetised using an overdose injection of pentobarbital-sodium anaesthesia (300mg/kg body weight, Merckle). Brains were removed, embedded in tissue freezing medium (Leica) and immediately flash-frozen in 2-methyl-butane at –40°C (5min) for short-term storage at –70°C or for instant sectioning. Serial cryostat sections (20µm) of the septal portion of the hippocampus were cut (Leica CM 3050S, Leica) and mounted on autoclaved polyethylene terephthalate- (PET-) foil stretched on a metal frame (Leica). Sections were then fixed in ice-cold acetone for 3min, dried on a heater at 40°C for 5min, and subjected to LMD and qPCR. Using LMD three microdissected OML per animal were collected and pooled for qPCR analysis.

Control of lesion quality

The quality of the entorhinal cortex lesion is critical for this study. Only animals with a complete entorhinal cortex lesion and without damage of the dentate gyrus were used for further analysis. Both, the completeness of the lesion and the intactness of the dentate gyrus were scrutinized in three steps:

1.) Rat brains were checked visually after perfusion and dissection. Only those animals were used for further analysis (horizontal sectioning) which did not show an unusually large hemorrhage within the first 10d after lesion. At later time-points (14d, 30d, and 6 month), a small and filiform glial scar had to be existent at the lesion site.

2.) The completeness of the ECL was verified in each animal macroscopically by sectioning (50µm) the lesion site horizontally on a vibratome (VT 1000S, Leica). Only those animals were used for further analysis (staining of transversal sections) which had received a complete perforant pathway transection, and which did not show any damage to the dentate gyrus. For a complete perforant pathway transection, the two unilateral electrolytic cuts had to be placed in the frontal and in the sagittal plane between the entorhinal area and the dentate gyrus.

3.) The completeness of the ECL was verified histochemically using frontal sections of lesioned rat hippocampus. For animals perfused at 1d, 4d, and 7d post lesion, the Fluoro-Jade B procedure was used. This procedure labels degenerating axons and their terminals with the anionic fluorescein derivative Fluoro-Jade B, and can be employed to detect axonal degeneration in the OML (Anderson et al., 2005; Schmued and Hopkins, 2000; Savaskan et al., 2000a). For animals surviving 10d, 14d, 30d, and 6 months, the classical acetylcholinesterase (AChE) procedure, which labels cholinergic fibers, was used to verify lesion completeness (Lynch et al., 1972; Zimmer et al., 1986). Both procedures have been used to demonstrate lesion quality after ECL in rats. The stainings indicate the extent of axonal denervation (Fluoro-Jade B procedure) and reinnervation (AChE histochemistry) following ECL. An enhanced staining in the OML indicates degenerated or sprouted axons, respectively. Every tenth section was processed for the Fluoro-Jade B procedure or AChE histochemistry. Only those animals were included in the study that showed increased Fluoro-Jade B staining or the typical AChE band in the OML (Fig. 5 b, d).

Fluoro-Jade B procedure

Transversal sections were mounted on slides and dried overnight (RT). Then, sections were incubated in a solution of 1% NaOH in 80% Ethanol (5min), hydrated in graded Ethanol and distilled water (70%, 50%, each: 2min) before incubating in a 0.06% potassium

permanganate solution (8min). After rinsing in distilled water (2min), sections were incubated (20min) in a 0.0004% solution of Fluoro-Jade B (Histo-Chem Inc. Jefferson, Ar, USA). Finally, sections were rinsed thoroughly in distilled water (4 x 2min), dried on a slide warmer (10min), and coverslipped with anti-fading fluorescent mounting medium (DAKO© Fluorescent Mounting Medium, Dako, Hamburg, Germany). Sections were photographed by an attached Spot2 digital camera (Diagnostics Instruments, Sterling Heights, MI, USA) on a Fluorescent Microscope (Olympus BX-40, Color View, Hamburg, Germany).

AChE procedure

Frontal sections were mounted onto slides and dried overnight. Then, sections were rinsed in 0.1M maleic buffer (2 x 4min), and incubated (2h) in 0.1M maleic buffered acetylcholine iodide incubation solution. After rinsing in 0.1M Tris buffered saline (TBS, ph 7.6, 3 x 4min), sections were incubated (10min) in Tris buffered 0.5% cobalt chloride and rinsed again in TBS (3 x 4min). The labeling was visualised with a nickel/DAB solution (0.005% 3,3`-diaminobenzidine, 0.005% nickel-ammonium sulfate, 0.006% cobalt chloride, 0.001% H2O2 in 0.1M PB), which resulted in a deep-blue labeling of the cholinergic neurons. The reaction was stopped by rinsing the sections in TBS. Finally, sections were dehydrated in graded Ethanol and Toluol (70%, 80%, 95%, 100%, 100%), and coverslipped with Hyper-Mount™ (Shandon Inc. Pittsburgh, PA, USA).

Incubation solution for AChE histochemistry

The Maleic buffered acetylcholine iodide incubation solution (0.1M, pH 8.0) contains:

360ml	maelic buffer (1.96% di-Sodiummaleate dihydrate)
40ml	H_2O
0.003g	Promethazine Hydrochloride (Sigma, Taufkirchen, Germany)
0.56g	di-Sodium Maleate 2-hydrate (Merck, Darmstadt, Germany)
0.059g	tri-Sodium Citrate 2-hydrate (Roth, Karlsruhe, Germany)
0.03g	Cupric (II) Sulfate 5-hydrate (AppliChem)
0.006g	Potassiumferricyanide (Merck)
0.021g	Acetylcholine Iodide (Sigma)

Fluoro-Jade B and AChE staining on frontal sections of rat dentate gyrus

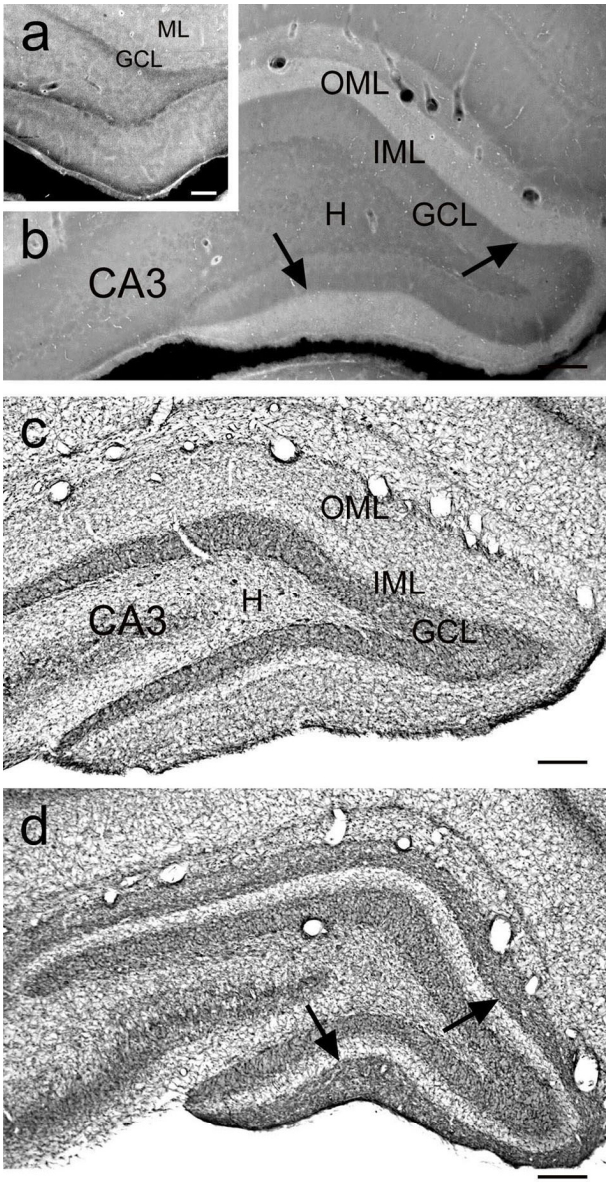

Figure 5 *Fluoro-Jade B and acetylcholinesterase histochemistry reveal the extent of axonal denervation and reinnervation following entorhinal cortex lesion*

a, b. Fascia dentata of a control animal (a) and an animal 4d after ECL (b) stained for Fluoro-Jade B. Note the enhanced fluorescent Fluoro-Jade B labeling in the denervated OML of the lesioned animal.

c, d. Control animal (c) and an animal 30d after ECL (d). In the lesioned animal, a dense acetylcholinesterase-positive fiber band is present in the OML. Note the sharp border (arrows) between the OML and the non-denervated IML. Scale bars: a:100µm, b-d: 150µm.

CA3: hippocampal subfield CA3, ECL: entorhinal cortex lesion, GCL: granule cell layer, H: hilus, IML: inner molecular layer, ML molecular layer, OML: outer molecular layer

Combined lasermicrodissection (LMD) and quantitative RT-PCR (qPCR)

Laser microdissection (LMD) is a research tool, which allows the isolation of specific tissue from a section of complex, heterogeneous tissue under direct microscopic visualisation. The combination of LMD with qPCR is a highly sensitive technique for studying gene expression, particularly in tissue where low expression changes are suspected that may otherwise escape detection (Ando et al., 2003; Prosniak et al., 2003). In this project, an optimized protocol of combined LMD/qPCR for microdissecting cell layers in the rat CNS was used (Burbach et al., 2004; Burbach et al., 2003). The Leica AS LMD system (Leica Microsystems, Wetzlar, Germany) was used to microdissect the OML of frontal brain sections. Subsequently, the Abi Prism 7000 Sequence Detection System (Applied Biosystems) was used to quantify the amount of NG2 mRNA in the microdissected tissue piece (Fig. 6).

Flow chart of the combined LMD and qPCR protocol

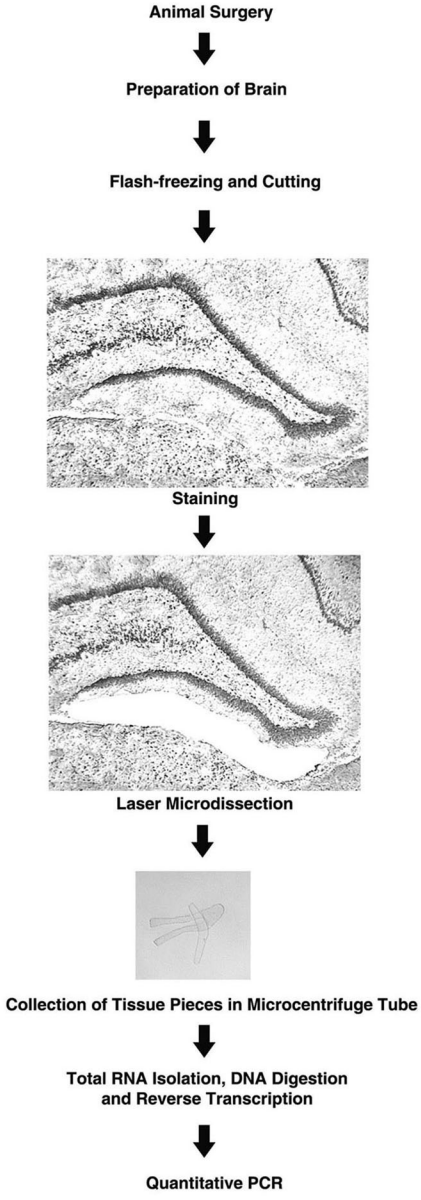

Figure 6 *Flow chart showing the important steps of the combined lasermicrodissection with subsequent qPCR protocol. Modified from Burbach (Burbach et al., 2003)*

First, transversal brain sections were stained at room temperature (RT, 1min) with a 1% toluidine blue solution (Merck, Darmstadt, Germany) dissolved in RNAse-free DEPC-treated water which was filtered through a 0.22µm Millex™ GP filter (Millipore, Eschborn, Germany). Then, the sections were briefly rinsed in DEPC water. After differentiation in 75% ethanol in DEPC (3min), sections were dried on a heater at 40^0C (5min) and immediately subjected to LMD. The PET-foil frames were mounted on a Leica AS LMD system with the section facing downwards. After adjusting intensity, aperture, and cutting velocity, the pulsed UV laser beam was directed along the border of the OML. Under direct microscopic visualisation the area of the infrapyramidal OML was dissected (Fig. 6). Using a 20x objective, settings were as follows: aperture 10, intensity 45, speed 6, and offset 39.

The microdissected tissue was transferred by gravity alone into a microcentrifuge tube cap placed directly underneath the section. Per animal three microdissected OML were collected. The tube cap was filled with a guanidine isothiocyanate (GITC) containing buffer (Buffer RLT, RNeasy Mini Kit, Qiagen, Hilden, Germany) to ensure isolation of intact RNA. Tissue collection was verified by inspecting the tube cap at higher magnification. Microcentrifuge tubes were immediately transferred on ice after tissue collection followed by three freeze-thaw cycles in a dry-ice/ethanol bath .

Protocol for RNA isolation using the LMD and RNeasy® Micro Kit (Quiagen)/TaqMan Reverse Transcription Reagents (Applied Biosystems)

Total RNA was isolated using the RNeasy® Mikro Kit (50) (Qiagen) according to the manufacturer's recommendations including on-columm (RNeasy MinElute™ Spin Column, Quiagen) DNAse treatment and a 2 x 30µl elution step. Finally, RNA was reverse transcribed using TaqMan Reverse Transcription Reagents (Applied Biosystems, Darmstadt, Germany).

RNeasy® Micro Kit (50) contains:

Rneasy MinElute™ Spin Columns in collection tubes
Collection tubes (1.5ml, 2ml)

Buffer RLT (contains guanidine isothiocyanate, GITC)
Buffer RW1 (contains ethanol)
Buffer RPE (supplied as concentrate, before usage adding 100% ethanol)
RNAse free water (bottle and tube)
Buffer RDD (completely unknown composition)
RNase free DNase
Carrier RNA, poly-A

The mastermix for the reverse transcription reaction using 30µl solution of isolated RNA using TaqMan Reverse Transcriptase Reagents (Applied Biosystems) contains:

7.5µl	10 x RT buffer
16.5µl	$MgCl_2$
15µl	dNTP
3.75µl	Random Hex.
1.5µl	RNase inhibitor
1.875µl	Reverse Transcriptase

Following is the detailed protocol developed for RNA isolation using the LMD and RNeasy® Micro Kit (50), inclusive DNase digestion and reverse transcriptase reaction using TaqMan Reverse Transcription Reagents (Applied Biosystems, Darmstadt, Germany). The protocol bases on manufacturer's recommendations. However, it was developed specifically for rat brain material.

1. Pipet (Eppendorf Pipet, Eppendorf, Wesseling-Berzdorf, Germany)
   30µl RLT Buffer (with 1% β-mercaptoethanol, AppliChem) in the cap of a microtube.
2. Lasermicrodissect the tissue and collect the tissue piece in the microtube cap
3. Transfer the probe immediately on ice
4. Spin down (Centrifuge 5417 R, Eppendorf)
5. Freeze-thaw the probe 3 times in dry ice / ethanol bath
6. Add the probe in 320ml RLT buffer and mix it carefully by up-down pipeting

7. Add lysate on column, spin down (20sec, 10000 revolutions per minute (rpm), RT), and trash discharge
8. Add 350µl RW1 buffer on column, spin down (15sec, 10000rpm, RT), and trash discharge
9. Mix 10µl DNaseI (dissolved in 550µl RNase free water) and 70µl RDD buffer very carefully by swivel the tube
10. Add the DNaseI solution on column incubating for 20min (RT)
11. Add 350µl RW1 buffer on column, spin down (15sec, 10000rpm, RT), and trash discharge
12. Put the column in a new tube, add 500µl RPE buffer, spin down (20sec, 10000rpm, RT), and trash discharge
13. Add 500µl RPE buffer, spin down (2min, 10000rpm, RT), trash discharge, and spin again (1min, 14000rpm, RT)
14. Put the column in a new tube, add 30µl RNase free water incubating for 5min (RT), and spin down (1min, 12000rpm, RT)
15. Put the discharge on the column, and spin down (1min, 12000rpm, RT)
16. Add 46.125µl mastermix to the 30µl discharge for the reverse transcriptase reaction and mix it carefully.
17. Reverse transcriptase reaction occurs during incubation for 10min at RT, and 1h at 48^0C (Thermomixer Compact, Eppendorf).
18. Finish procedure with heating 5min at 95^0C (Thermomixer Compact, Eppendorf), immediatly freezing on ice, and a final down spin.

Quantitative reverse transcription-PCR (qPCR)

CDNAs were subjected to qPCR utilizing the Abi Prism 7000 Sequence Detection System (Applied Biosystems) and TaqMan Universal PCR Master Mix (Applied Biosystems). NG2 primers and an intron-spanning probe bridging the border between Exon 6 and Exon 7 of the NG2 gene were selected using the Primer Express software (Applied Biosystems). This ensured the specific detection of rat NG2 mRNA without amplification of genomic DNA. This region corresponds to the extracellular tail of the mature NG2 protein. For normalization of C_T-values to an endogenous control, eukaryotic 18S ribosomal RNA primers and probe were used. The probes were labeled with 6-carboxyflurescein (FAM) at

the 3`end and with 6-carbox-tetramethyl-rhodamine (TAMRA) at the 5`end. The following rat-specific primers were used: NG2: 5`-TGG ACA GCG GGC TTG TG-3` (sense), 5`-CGC CAT CAG AGA GGT CGA A-3` (antisense), 5`-TTC TCA CAG AGA GGA GCC CTG GAA GG-3` (probe). 18S: 5'-CGG CTA CCA CAT CCA AGG AA-3' (sense), 5'-GCT GGA ATT ACC GCG GCT-3` (antisense), 5`-TGC TGG CAC CAG ACT TGC CCT C-3` (probe).

For amplification of target sequences, a standard amplification program was used: 1 cycle of 50^0C/2min, 1 cycle of 95^0C/10min, 45 cycles of 95^0C/15sec, and one cycle of 60^0C/1min Then, a relative quantitation of target cDNA expressed in relative x-fold differences was performed. All quantifications were normalized to the endogenous control (18S rRNA) to account for variability in the initial concentration and in the conversion efficiency of the reverse transcription reaction. For relative quantification of NG2 mRNA expression following ECL, the NG2 mRNA level measured in the OML of non-lesioned control animals was set as reference level (equivalent: one). NG2 mRNA levels measured in lesioned animals were expressed as x-fold changes compared to the reference (control) level.

Statistical analysis was performed using SPSS for Windows (SPSS, München, Germany). The non-parametric Kruskal-Wallis-test was used to compare differences among the groups investigated and pair-wise analysis was performed using the Mann Whitney U-test. Differences were considered statistically significant if the p-value was $p \leq 0.05$.

Immunohistochemistry

In this study, standard immunohistochemical procedures were employed. These involved: (1) specific antigen binding by a primary antibody, (2) binding of a secondary antibody, either a fluorocrome conjugated or a biotin-conjugated antibody to the tissue bound antibody-antigen complex, and finally (3) detection of the tissue bound biotin using the well established avidin-biotin-peroxidase system, and Diaminobenzidine (DAB) as a chromogen (Hsu et al., 1981).

Optimal conditions for the immunohistochemical detection were determined for each individual antibody. Conditions depended on antigen availability, antigen antibody affinity,

antibody type, and detection enhancement method. The working dilutions for the antibodies were optimized for each system in which they were employed. The working dilution was considered optimal if a strong specific antigen staining and a weak non-specific background, i.e. a good signal-to-noise ratio, was obtained.

Staining protocols

For light microscopy, free-floating sections were washed in phosphate buffered saline (PBS) and incubated in PBS containing 10% methanol and 3% hydrogen peroxide (H_2O_2) for 0.5h to reduce endogenous peroxidase activity. Then, the sections were washed (3 x 4min) and incubated in 5% bovine serum albumin (BSA, 1h) to reduce the unspecific staining. Subsequently, the sections were incubated for two days at 4°C in the primary antibody solution (polyclonal rabbit anti-NG2, 1:1000 in 0.1M PBS, containing 1% BSA, Chemicon, Temecula, USA). For the immunohistochemical detection of the primary antibody, a secondary biotinylated antibody was used (1:200, RT, biotinylated anti-rabbit; Vector Laboratories, Burlingame, USA). Then, sections were washed (3 x 4min)and transferred to ABC solution (ABC-Elite, Vector Laboratories) for 2h. After washing the sections in PBS, they were stained with a solution containing 0.0025% DAB, 0.005% ammonium-nickel-sulphate, and 0.006% cobalt-chloride activated with 0.001% H_2O_2. Finally, the sections were mounted onto slides, dehydrated through an ascending ethanol series, and coverslipped. Sections were digitally photographed (Olympus BX-61).

Double-immunofluorescence was employed for further characterization of NG2-positive cells. Free-floating sections were washed in PBS (3 x 4min), incubated in 5% BSA (1h) to reduce unspecific staining. Subsequently, they were incubated in either anti-NG2 mouse or rabbit antiserum (1:1000, 1% BSA, 4°C, 2d, Chemicon), depending on the species in which the second antiserum for the second marker was generated. After washings in PBS (3 x 4min), sections were incubated with an Alexa 568-labeled goat anti-rabbit or anti-mouse secondary antibody (1:1000, 1% BSA, RT, 2h, Molecular Probes, Eugene, USA). Then, sections were incubated (24h, 4°C) with the following glial markers:

1.) The astrocyte marker mouse anti-glial fibrillary acidic protein (GFAP, 1:1000, Sigma-Aldrich, Taufkirchen, Germany), or the astrocyte marker mouse anti-S100

Beta (1:1000, Sigma) which is a marker for the calcium binding protein found in the cell bodies of astrocytes.

2.) The microglia/macrophage marker rabbit anti-ionised calcium binding adapter molecule 1 (IBA1, 1:1000, 1% BSA, Wako Chemicals, Neuss, Germany).

3.) With the adult oligodendrocyte marker mouse anti-myelin phospholipid protein (PLP, 1:1000, Serotec, Kidlington, UK) or with the oligodendrocyte precursor marker, rabbit anti-platelet-derived-growth factor-receptor-α (PDGFR-α, 1:1000, Abcam, Cambridge, UK)

After washing (0.1M PBS, 3 x 4min), sections were incubated (2h, RT) with an Alexa 488 labeled goat anti-rabbit (for IBA1) or a goat anti-mouse antibody (for PLP and GFAP). Finally, sections were washed (3 x 4min), transferred onto glass slides, and mounted under glass coverslips with anti-fading fluorescent mounting medium (DAKO©, Dako). Double-labelled sections were analysed with a Zeiss LSM 510 laser scanning microscope (Zeiss, Jena, Germany) using a Zeiss 20 x lens (NA 0.75) and 7 x digital zoom.

For immunohistochemical detection of BrdU-labeled cell nuclei, free floating sections were preincubated in 15% methanol and 3% H_2O_2 (30min) followed by washing in PBS (3 x 7min). DNA was denatured to expose the antigen by incubation in 2x sodium saline citrate (SSC, 0,3M NaCl and 0,03M sodium citrate,) for 15min at RT, then for 5min at 60°C, followed by incubation in 2N HCl for 30min at 37°C. Acid was neutralized in 0,1M borate buffer (pH 8.5, 10min) and washed in PBS (3 x 7min). The sections were blocked with 10% goat serum and 10% bovine serum albumin in PBS (1h). Then, sections were incubated for double-labeling with mouse monoclonal anti-BrdU antibody (1:500, Amersham, Buckinghampshire, UK) and rabbit polyclonal antibody against NG2 (1:500, Chemicon) at 4°C overnight. After being washed in PBS immunofluorescence staining was performed with species specific Alexa fluor secondary antibodies (1:1000, Molecular Probes) for 2h. Finally, sections were washed (3 x 7min) and mounted under glass coverslips using fluorescent mounting medium (DakoCymation).

Buffer solutions

The following buffer solutions were used:

1.) PBS (phosphate buffered saline) is made up of 2 different phosphate-sodium solutions, and contains 0.9% NaCl (AppliChem, Darmstadt, Germany). Used for applications at 0.1M:
Stocksolution: 0.4M (pH 7.4)
Solution 1 (0.4M): 7.12% Na_2HPO_4 x $2H_2O$
Solution 2 (0.4M): 5.5% NaH_2PO_4 x H_20

2.) TBS (Tris buffered saline) 0.05M, pH 7.4:
0.6% Tris (Tris (hydroxymethyl)methylamine, $C_4H_{11}NO_3$, AppliChem), 0.9%, NaCl,
0.001% Thimerosal ($C_9H_9HgNaO_2S$, AppliChem)

Transmission electron microscopy

For electron microscopy, NG2-immunostained sections were processed according to standard procedures (Bolam, 1992). In short: NG2 immunostained sections were osmicated (0.5% OsO_4 in 0.1M PBS, 30min), washed in PBS (3 x 5min), and dehydrated in ethanol (30%, 50%, 70%, each: 5min). Then, sections were contrasted (70% ethanol containing 1% uranyl acetate) and onward dehydrated in an ascending ethanol series (80%, 90%, 96%, 100%, each: 10min). Then, the sections were embedded two times in propylene oxide (5min), followed by a mixture of propylene oxide/durcopan (1:1, 1h), and finally, in 100% Durcopan over night (Durcopan ACM-Kit 44610-1EA, Fluka, Taufkirchen, Germany). For sectioning, sections were embedded in blocks and ultrathin sections were collected on single-slot Formvar-coated copper grids, and examined using a Zeiss electron microscope (EM 109, Zeiss).

Bromo-deoxyuridine injection

5´-bromo-2´-deoxyuridine (BrdU) is a thymidine analog which is selectively incorporated into the DNA of proliferating cells providing a marker for the DNA being replicated. The number, and the identity of proliferating cells can be detected in tissue sections by, e.g. immunoflurescensce staining. In this study, BrdU-labeling was employed to detect NG2-cells generated after ECL. Control and lesioned rats received intraperitoneal injections of BrdU (Sigma-Aldrich; BrdU dissolved in 0.9% NaCl with 0,025M NaOH; 50mg/kg body weight) for 9 days, beginning on the day of lesioning. On day 10 after ECL animals were deeply anesthetized and perfused (4% paraformaldehyde, 0.1 M PB).

Digital processing of illustrations

All photographic images were obtained using a digital camera and subsequently processed using a commercial available graphics software (Adobe Photoshop 6.01). Single fluorescent images of the same section were digitally superimposed. All images required minor adjustment of contrast, brightness, and sharpness. No additional image alteration was performed.

Results

Amount of NG2 mRNA is significantly increased in the denervated outer molecular layer

To determine whether and to which extent the expression of NG2 mRNA is regulated following entorhinal denervation, the amount of NG2 mRNA was quantified in the OML at different time-points following ECL. Laser microdissection of the OML was employed to collect tissue specifically from the zone of denervation and qPCR was used to measure NG2 mRNA levels in these tissue samples (Fig. 7 b, c).

OMLs from three section per animal were collected. In total, four animals were used per time-point. The OMLs of the non-lesioned animals were used as controls and the NG2 level of these tissue samples was used as reference level (equivalent: one). NG2 mRNA expression levels were measured at 6h, 12h, 2d, 4d, and 7 days post lesion and compared to control levels (Fig. 7 c). Relative quantification of NG2 mRNA levels revealed no significant increase of NG2 mRNA at 6h and 12h. However, at 2 days post lesion and 4 days post lesion a 2.5-fold and a 2.0-fold increase of N G2 mRNA expression levels was detected, respectively (Fig. 7 c). At 7 days post lesion, NG2 mRNA expression had returned to control values (Fig. 7 c).
In summary, the amount of NG2 mRNA in the denervated OML was significantly increased by 2 and 4 days post lesion compared to amount of NG2 mRNA in control rats.

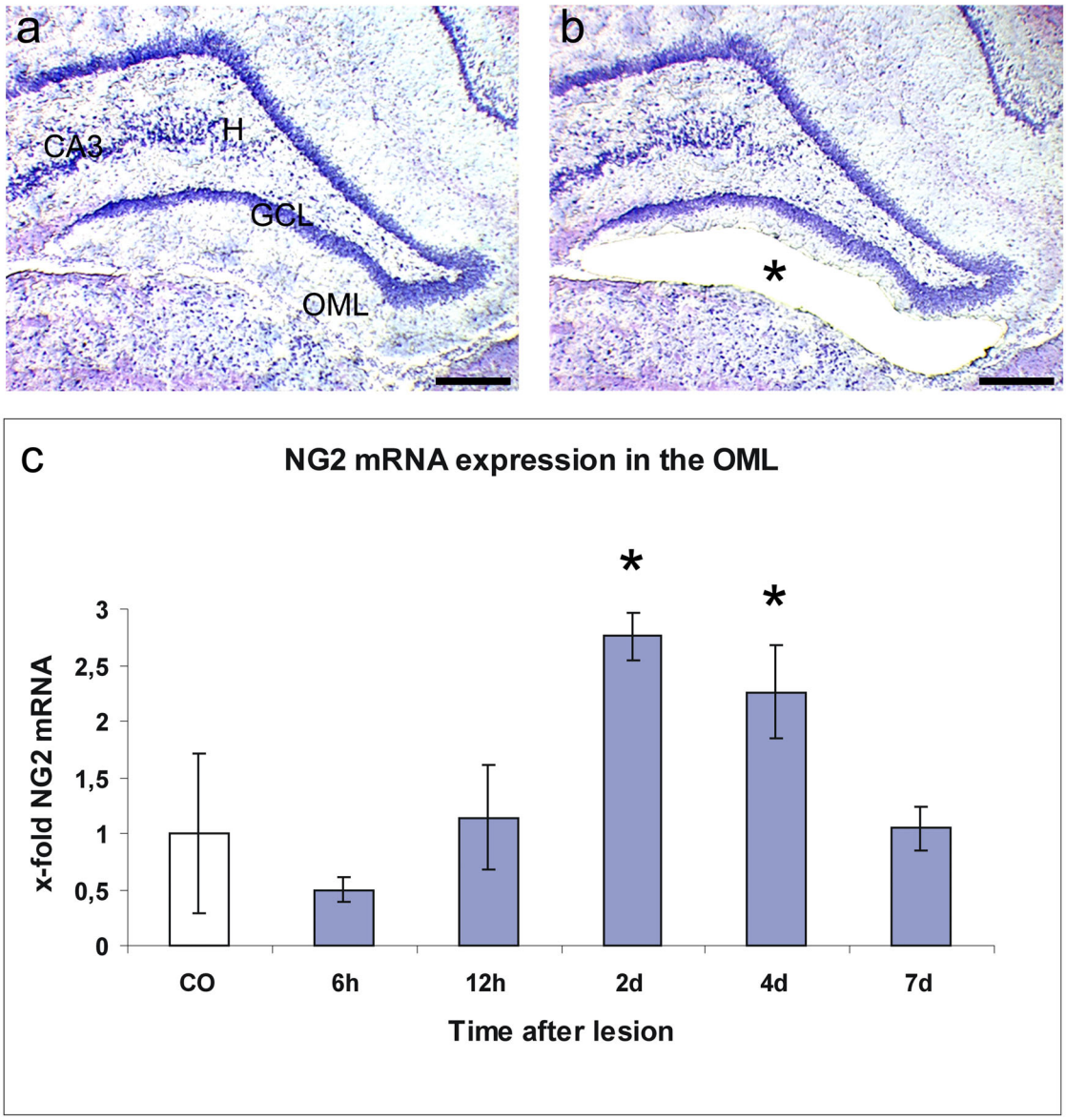

Figure 7 Significant increase of NG2 mRNA expression levels in the outer molecular layer following entorhinal cortex lesion

a, b. Frontal section of the rat fascia dentata stained with toluidine blue. Same section before (a), and after (b) LMD of the OML. The asterisk indicates the area of the collected tissue piece that had been cut.

c. Quantitative RT-PCR for NG2 mRNA in microdissected OMLs. Relative quantification of NG2 mRNA expression levels showed statistically significant increases ($p<0.05$) of NG2 mRNA by day 2, and day 4 post lesion compared to controls (CO). Error bars: standard deviation (SD), Scale bars: 200µm

CA3: hippocampal subfield CA3, ECL: entorhinal cortex lesion, GCL: granule cell layer, H: hilus, IML: inner molecular layer, LMD: lasermicrodissection, OML: outer molecular layer.

NG2-positive cells are regularly distributed in the dentate gyrus of control rats

In adult rats without lesion, NG2-positive cells were found throughout the entire fascia dentata. These cells appeared to be regularly distributed throughout the dentate gyrus (Fig. 8 a). Numerous NG2-positive cells were present in all regions of the dentate gyrus, without any conspicuous accumulation in any layer. Additionally, no differences in the intensity of the NG2 immunostaining could be observed between the layers of the dentate gyrus.

Within the molecular layer the distribution of NG2-positive cells was also regular (Fig. 8 a, c). The molecular layer could not be subdivided into the IML and the OML, because no border between the IML and OML was detectable on the basis of the NG2 immunostaining (Fig. 8 c). In the granule cell layer, NG2-positive cell bodies were infrequently found. The NG2-positive immunostaining, which was observed in the granule cell layer, usually originated from pervading processes of NG2-positive cells that were located in neighbouring layers: the subgranular zone and the IML. In the subgranular zone some NG2-immunopositive cells were found. Higher magnification revealed the typical cellular morphology of single NG2-positive cells: Both, in rats without lesion and in rats with ECL, NG2-positive cells exhibited an oval cell body that measured 10 - 12µm in diameter. From the NG2 cell soma, several radially oriented processes extended outwards in all directions. The processes of NG2-positive cells were highly ramified and frequently more than 60µm long (Fig. 8 c). The intensity of the immunostaining of NG2-positive cells was very similar in all layers, i. e. no difference in the staining intensity of single NG2-positive cells was observed between the layers of the fascia dentata (Fig. 8 a, c). In contrast to lesioned rats, no NG2-positive staining was observed in the neuropil surrounding NG2-positive somata and processes in control rats (Fig. 8 a, c).

Staining of NG2-positive cells is enhanced in the denervated outer molecular layer

The distribution and staining pattern of NG2-positive was investigated at different time-points following lesion (1, 4, 7, 10, 14, 30 days post lesion, and 6 month post lesion). Following ECL, the regular pattern of NG2-immunostaining in the dentate gyrus was changed:

At 1 day post lesion, an increase in NG2-immunostaining was barely detectable in the molecular layer. Only a weak difference in the intensity of the immunostaining was observed within the molecular layer, e.g. between the OML and IML (Fig. 8 d). However, at 4 days post lesion NG2-immunostaining was qualitatively enhanced in the denervated OML, whereas the intensity of NG2-immunostaining in the IML was not (Fig. 8 e). In contrast to the OML, the intensity of NG2-immunostaining in the IML appeared to remain at control levels. A further qualitative increase in NG2-immunolabeling was observed in the OML at 7 days post lesion, and at 10 days post lesion (Fig. 8 f, g). At 10 days post lesion, the immunostaining for NG2 reached its maximum intensity in the OML (Fig. 8 b, g). Thereafter, at 14 days post lesion and at 30 days post lesion, NG2-immunolabeling in the OML gradually decreased (Fig. 8 h, i), and returned to control levels within 6 month post lesion (Fig. 8 j).

As described above, the intensification of NG2 immunostaining was observable specifically in the denervated OML. In the other layers of the dentate gyrus, e.g. the IML, no enhanced immunostaining was detectable at any time-point following the lesion. In other words, enhanced NG2-immunostaining in the denervated OML was restricted to the denervated OML, and was not found in other layers of the fascia dentata. Since the increase in NG2-staining in lesioned animals was highly localized to the denervated OML, the OML could readily be delineated from the non-denervated IML (Fig 8 b). Hence, at 4, 7, and 10 days post lesion, the border between the OML and the IML was clearly discernable (Fig. 8 e, f, g).

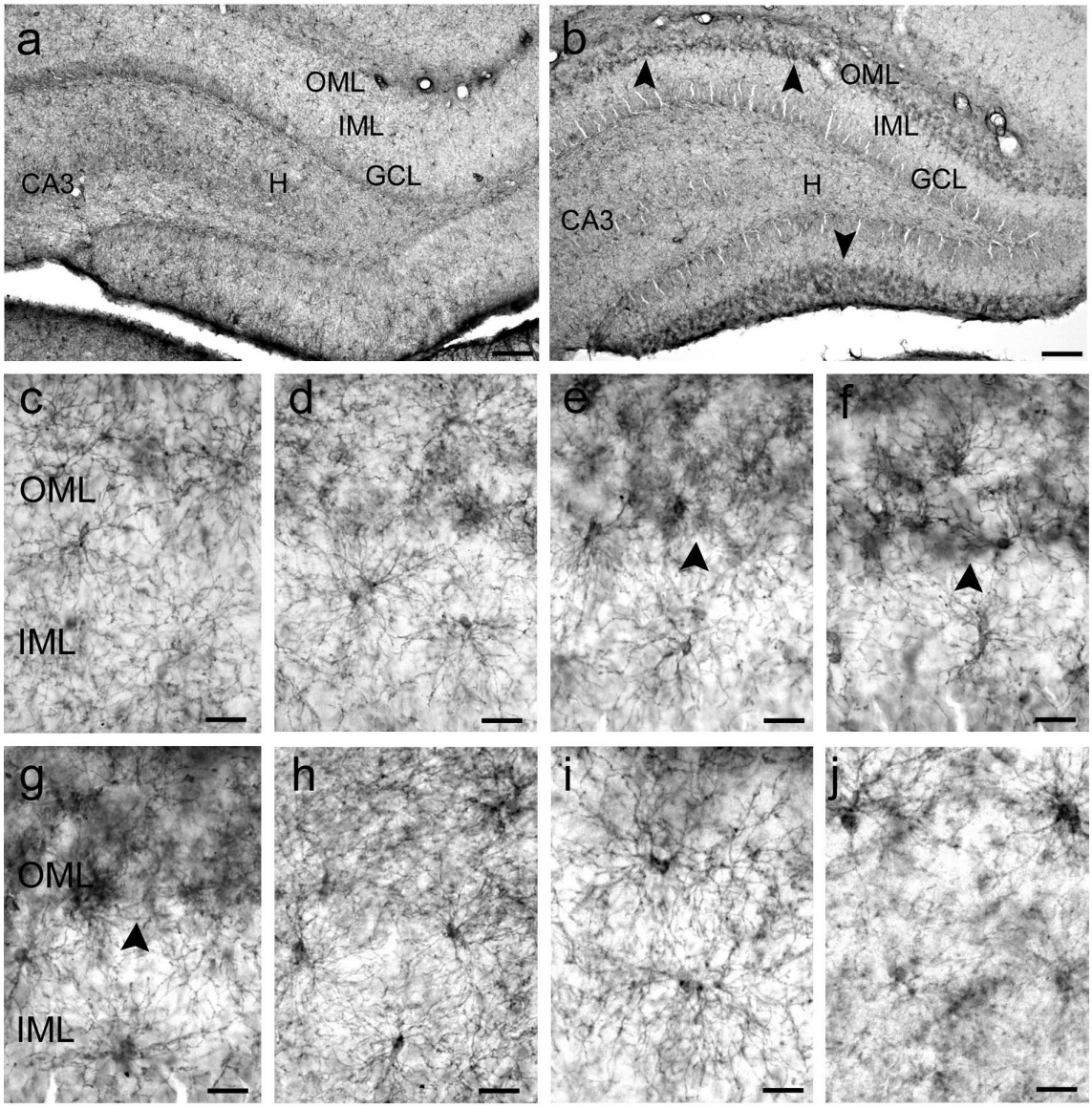

Figure 8 *NG2 immunohistochemistry of the rat fascia dentata at different time points following lesion*

a, b. Fascia dentata of a control rat (a) and a rat 10 days after ECL (b) stained for NG2. Whereas NG2-immunostaining is fairly homogeneous in the control (a), it is clearly enhanced in the OML of the lesioned animal (b). Note that the increased immunolabeling of the OML sharply delineates the denervated zone from the non-denervated IML (arrowheads).

c – j. Portions of the fascia dentata of a control animal (c) and animals at different time-points following lesion. 1 day (d), 4 days (e), 7 days (f), 10 days (g), 14 days (h), 30 days (i), and 6 months (j). Compared to controls, NG2-immunostaining is qualitatively enhanced in the OML by 1dpl, strongest around 10dpl, and returned to control levels within 6 month pl. Scale bars: a, b: 200µm; c - j: 30µm

CA3: hippocampal subfield CA3, dpl: days post lesion, ECL: entorhinal cortex lesion, GCL: granule cell layer, H: hilus, IML: inner molecular layer, OML: outer molecular layer, pl: post lesion.

The neuropil of the denervated outer molecular layer is enriched with NG2 following entorhinal cortex lesion

At higher magnification, the morphology of single NG2-positive cells could be analysed. At this level of resolution, "cloudy" NG2-positive material was revealed in the neuropil surrounding the NG2-positive cell bodies and processes following ECL (Fig. 9 c). In control animals without lesion, no NG2-immunostaining was observed in the tissue surrounding the NG2-positive cell bodies (Fig. 9 a). Likewise, the tissue between their processes was unstained. In the control situation, the NG2-positive cells showed a clear and distinct staining of their cell bodies and processes (Fig. 9 a). In contrast, a dense cloudy staining appeared around NG2-positive cells and their processes between 4 days post lesion and 14 days post lesion (Fig. 8 e - g). This cloudy staining emerged exclusively in the OML. It increased with time following ECL between 1, 4, and 7 days, and reached a maximum of intensity at 10 days post lesion (Fig. 8 b, g, 9 c). Finally, at 6 months post lesion the cloudy staining had disappeared around the NG2-positive cells in the OML (Fig. 8 j, 9 b).

The appearance of these NG2-positive clouds suggest that NG2 might be released into the ECM of the denervated OML. To test this, electron microscopy was performed of NG2-positive cells in the denervated OML 10 days post lesion.

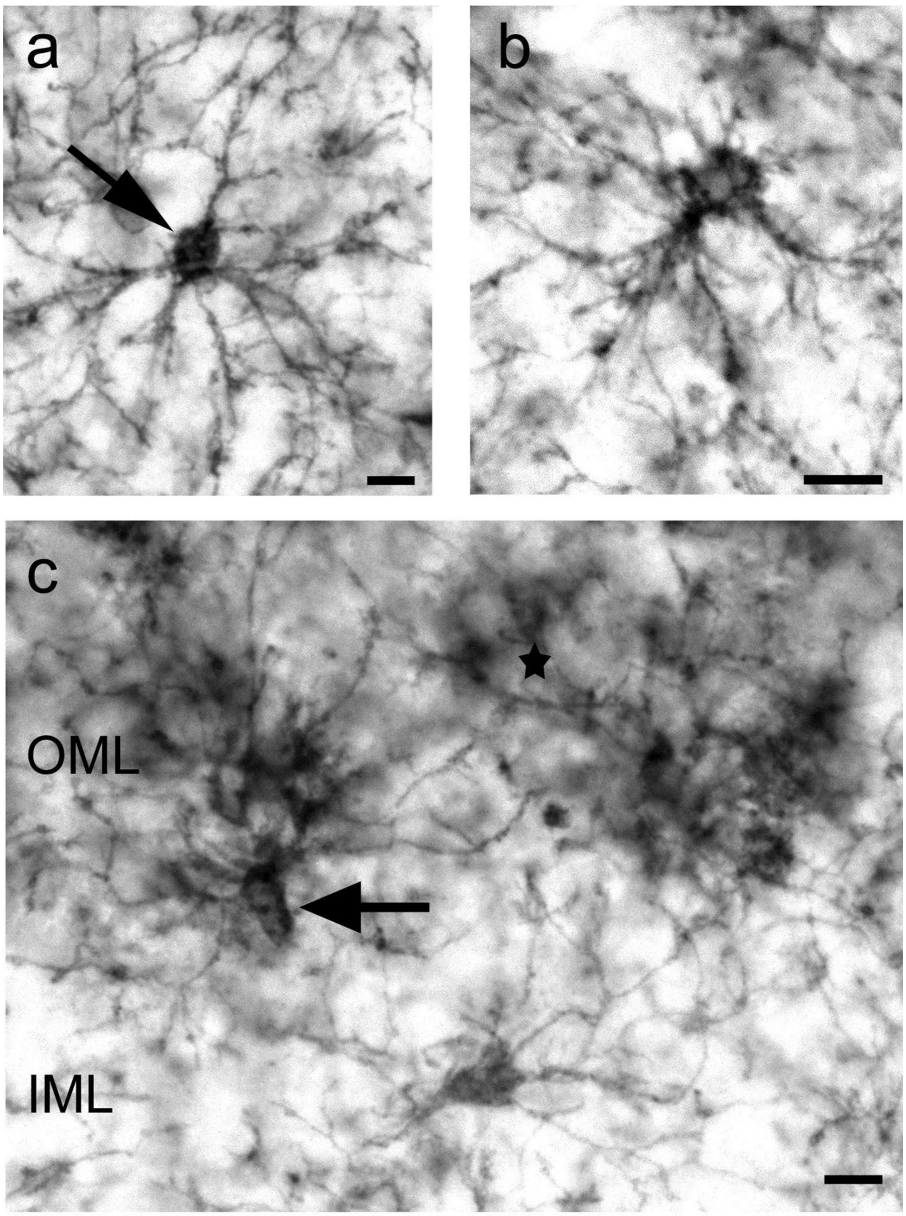

Figure 9 *Morphology of NG2-positive cells in the outer molecular layer of control and lesioned rats*

a. NG2-positive cell in the OML of a control animal. The cell body (arrow) and several processes of the cell are distinctly labeled. Note, that the space between the processes of the NG2-positive cell does not contain immunocytochemically detectable amounts of NG2.

b. NG2-positive cell in the OML at 6 month pl. The morphology of this cell closely resembles that of the NG2- positive cells found in controls.

c. NG2-positive cells in the OML and IML at 10dpl. The somata of individual NG2-positive cells can readily be recognized (arrow). NG2-positive processes located in the OML are surrounded by clouds of NG2-immunopositive material (asterisk), suggesting that NG2 is secreted into the surrounding extracellular matrix. Scale bars: 10μm; dpl: days post lesion, IML: inner molecular layer, OML: outer molecular layer, pl: post lesion

Electron microscopy of NG2-positive cells in the outer molecular layer: Immunoprecipitate in the extracellular matrix surrounding glial and neuronal profiles

Electron micrographs of NG2-positive cells in the OML 10 days post lesion revealed a close association of NG2-immunoprecipitate with glial surfaces. Characteristically, immunoprecipitate was found on the plasmalemma of NG2-positive glial cells in the denervated OML (Fig. 10 a - c). These cells typically showed a fairly large (8 x 12µm) and more or less ovoid nucleus surrounded by a thin ring of cytoplasm (10 a). From the cell soma several NG2-immunopositive processes extended outwards into the surrounding neuropil (10 a, b, c). In some cases, a weak staining of NG2-immunoprecipitate attached to intracellular membranes was also observed (Fig. 10 b). To verify this observation, further analysis using immunogold techniques will be required. Interestingly, immunoprecipitate was also observed in the extracellular matrix surrounding various profiles in the neuropil (Fig. 10 d, e). NG2- immunoprecipitate was closely associated with glial as well as neuronal structures. It could be observed in the extracellular matrix surrounding synapses (Fig. 10 d), degenerating boutons (Fig. 10 d), spines (Fig. 10 d, e), and dendrites (Fig. 10 f).

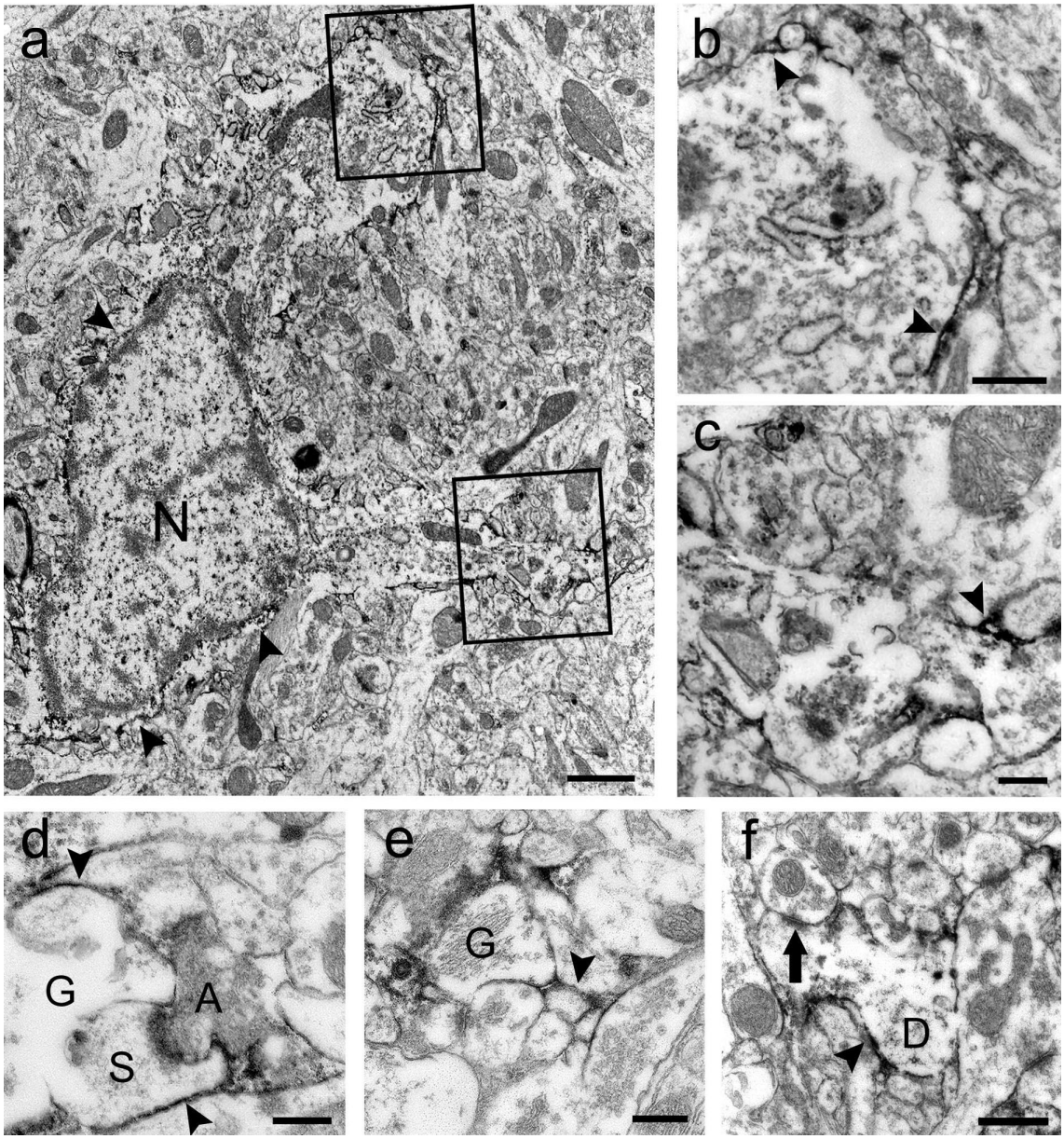

Figure 10 *Electron micrographs of NG2-immunopositive structures in the outer molecular layer 10 days after entorhinal cortex lesion*

a. Electron micrograph of a NG2-positive cell in the OML following ECL. The soma is indicated by arrowheads. Boxed areas are shown at higher magnification in b, and c.

b, c. Higher magnification of the NG2 processes in a (framed areas). The NG2-immunoprecipitate is associated with the plasmalemmal surface of the cell (arrowheads).

d. NG2-immunoprecipitate (arrowheads) found in the extracellular matrix surrounding a degenerating axon terminal (A), which forms a synapse with a postsynaptic spine (S). A glial process (G) is present in the vicinity.

e. NG2-positive material is found in the neuropil (arrowhead). The surface of numerous profiles is labeled.

f. NG2-immunoprecipitate surrounds a putative dendrite (D). A presynaptic terminal forms a synapse (arrow) with the dendritic shaft.

Scale bars: a: 2µm; b: 0.25µm, c: 0.1µm; d: 0.25µm; e: 0.25µm; f: 0.5µm;
ECL: entorhinal cortex lesion, OML: outer molecular layer

NG2-positive cells do not express astroglial, microglial, and adult oligodendroglial markers

In order to determine the cellular identity of the glial cell type producing NG2 in the denervated zone following ECL, double-immunofluorescence was used. NG2 immunostaining was combined with the following markers for glial cell types that could produce NG2 in the brain:

1.) GFAP and S100 Beta for astrocytes. The staining of GFAP- positive astrocytes depends on the phosphorylation status of the glial fibrillary acid protein (Stichel et al., 1991). For this reason, double-labeling experiments were also performed with the astrocyte marker S100 Beta.

2.) IBA1 for microglia/macrophages

3.) PLP for adult oligodendrocytes, and PDGFR-α for oligodendrocyte precursor cells.

Sections were analysed using confocal microscopy to unequivocally identify double-labelled cells. In case of the PDGFR-α/NG2 double-immunofluorescence experiments, no specific PDGFR- α staining of oligodendrocyte precursor cells could be obtained (data not shown). In case of the S100 Beta/NG2 double-immunofluorescence experiments, S100 Beta-positive astrocytes were labeled, but these cells were not double-labelled with NG2 (data not shown). Thus, no conclusive results were obtained in these experiments.

In the double-labeling experiments of NG2 with one of the other three glial markers (i.e. GFAP, IBA1, PLP) no colocalisation was found. Although the OML of control and EC-lesioned rats was carefully scanned and all cell populations were robustly stained, no double-labeling of NG2-positive cells with any of these three markers was observed in the molecular layer (Fig. 11 a - f). This suggests, that NG2-positive cells do not express typical markers for astrocytes, microglia/macrophages or adult oligodendrocytes in the unlesioned as well as the denervated dentate gyrus. Thus, NG2-positive cells could represent a distinct glial cell type.

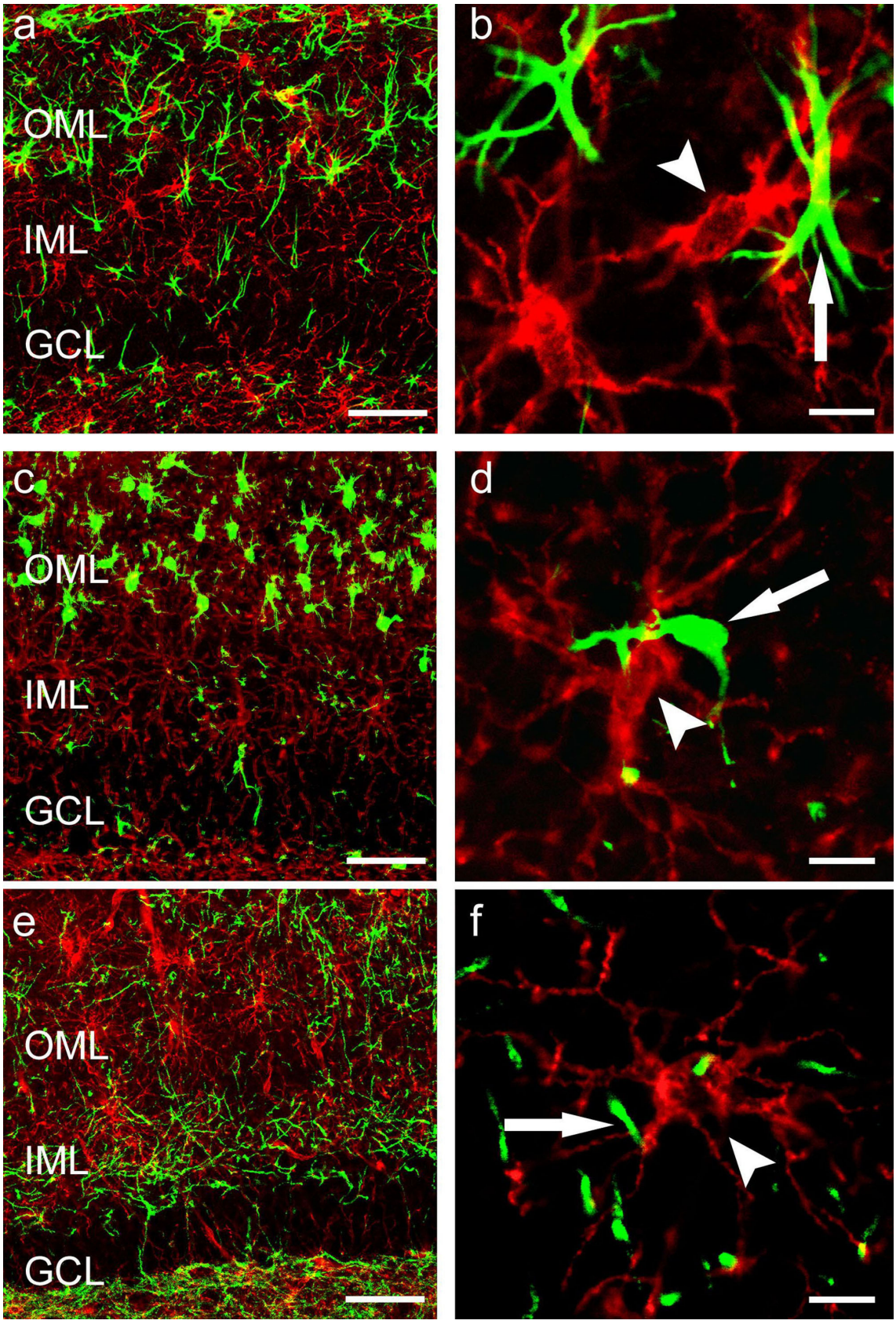

Figure 11 *Double-labelings of NG2-positive cells in the fascia dentata 10 days post lesion for astroglial (GFAP), microglial (IBA1), and oligodendroglial (PLP) markers*

a, b. Portion of the fascia dentata stained for NG2 (red) and the astroglial marker GFAP (green). Confocal microscopy revealed that NG2 did not label astrocytes.

c, d. Portion of the fascia dentata stained for NG2 (red) and the microglial marker IBA1 (green). NG2 and IBA1 labelled distinct cell populations.

e, f. Portion of the fascia dentata stained for NG2 (red) and the oligodendroglial marker PLP (green). There was no overlap of NG2 and PLP-positive structures.

Scale bars: a, c, e: 150µm; b, d, f: 10µm.
GFAP: glial fibrillary acid protein, IBAI: ionised calcium binding adapter molecule 1; PLP: myelin phospholipid protein

A subpopulation of NG2-positive cells is generated postlesion in the outer molecular layer

To receive an impression whether the increase of NG2 mRNA and the accumulation of NG2-immunoreactivity in the OML could be caused, at least in part, by the generation of new NG2-positive cells, incorporation of BrdU was studied after ECL. Control rats and EC-lesioned rats received BrdU injections for 9 consecutive days (beginning on the day of lesioning) and were transcsardially perfused at day 10 post lesion. Then, sections were double-labeled for NG2 and BrdU, and analysed using confocal microscopy to unequivocally identify double-labelled cells. In control rats, no NG2/BrdU double-labeled cells were found in the OML. In these animals, BrdU-positive cells were rarely found in the subgranular zone, the zone of hippocampal neurogenesis. None of these cells was NG2-positive (Fig. 12 a).

In contrast, in rats with ECL several BrdU/NG2-double-labeled cells were additionally found in the OML indicating that some NG2-positive cells are generated post lesion (Fig. 12 b). This suggests that generated cells could contribute to the increase of NG2 mRNA and protein observed in the denervated OML of the dentate gyrus.

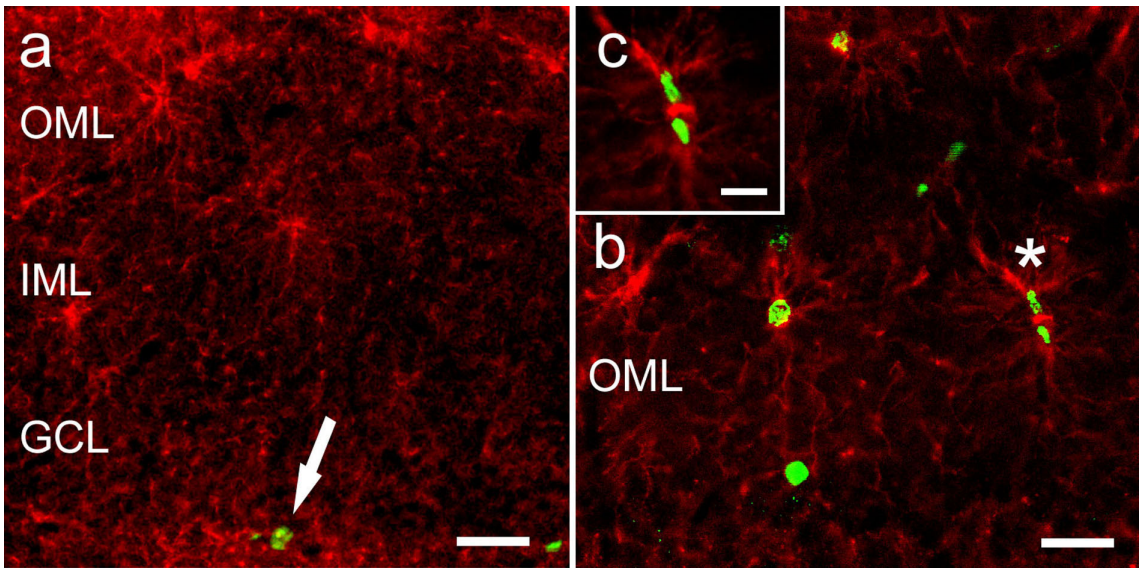

Figure 12 *Double-labeling of NG2-positive cells with the proliferation marker 5′-bromo-2′-deoxyuridine in the dentate gyrus of unlesioned control and after 10 days post lesion*

a. Portion of a non-denervated rat fascia dentata (control) stained for NG2 (red) and the proliferation marker BrdU (green). Confocal microscopy revealed no double-labeled cells. The arrow points to a BrdU-positive nucleus in the subgranular zone.

b, c. Portion of a denervated rat fascia dentata (10dpl) stained for NG2 (red) and the proliferation marker BrdU (green). Confocal microscopy revealed some double-labeled cells in the OML. The asterisk indicates two NG2/BrdU double-labeled cells. These cells were generated during the postlesional period. Higher magnification of these NG2/BrdU double-labeled cells shown in c.

Scale bars: a, b: 50µm, c: 20µm
BrdU: 5´-bromo-2´-deoxyuridine, dpl: days post lesion, ECL: entorhinal cortex lesion, OML: outer molecular layer

Discussion

Summary of results

To study the role of the CSPG NG2 for the reorganization of the adult dentate gyrus following ECL, the distribution pattern of the CSPG NG2 was analysed at different time-points post lesion on the mRNA and protein levels. Electron microscopy was performed to localize NG2 on the ultrastructural level. NG2 producing cells were further characterised using double-labeling with glial markers for astrocytes, microglia/macrophages and adult oligodendrocytes. Finally, it was investigated whether some of the NG2-positive cells are generated post lesion using the proliferation marker BrdU.

Lasermicrodissection and subsequent qPCR of the OML revealed a significant increase of the amount of NG2 mRNA at day 2 and day 4 post lesion. Immunohistochemistry of NG2 protein was highly increased specifically in the OML between day 4 and day 10 post lesion. This layer-specific increase resulted in a sharp NG2-positive border between the denervated OML and the non-denervated IML. Furthermore, a cloudy staining was clearly detectable around the cell bodies and processes of NG2-positive cells at 10 days post lesion. Ultrastructural analysis of NG2-positive cells at 10 days post lesion revealed NG2 immunoprecipitate not only on glial surfaces but also in the ECM surrounding neuronal and glial structures. The double-labelings of NG2-positive cells with glial markers for astrocytes, microglia/macrophages, and adult oligodendrocytes did not show any colocalisation of NG2-positive cells with GFAP, IBAI, or PLP. Finally, incorporated BrdU showed that some NG2-positive cells are generated following ECL.
After some methodological considerations, the data will be discussed in the context of the axonal growth regulating properties of NG2. Finally, a role for NG2 in the denervated dentate gyrus will be proposed.

Methodological considerations

Combined lasermicrodissection and quantitative RT-PCR

General aspects

Standard precautions during LMD and subsequent qPCR were reviewed in detail by Fink et al. (Fink and Bohle, 2002). Briefly, all solutions must be prepared with RNAse-free water and all nescessary equipments should be sterile. To effectively remove nucleic acid contaminations or RNAses, all contact areas must be carefully cleaned, e.g. with the commercially available RNAseZap (Sigma). To decrease the risk of contamination with nonnative DNA and as a result false positive findings, tissue preparation, microdissection, and RNA isolation should be separated from RT-PCR reactions. Therefore, work should only be done in separately designated RNA-areas, including the site where microdissection is performed, each equipped with a separate set of tools. Certainly, standard molecular biology rules must be followed. Noteworthy, the requirement of the toluidine blue solution is to be filtered in order to ensure the application of a sterile solution onto the slide. Furthermore, PET-foils stretched on a metal frame need to be autoclaved to avoid tissue contamination. To ensure that each single frame remains sterile, it is advisable to wrap each slide in an aluminium foil before autoclaving. Finally, the whole LMD procedure should last no longer than 2h to minimize the risk of RNA degradation. If all of these rules are observed, RNA degradation can be minimized.

Morphology, section thickness, and staining procedure

By using the Leica AS LMD system one is restricted to sections placed directly on a PET-foil stretched on a metal frame. Therefore, it was necessary to verify the morphology and quality of sections mounted on foil after application of standard histochemical stains. The morphological quality of sections after staining depended on their thickness, with thinner sections resulting in better quality. Therefore, section thickness should be minimized to improve staining. However, to reduce the time needed to collect a sufficient amount of tissue, section thickness should be maximized. The maximal section thickness itself was, in turn, limited by the force of the ultraviolet laser beam. Taken together, section thickness had to be balanced between requirements for good morphological quality and the maximal yield of tissue during LMD. Optimal results lead to a good staining and a good mRNA amount during LMD, and could be achieved using brain sections with a thickness of 20µm.

These sections were mounted on PET-foil, fixed with acetone, and finally stained with a modified toluidine blue staining, based on the original protocol by Romeis (1968). The fixation of the sections with acetone has, compared to non-alcoholic fixatives, a low rate of nucleic acid degradation and a preservative effect on RNA (Goldsworthy et al., 1999), and offered best morphological results with the toluidine blue staining. Since RNA degradation is known to occur primarily in an aqueous environment, staining time in watery solution could be decreased to 1min with no effect on staining quality. Furthermore, drying sections on a heater at 40^0C for 5min after staining procedure reduced the exposure of RNA to aqueous media, thereby minimizing RNA degradation during LMD preparation steps.

Total RNA isolation, reverse transcription, and quantitative RT-PCR

In this study the RNeasy Mini Kit (Quiagen) was used. No differences in RNA extraction were observed between the commercially available kits (ProPure, Arcturus; RNeasy Mini, Quiagen) or employing phenol/choloroform extraction utilizing Tri Reagent (Sigma) (Burbach et al., 2003). However, the RNeasy Mini Kit is advantageous since it offers the possibility for on site DNA digestion. This reduces the risk for RNA degradation because a DNA digestion step can be avoided. This step is necessary to avoid genomic DNA contamination if primers are selected within one 3´ exon region of the gene of interest and RNA and genomic DNA are not distinguishable during PCR. In this study, however, NG2 primers and an intron-spanning probe bridging the border between Exon 6 and Exon 7 of the NG2 gene were selected ensuring the specific detection of rat NG2 mRNA without amplification of genomic DNA. Additionally, the use of random hexamers is advisable, since this assures the transcription of all fragments even if minimal RNA degradation is present.

Quantitative RT-PCR after LMD of defined cell populations is a recently developed technique employed for gene expression profiling (Fink et al., 1998; Fink and Bohle, 2002). In this study, the relative quantification of gene expression levels was established by calculating fold-differences between control and target samples, since the absolute quantification of gene expression changes does not offer any further scientifically relevant information. After confirming the reproducibility of several assays utilizing standard curve analysis, gene expression was calculated by applying the comparative C_T-method using

arithmetic formulas to achieve the same result as the standard curve method (Applied Biosystems, User Bulletin #2: Relative Quantification of Gene Expression). This approach is particularly valuable if cDNA amounts are limited, which is the case if limited amounts of lasermicrodissected tissue are available.

Immunohistochemistry

In the presented study two different types of NG2 antibodies were used: a polyclonal and a monoclonal antibody. In short, polyclonal antibodies are usually raised in rabbits and produced by different b-lymphocytes. As a consequence, polyclonal antibodies are immunochemically heterogenous and react to different epitopes of the antigen against which they were generated. Monoclonal antibodies are usually raised in plasma cell clones (hybridoma cells) of mice and are immunochemically identical. Therefore, they react to only a single epitope of the antigen against which they were generated. The specificity of an antibody needs to be verified prior to its use and there are different methods to test its specificity. One method is the classical process of elimination, in which the staining procedure is performed sequentially without the individual reagents of the specific protocol. Additionally, one could perform pre-adsorptions experiments with the specific peptide or the protein that was used to generate the antiserum or the monoclonal antibody. The antibody should bind to this peptide which should result in an absence of staining.

The immunogenic peptides that were used to genereate the NG2 mono- and polyclonal antibodies were not commercially available. Therefore, the specificity of the NG2 antibodies was tested using the classical process of elimination, i. e. either the primary or the secondary antibody were omitted. Furthermore, the specificity of the commercially available polyclonal NG2 antibody (Chemicon) has been repeatedly tested using Western blot analysis (Davies et al., 2004). Additionally, the distribution of NG2-positive cells observed with the Chemicon antibody corresponds to the distribution of NG2-positive cells in the hippocampus as published by others, using the antibody generated by Stallcup (Bu et al., 2001). The monoclonal antibody against NG2 (Chemicon) used in the study was generated against a cell line expressing a truncated form of NG2, which is > 280kDa. The specific epitope of NG2 recognizing by the monoclonal antibody has not yet been published, but immunostainings of control sections using the monoclonal antibody resulted in the same cellular labeling pattern as with the polyclonal antibody. However,

immunostainings of denervated sections did not show the "cloudy precipitate", suggesting that the monoclonal antibody does not recognize all forms of proteolytically cleaved NG2.

Does NG2 affect axons within the zone of denervation? Regulation of branching and axonal complexity

In previous studies, changes of NG2-positive cells were studied at lesion sites and correlated with cellular and axonal growth processes occurring in the microenvironment of the glial scar (Norton et al., 1992; Kreutzberg, 1996; Levine et al., 2001; Chen et al., 2002a). Jones and coworkers (2002) reported that NG2-positive cells accumulated in the glial scar after spinal cord injury within 1 day, reached a maximum by 7 days, and remained elevated for weeks thereafter (Jones et al., 2002). Since the upregulation of NG2 temporally and spatially coincided with axonal regeneration failure and since NG2 has strong axon growth-inhibiting properties *in vitro*, it was suggested that NG2 could be a major obstacle for axonal regeneration (Fidler et al., 1999; Levine et al., 2001; Jones et al., 2003; Sandvig et al., 2004).

In the present work the ECL model was used to study postlesional changes of NG2 in a denervated zone: the denervated OML of the fascia dentata. Following ECL entorhinal axons degenerate resulting in a denervation of their target zone. Fibers from other brain areas that terminate in the OML (e.g. fibers from the contralateral entorhinal cortex and fibers from the medial septum) sprout new axon collaterals between 7 to 10 days post lesion. By 30 days post lesion these fibers have reinnervated the denervated OML and the sprouting process is complete (Lee et al., 1977; Loesche and Steward, 1977; Nadler et al., 1977; Deller and Frotscher, 1997).

A significant upregulation of NG2 mRNA was observed at day 2 and day 4 post lesion specifically in the denervated OML. At the protein level, NG2 accumulation was clearly detectable by day 4 post lesion, reached a maximum by 10 days post lesion and returned to control levels between 30 days and 6 months post lesion. Thus, NG2 was strongly upregulated within the denervated OML of the fascia dentata during the period of lesion-induced axonal growth. Interestingly, the time course of NG2 mRNA upregulation and of NG2 protein accumulation in the OML, are different. Whereas NG2 mRNA is maximally increased at 2 and 4 days post lesion, the immunohistochemically detectable maximum of NG2 protein accumulation is not until 10 days post lesion. However, this time span have

been shown for several CSPGs or ECM molecules in the denervated OML and could be explained by (1) the complex synthesis of chondroitinsulfate proteoglycans and by (2) the accumulation of the protein in the ECM of the denervated OML (Haas et al., 1999; Thon et al., 2000). Whereas mRNA levels may return to normal levels after day 4, the the full-length CSPG may accumulate in the denervated zone. In any case, NG2 mRNA amount and NG2 immunoreaction was significantly increased in the denervated OML during the time of collateral sprouting. Thus, accumulation of NG2 does not seem to impede this form of axonal growth, and collateral sprouting does occur in spite of the presence of NG2 in the zone of denervation.

The coincidence of NG2 upregulation and axonal regeneration failure at a CNS lesion site in the spinal cord on the one hand, and the coincidence of NG2-upregulation and robust sprouting in the denervated OML on the other hand seem to contradict each other. In the former case, NG2 correlates with the inhibition of axonal growth whereas in the latter case, NG2 correlates with the permission or the promotion of axonal growth. However, it should be kept in mind that the two types of axonal growth differ: Whereas axonal regeneration in the spinal cord following lesion is characterized by long-distance growth of injured axons, axonal sprouting is characterized by short-distance growth and axonal branching of unlesioned fibers. Nevertheless, injured axons which fail to regenerate across a lesion site exhibit a growth behaviour similar to that of sprouting axons following ECL. They grow for short distances towards the lesion site and branch off several sprouts before ceasing growth, a process often referred to as "regenerative sprouting" (Moore, 1974; Steward, 1994).

In the dentate gyrus, a significant increase of the degree of branching and total fiber length for single fibers as well as an increase in the density of axonal boutons was also demonstrated following ECL using the anterograde Phaseolus vulgaris leucoagglutinin (PHAL) (Deller et al., 1995a). Furthermore, these studies revealed alterations in the normal fiber morphology: Some sprouting axons develop a large number of short, truncated axonal processes (axonal extensions), and other fibers gave rise to conspicuous glomerular or tangle-like formations (Deller et al., 1995b; Deller et al., 1996c). The early appearance of immunoreactivity of NG2 in the OML between 4 and 14 days following lesion correlates with the proliferation of presynaptic processes, which begins between 4 and 6 days post lesion, and which is almost complete by 12 days post lesion (Steward, 1991). Thus, NG2

protein levels are highest in the OML precisely during the time period after ECL, in which most of the new terminals are formed, and are back to control levels when the time of reactive terminal formation is over. Davies and co-workers (1999) noted that the branching behaviour of regenerating axons *in vivo* correlates with the amount of CSPGs in the tissue. Regenerating axons that grow towards the lesion site grow up a gradient of increasingly CSPG-rich extracellular matrix. Since these axons become more branched as they enter more deeply into the CSPG-rich environment, they proposed that increasing amounts of CSPGs could promote the formation of axonal sprouts before acting as a stop signal at very high concentrations (Davies et al., 1999).

Thus, the effect of NG2 on growing axons at a lesion site and in denervated areas of brain could depend on the amount of NG2 in the tissue: Whereas NG2 levels at a lesion site increase continuously towards the lesion centre, resulting in regeneration failure, moderate amounts of NG2 in a denervation zone would increase the branching of axons and permit local axonal growth.

Does NG2 affect axons outside the zone of sprouting? Regulation of a layer-specific sprouting response

The previous paragraph focused on the putative biological effects of NG2 on sprouting axons *within* the zone of denervation, where NG2 may cause the branching of axons while permitting local axonal growth. However, the layer-specific pattern of NG2 upregulation not only correlates with axonal growth in the OML but also with the failure of ingrowth of commissural/associational fibers from the adjacent non-denervated IML into the denervated zone. As shown in studies using anterograde PHAL tracing following ECL (Deller et al., 1995a; Deller et al., 1996a; Deller et al., 1996c; Frotscher et al., 1997), the denervated zone is only reinnervated by fibers that are present in the OML already before lesioning. Fibers from the IML fail to invade the denervated zone.

A large amount of studies show that CSPGs and ECM molecules define boundaries for growing axons (Faissner and Steindler, 1995; Pearlman and Sheppard, 1996; Margolis and Margolis, 1997; Wilson and Snow, 2000; Bovolenta and Fernaud-Espinosa, 2000; Rauch, 2004; Rhodes and Fawcett, 2004). These studies largely agree that CSPGs do not simply prevent axonal growth. Rather, it was demonstrated that the effects of CSPGs on axonal

growth depend on the contextual situation, i.e. the presence and relative concentration of other growth regulating molecules, the expression of relevant receptors, and on the amount of CSPGs present in the matrix (Snow and Letourneau, 1992; Davies et al., 1999; Deller et al., 2001). Interestingly, in cases in which growing axons were given a choice situation between a CSPG-rich and a CSPG-poor environment, axons preferred to grow on CSPG-poor substrates (Snow et al., 1990; Snow and Letourneau, 1992; Anderson et al., 1998; Snow et al., 2002).

From the point of view of axons located in the IML, the situation following ECL is very similar to that of axons in a choice situation *in vitro*: These fibers are present within a CSPG-poor zone and would have to cross a CSPG-rich border in order to enter the denervated OML. Since a CSPG-rich border strongly repels growing axons (Snow and Letourneau, 1992), it is conceivable that this border could prevent the ingrowth of IML fibers into the denervated OML, thereby creating the layer-specific sprouting pattern observed after ECL.

Secretion of cleaved NG2 into the zone of sprouting

The dentate gyrus at 7 and at 10 days following lesion exhibited not only a strong staining of the NG2-positive cells in the denervated OML but also a strong immunostaining of the ECM. This staining of the ECM was only detectable in the denervated zone. Electron microscopy at 10 days post lesion in the OML revealed immunoprecipitate both on the plasmalemmal surface of NG2-positive glial cells and in the extracellular space surrounding NG2-positive cells. Interestingly, NG2-immunoprecipitate was also found in the surrounding of neuronal profiles, in association with vesicular structures within the processes, and around degenerating axon terminals. To pinpoint the precise subcellular localization of NG2 within the cells and on intracellular membranes, it would be necessary to perform immunogold-labeling. Concerning the localization of NG2 in the ECM, however, it is known that NG2 can be cleaved via cell surface proteolysis, probably by the matrix metalloproteinase MMP9 (Nishiyama et al., 1995). Furthermore, cleaved NG2 is found in the zone of glial scars in the spinal cord (Asher et al., 2001; Jones et al., 2002). The data suggests that NG2 is cleaved in the zone of sprouting following lesion of the entorhinal cortex with the following sequence of events: (1) In reaction to the injury, NG2

is first cleaved, (2) released into the ECM, and (3) these short forms of NG2 attaches to neuronal structures.

Regulation of ECL induced axonal growth: the role of the extracellular matrix

As has been pointed out in the previous paragraphs, the effect of the extracellular matrix on growing axons depends considerably on the relative contributions as well as the order of assembly of the various proteoglycans and glycoproteins. Therefore, it should be kept in mind that NG2 is only one of several CSPG species found in the extracellular matrix, of both, the normal and the injured brain. Following ECL, layer-specific upregulation of several CSPGs was previously observed in the OML. Among these molecules, neurocan (Haas et al., 1999), brevican (Thon et al., 2000), and DSD-1 proteoglycan (Deller et al., 1997) were prominent. The relative contribution of each of these molecules to the regulation of lesion-induced axonal growth *in vivo* remains unknown. Therefore it may be plausible that the removal of only one of these CSPGs from the extracellular matrix has little or no effect on the regulation of lesion-induced axonal growth because the presence of the other CSPGs may compensate for this loss. It has been recently shown that axonal regeneration is not enhanced following spinal cord injury in NG2-null mice (de Castro et al., 2005). NG2 most likely acts in concert with and probably depends on the order of assembly and concentration of other extracellular matrix molecules.

NG2-positive cells: a fourth glial cell type in the brain of adult rats ?

The identity of NG-positive cells under normal conditions as well as in different lesion models is under strong debate (Greenwood and Butt, 2003). In general, NG2-positive cells are discussed as a glial cell type. *In vitro*, their immunohistochemical identity depends on the tissue culture conditions they were exposed to (Levine and Stallcup, 1987). In CNS cultures containing serum, NG2 cells abundantly express GFAP and have a stellate morphology indicating that these NG2-positive cells correspond to the type 2 astrocytes first described in culture of optic nerve (Raff et al., 1983). In serum-free CNS cultures NG2-positive cells are GFAP-negative but Galactocerebroside C (GalC)-positive. GalC is a marker that binds specifically to oligodendrocytes and Schwann Cells (Levine and Nishiyama, 1996). The ability of NG2 cells to develop into either astrocytes or oligodendrocytes has been investigated in detail in the rat cerebellum by Levine et al.

(Levine and Card, 1987). Additionally, NG2 cells are discussed as oligodendrocyte precursor cells (Chang et al., 2000; Chen et al., 2002a; Peters and Sethares, 2004b), microglia/macrophages (Kreutzberg, 1996; Bu et al., 2001; Jones et al., 2002) or as a completely novel glial cell type (Butt et al., 2002). Belachew and coworkers (2003) have shown that NG2-positive cells in the CNS can generate various cell types including oligodendrocytes, astrocytes, and neurons (Belachew et al., 2003). In other studies NG2-positive cells are discussed as a type of neuronal cells (Aguirre et al., 2004). However, despite these controversies, there is general agreement, that NG2-positive cells contribute to the formation of glial scars (Fawcett and Asher, 1999; Chen et al., 2002b; Ughrin et al., 2003).

Due to the distribution of NG2-positive cells in the dentate gyrus of adult rats it can be excluded, that within the hippocampus formation NG2-positive cells are neurons (Amaral and Witter, 1995). To further enlighten the identity of NG2-positive cells in the dentate gyrus following ECL, double-labelings of NG2 with several glial markers for astrocytes (GFAP, S100 Beta), microglia/macrophages (IBAI), adult oligodendrocytes (PLP), and oligodendrocyte progenitors were performed.

The data show that NG2 is not produced by reactive astrocytes. No colocalisation of NG2-positive cells and GFAP-positive or S100 Beta-positive cells was found in the dentate gyrus, neither in controls without lesion nor at any time-point of investigation following lesion. Even though other CSPGs (e.g. Neurocan) are produced by reactive astrocytes following ECL (Haas et al., 1999; Deller et al., 2001), and although Alonso (2005) has shown that GFAP-positive astroglial cells found in the glial scar after stab wound injury may be recruited from resident NG2-positive cells until 6 days post lesion (Alonso, 2005), NG2-positive cells are not GFAP-positive at any time point following lesion in the rat dentate gyrus.

Jones and co-workers (2002) observed NG2-positive cells expressing microglial/macrophages markers, which takes part in formation of the glial scar following lesion of the spinal cord, and inhibit axonal growth (Jones et al., 2002). Therefore, it was tested if NG2-positive cells could be found in a zone of denervation and layer-specific sprouting. However, no colocalisation of NG2-positive cells with microglia/macrophages could be observed neither in control situation nor in the denervated OML of the dentate

gyrus following ECL. Since NG2-positive cells were found in a large number around demyelinating lesions, they may play a role in the replacement of oligodendrocytes that have been lost to the disease (Gensert and Goldman, 1997; Levine et al., 2001; Tanaka et al., 2003; Alonso, 2005). However, the double-labelings of NG2-positive cells and the oligodendrocyte marker PLP revealed no colocalisation of their processes in the dentate gyrus neither before nor following ECL.

The double-labelings of the glial cell types the dentate gyrus showed that NG2 is neither produced by astrocytes, nor by microglia/macrophage, nor or by adult oligodendrocytes. Therefore, based on the double-labeling data, NG2 cells in the dentate gyrus do not seem to be a subpopulation of one of the three classical glial cell types. This suggests that NG2 cells may indeed be a fourth glial cell type as proposed by others (Butt et al., 2002). However, further investigations will be necessary to solve this question definitely.

A subpopulation of NG2-positive cells found in the denervated OML was generated postlesion as judged by the incorporation of BrdU. These NG2/BrdU-positive cells were primarily observed in the denervated OML. In addition, some BrdU-positive cells (immunonegative for NG2) were also found in the subgranular zone, the zone of neurogenesis in the adult dentate gyrus (Cameron et al., 1995; Gould et al., 1999; Kempermann et al., 2004; Shukla et al., 2005). These data are in line with previous studies reporting proliferation following ECL of glial cells (Gall et al., 1979; Avendano and Cowan, 1979; Hailer et al., 1999) and neurons (Cameron et al., 1995). Since the postlesional generation of NG2-positive cells in the adult brain has recently been demonstrated at lesion sites using other lesion models (Alonso, 2005; Dzwonek, 2005), the data of this study suggest that NG2-positive cells are generated also in denervated areas of the adult brain. Although the generation of NG2-positive cells in the denervated OML can be concluded with confidence on the basis of the presented data, several questions remain: Which are the cells that give rise to NG2-cells post lesion? Are subgranular precursor cells involved in the generation of NG2-positive cells or do NG2-cells proliferate within the denervated zone? Do newly generated NG2-cells differentiate into other glial cell types or do they remain a "fourth glial cell type", as suggested by others (Nishiyama, 2001; Butt et al., 2002; Greenwood and Butt, 2003). These questions will have to be addressed in future studies aimed at the role of glial proliferation and neurogenesis in dentate gyrus following ECL.

Summary and conclusion

To test whether the CSPG NG2 is involved in the regulation of axonal growth processes in denervated areas of the brain, NG2-positive cells in the denervated rat fascia dentata following ECL were analyzed and data were correlated with the lamina-specific sprouting response of uninjured fibers. The main findings of the study can be summarized as follows:

1.) NG2 is upregulated in the denervated OML of the fascia dentata by a subpopulation of NG2-positive glia and probably secreted into the ECM. Importantly, the time course of NG2-upregulation coincides with the time course of axonal sprouting. Therefore, the data suggest that NG2 does not prevent lesion-induced axonal growth within the denervated zone. Rather, NG2 could be involved in the regulation of axonal branching within the denervated OML.

2.) The upregulation of NG2 occurs in a conspicuous spatial pattern: NG2 is selectively upregulated within the denervated OML, and therefore, a NG2-boundary is formed towards adjacent non-denervated areas. This pattern of NG2-upregulation correlates with the layer-specific pattern of the sprouting response, and suggests that NG2 could prevent fiber ingrowth into the denervated zone from the outside, i.e. from adjacent non-denervated areas of the dentate gyrus.

3.) NG2-positive cells do not coexpress markers for astrocytes, microglia/macrophages, and adult oligodendrocytes. Therefore, NG2-positive cells could be a fourth glial cell type in the dentate gyrus that participates in the glial reaction to entorhinal denervation.

4.) A subpopulation of NG2-positive cells is generated postlesion and primarily found in the denervated OML. Additional studies will be necessary to unravel their role in the reorganization of the dentate gyrus following entorhinal denervation.

Taken together, these data suggest that -in combination with other CSPGs and ECM molecules- NG2 could be responsible for the regulation of layer-specific sprouting in the dentate gyrus following ECL. NG2 may regulate axonal growth in the denervated OML of

the fascia dentata (1) by increasing branching within the denervated zone, and (2) by contributing to the CSPG barrier formed between the IML and OML.

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Publications

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Zusammenfassung (Deutsch)

Die Reorganisation der Fascia dentata der Ratte nach entorhinaler Denervierung: eine regulatorische Rolle für das Chondroitinsulfat Proteoglykan NG2

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Ziel dieser Arbeit war es, die Regulation axonaler Wachstumsprozesse und die Bedeutung des wachstumshemmenden Chondroitinsulfat Proteoglykans (CSPG) NG2 bei plastischen Umbauvorgängen des ZNS besser verstehen zu können. Die zu Grunde liegende Frage war, ob NG2 an der Regulation axonaler Wachstumsprozesse in denervierten Zonen des ZNS beteiligt ist. Hierfür wurden die zeitlichen und regionalen Veränderungen von NG2 in der Fascia dentata der Ratte vor und zu verschiedenen Zeitpunkten nach einer Läsion des entorhinalen Kortex untersucht. Die Expression von NG2 wurde auf mRNA-, Protein- und ultrastruktureller Ebene bestimmt und mit dem postläsionalen Sprossungsverhalten unverletzter Axone in der denervierten äußeren Molekularschicht (OML) der Fascia dentata korreliert. Abschließend wurde die Identität NG2-produzierender Zellen analysiert und untersucht, ob es zu einer Neubildung von NG2-positiven Zellen nach der Läsion kommt. Die Ergebnisse zeigten eine Hochregulierung von NG2 auf mRNA und auf Protein Ebene, die räumlich auf die deafferenzierte OML begrenzt war. Auf ultrastruktureller Ebene war in der OML NG2-positives Immunoprezipitat nicht nur auf glialen Zelloberflächen und in der extrazellulären Matrix zu sehen, sondern auch in engem Kontakt zu neuronalen Strukturen. Kolokalisationen von NG2-positiven Zellen mit anderen glialen Zellen (Astrozyten, Mikroglia/Makrophagen und Oligodendrozyten) wurden weder vor noch nach der Läsion gefunden, sehr wohl jedoch einige postläsional neu generierte NG2-positive Zellen.

Zusammenfassend lässt sich sagen, dass alle postläsionalen Veränderungen von NG2 (1.) räumlich auf die denervierte OML begrenzt waren und (2.) zeitlich mit der Sprossungsantwort unverletzter Fasern übereinstimmten. Dies legt nahe, dass NG2 an der Regulation axonaler Wachstumsprozesse in denervierten Schichten des ZNS beteiligt ist. Ausserdem wird vermutet, dass NG2 postläsional gespalten wird und, dass NG2-positive Zellen ein von Astrozyten, Mikroglia/Makrophagen und Oligodendrozyten differenter glialer Zelltyp sind.

Einleitung

Die Läsion des entorhinalen Kortex (ECL) der Ratte führt zu einer Degeneration der durchtrennten Fasern des *Tractus perforans* und damit zu einer Deafferenzierung der distalen Dendriten der OML der Fascia dentata. Die denervierten Körnerzellen werden anschließend von anderen, unverletzten Fasersystemen reinnerviert. Auffällig bei diesem

postläsionalen Sprossungsverhalten unverletzter Axone ist ihre strenge Orientierung an der existierenden Schichtung der Fascia dentata. Es lässt sich folgendes beobachten:

1.) Nur diejenigen Fasersysteme beteiligen sich an der Reinnervation der OML, die schon vor der Läsion innerhalb der deafferenzierten Schicht zu finden waren. Es sprossen beispielsweise Fasern des kontralateralen *Tractus perforans* oder Fasern des medialen Septums.

2.) Die sprossenden Fasern bleiben streng innerhalb der existierenden Faserschichtung auf die denervierte OML begrenzt. Weder wachsen die Fasern der OML in die innere Molekularschicht (IML) ein, noch dringen umgekehrt Fasern der IML in die OML ein.

Verantwortlich für diese schichtenspezifische Sprossung sollen, neben intrinsischen Molekülen der Neurone selbst, extrazelluläre Moleküle in der Umgebung der sprossenden Axone sein. Die OML wird postläsional mit verschiedenen Molekülen angereichert, wie z.B. Zelladhäsionsmolekülen, verschiedenen Wachstumsfaktoren und Zytokinen. Durch diese Anreicherung der extrazellulären Matrix (EZM) mit verschiedenen wachstumsfördernden und wachstumshemmenden Molekülen könnte der Sprossungsprozeß der unverletzten Axone auf die deafferenzierte Schicht begrenzt werden.

Die vorliegende Arbeit untersucht das wachstumshemmende CSPG NG2. Moleküle dieser Gruppe sind dafür bekannt, lokal axonale Wachstumsprozesse zu beeinflussen. NG2 ist ein transmembranes, ca. 300kDa großes Molekül mit inhibitorischen Eigenschaften, wie verschiedene *in vitro* Untersuchungen an auswachsenden Axonen zeigen konnten. *In vivo* ist es vor allem in der sogenannten „glialen Narbe“ nach einer Verletzung des Nervensystems zu finden und soll hauptverantwortlich für das Regenerationsversagen durchtrennter Rückenmarksneurone sein, welche die gliale Narbe nicht durchwachsen können. Die Identität NG2 produzierender Zellen wird in der aktuellen Literatur kontrovers diskutiert. Im allgemeinen werden NG2-positive Zellen als Oligodendrozyten Vorläuferzellen angesehen, so wird beispielsweise auch der kommerziell erhältliche Antikörper gegen diesen Zelltyp deklariert. Einige Studien zeigen NG2 allerdings auch auf Mikroglia/Makrophagen in der glialen Narbe nach Rückenmarksverletzungen und es ist eine mediumsabhängige Entwicklung von NG2-positiven Zellen zu entweder Astrozyten oder Oligodendrozyten *in vitro* nachgewiesen worden.

Um die Regulation axonaler Wachstumsprozesse nach einer Läsion des ZNS und den Beitrag von NG2 zu diesem plastischen Umbauprozessen besser verstehen zu können, wurden folgende Fragen bearbeitet:

1.) Wie sind (a) die mRNA und (b) das Protein von NG2 im Hippokampus nach entorhinaler Läsion räumlich und zeitlich verteilt?
2.) Wird NG2 postläsional in die EZM der OML abgegeben ?
3.) Welche Zellen exprimieren NG2 im Hippokampus der Ratte?
4.) Werden NG2-positive Zellen im Hippokampus postläsional generiert?

Material, Methoden und Ergebnisse

Um die äußere Molekularschicht der Fascia dentata zu denervieren, wurde bei Ratten eine unilaterale Läsion des entorhinalen Kortex durchgeführt. Diese durchtrennt den *Tractus perforans*, dessen Fasern in der OML des Hippokampus enden. Da die Vollständigkeit der Läsion die Grundlage für alle nachfolgenden Versuche bildet, wurden die läsionierten Hirne wie folgt kontrolliert: Zuerst wurden sie bei der Entnahme makroskopisch auf Blutungen untersucht. Zu frühen Zeitpunkten nach der Läsion (1, 4, und 7 Tage (d)) wurden nur Hirne ohne auffällige Blutung weiterverwendet. Zu späteren Zeitpunkten nach der Läsion (10, 14, 30d und 6 Monate), musste für die Weiterverwendung eine sehr feine Narbe an der Läsionsstelle zu sehen sein. Diese Hirne wurden außerdem an der Läsionsstelle horizontal geschnitten und mussten einen unbeschädigten Hippokampus aufweisen. Abschließend wurden dann Frontalschnitte des Hippokampus entweder mit dem Fluoreszensmarker Fluoro Jade B (markiert degenerierende Axone zu frühen Zeitpunkten nach Läsion) oder mit Acetylcholinesterase (markiert sprossende cholinerge Fasern zu späten Zeitpunkten nach Läsion) gefärbt. Bei beiden Färbungen musste die OML deutlich kräftiger gefärbt sein, als die nicht-deafferenzierte IML.

1a.) Zur Quantifizierung der NG2 mRNA Expression wurde Lasermikrodissektion mit anschließender quantitativer RT PCR (qPCR) kombiniert. Zu verschiedenen Zeitpunkten (Kontrolle ohne Läsion, 6h, 12h, 2d, 4d, 7d nach Läsion) wurde die denervierte OML mit dem Laser ausgeschnitten, um dann die Menge an NG2 mRNA in genau dieser Zone mit

Hilfe der qPCR zu ermitteln. Diese ergab eine postläsionale Erhöhung der NG2 mRNA an Tag 2 (2,5-fach) und an Tag 4 (2-fach).

1b.) Zur Charakterisierung des Verteilungsmusters von NG2 auf Proteinebene wurden Frontalschnitte des Hippokampus immunhistochemisch auf NG2 angefärbt (Kontrolltiere ohne Läsion, 1d, 4d, 7d, 10d, 30d und 6 Monate nach der Läsion). In den Kontrolltieren waren NG2-positive Zellen qualitativ gleichmäßig im Hippokampus verteilt. Jedoch schon am ersten Tag ließ sich eine Anreicherung von NG2 in der denervierten OML erkennen. Diese NG2-positive Färbung in der OML verstärkte sich am 4. Tag nach Läsion, wiederum am 7. Tag und erreichte am 10. Tag nach der Läsion ein qualitatives Maximum. Am 30. Tag sowie auch 6 Monate nach der Läsion entsprach die Färbung der Kontrollsituation.

2.) Aufgrund des lichtmikroskopischen Verteilungsmusters von NG2 wurden einige Hirne elektronenmikroskopisch untersucht (10d nach der Läsion). Die elektronenmikroskopische Untersuchung von NG2-positiven Zellen zeigte Immunreaktivität auf glialen Zelloberflächen und im Extrazellularraum der OML, aber auch in engem Kontakt zu neuronalen Strukturen.

3.) Die Identität NG2 exprimierender Zellen sollte mit Hilfe von Fluoreszenz-Doppelmarkierungen von NG2-positiven Zellen mit GFAP-positiven Astrozyten, IBAI-positiven Mikroglia/Makrophagen und PLP-positiven Oligodendrozyten festgestellt werden. Jedoch ließen sich weder vor noch nach der Läsion Kolokalisationen von NG2-positiven Zellen mit einem dieser Marker für gliale Zelltypen nachweisen.

4.) Mit Hilfe des Zellproliferationsmarkers Bromdesoxyuridin (BrdU) sollte schließlich festgestellt werden, ob NG2-positive Zellen im Hippokampus postläsional generiert werden. Der Einbau von BrdU in NG2-positive Zellen wurde mit Fluoreszenz-Doppelfärbungen von NG2 und BrdU kontrolliert. Bei Tieren ohne Läsion waren nur wenige BrdU-positive (NG2-negative) Zellen in der subgranulären Zone zu finden. NG2/BrdU doppelmarkierte Zellen waren in Kontrolltieren nicht zu sehen. Zehn Tage nach der Läsion ließen sich jedoch einige neu gebildete NG2-positive Zellen (BrdU-positiver Zellkern) in der denervierten OML nachweisen.

Diskussion

Was könnte die auf die OML begrenzte Anreicherung von NG2 während des Sprossungsvorgangs bedeuten?

Wie anfangs beschrieben wird angenommen, dass CSPGs den Sprossungsprozess unverletzter Axone in der OML des Hippokampus beeinflussen können. Die Moleküle scheinen aber unterschiedliche Effekte auf sprossende Axone (a) innerhalb der deafferenzierten Schicht und (b) außerhalb dieser Schicht auszuüben. Die sprossenden Axone innerhalb der deafferenzierten OML befinden sich in einer Umgebung, die nicht nur mit NG2 angereichert ist, sondern auch eine Vielzahl anderer EZM-Moleküle, Zelladhäsionsmoleküle und Wachstumsfaktoren enthält. Da eine Reinnervation der Körnerzellen stattfindet, scheinen diese Moleküle in ihrer Gesamtheit die Axonsprossung in der OML zu unterstützen. Die Anwesenheit wachstumshemmender Moleküle, wie NG2, unterdrückt in dieser Zone die axonalen Wachstumsvorgänge nicht. So wurde in anderen Studien gezeigt, dass Axone in einer „wachstumshemmenden Umgebung“ wachsen können, wenn sie sich von Anfang an in dieser Umgebung befinden. Zusätzlich erhöht sich der Verzweigungsgrad wachsender Axone relativ zur Konzentration inhibitorischer Substrate, wie ebenfalls gezeigt wurde. Die Anreicherung mit inhibitorischem NG2 in der Sprossungszone der denervierten Fascia dentata könnte somit die Reinnervation der distalen Dendriten der Körnerzellen durch eine verstärkte Verzweigung sprossender Axone unterstützen. Für sprossende Axone außerhalb der deafferenzierten OML (Fasern der IML) allerdings ist eine mit wachstumshemmenden Molekülen angereicherte Schicht eine effektive Barriere, wie aus verschiedenen Studien bekannt ist. Für einwachsende Axone stellen solche mit wachstumshemmenden Molekülen angereicherten Zonen unüberwindbare Hindernisse dar. Die Anreicherung der OML mit NG2 könnte so erklären, wieso es nicht zur Einsprossung von Fasern aus angrenzenden Schichten kommt.

Welche Rolle könnte in der EZM angereichertes NG2 für axonales Wachstum nach einer Läsion spielen?

NG2 ist bekanntermaßen nur eines von verschiedenen Molekülen, die in der Umgebung von sprossenden Axonen zu finden sind. Der relative Beitrag der einzelnen Moleküle zur

Regulation des läsionsinduzierten axonalen Wachstums ist nicht geklärt. Da Axone von NG2 -/- Mäusen keine verbesserte Regeneration nach einer Rückenmarksverletzung zeigen, ist es wahrscheinlich, dass NG2 zusammen mit anderen EZM Molekülen agiert und die jeweilige Konzentration des einzelnen Moleküls den Einfluss auf die Regulation des axonalen Wachstums bestimmt. Postläsional gespaltenes und in die EZM abgegebenes NG2 könnte aber das schichtenspezifische axonale Wachstum unterstützen. Die elektronenmikroskopischen Daten dieser Arbeit zeigen NG2-positives Immunpräzipitat nicht nur auf glialen Zelloberflächen und in der umgebenden EZM, sondern auch in engem Kontakt zu neuronalen Strukturen. Dies stützt die Vermutung, dass NG2 postläsional gespalten und in die EZM abgegeben wird. Es ist bekannt, dass NG2 *in vitro* beispielsweise durch die Matrix Metalloproteinase 9 an seiner Membrandomäne gespalten werden kann. Wenn es auch *in vivo* in der Sprossungszone postläsional gespalten wird und dann an neuronale Strukturen bindet, verändert es die molekulare Zusammensetzung der OML und könnte auf diese Weise den Sprossungsprozess der Axone auf die deafferenzierte Zone begrenzen.

NG2-positive Zellen, ein neuer Zelltyp?

Im Unterschied zu anderen CSPGs wird NG2 nicht von reaktiven Astrozyten exprimiert. Es wurden weder pre- noch postläsional Kolokalisationen von NG2-positiven Zellen und GFAP-positiven Astrozyten gefunden. Andere Doppelfärbungen zeigten auch keine Doppelmarkierungen von NG2 und IBAI-positiven Mikroglia/Makrophagen, wie sie in der glialen Narbe nach Rückenmarksverletzungen gezeigt wurden. Ebenfalls konnten keine NG2/PLP-positiven Doppelmarkierungen gefunden werden, was auch adulte Oligodendrozyten als NG2 exprimierenden Zelltyp mit großer Sicherheit ausschließt. Ein neuronaler Zelltyp kann aufgrund des Verteilungsmusters der NG2-positiven Zellen ebenfalls ausgeschlossen werden. Mit den vorliegenden Daten kann nicht gesagt werden, von welchen Zellen NG2 exprimiert wird. Es kann auch nicht ausgeschlossen werden, dass die NG2-positiven Zellen einen gänzlich neuen glialen Zelltyp darstellen, wie aktuell in der Literatur diskutiert wird. Es kann aber gesagt werden, dass NG2 positive Zellen in der Fascia dentata der adulten Ratte ein von Neuronen, Astrozyten, Mikroglia/Makrophagen, und adulten Oligodendrozyten klar zu differenzierender Zelltyp sind. Ein Teil dieser NG2-positiven Zellen wird darüber hinaus erst postläsional generiert.

Zusammenfassend zeigen die in dieser Arbeit ermittelten Daten, dass die Hochregulierung und die Anreicherung von NG2 in der EZM sowohl räumlich als auch zeitlich mit dem Sprossen der Fasern in der OML und der dort stattfindenden axonalen Reorganisation korrelieren. Dies unterstützt die Vermutung, dass NG2 im Zusammenspiel mit anderen CSPGs und EZM Molekülen für die Regulation der schichtenspezifischen Sprossung im Gyrus dentatus der Ratte nach einer Läsion des entorhinalen Kortex verantwortlich ist. NG2 könnte (1) über die Erhöhung des Verzweigungsgrades der Fasern innerhalb der denervierten Zone und (2) über seinen Beitrag zu der molekularen Barriere zwischen der denervierten OML und der angrenzenden IML das Faserwachstum in der OML regulieren.

Lebenslauf

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