

Retinoic acid signalling after peripheral nerve injury

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1. General Introduction

It has been long recognised that damage to the adult mammalian central nervous system (CNS) is irreversible. However, it was not until early in the 20th century that Ramon y Cajal made the observation that while the CNS does not regenerate, the peripheral nervous system (PNS) of adult mammals does (Ramon y Cajal, 1928). He concluded that CNS axons do not regrow because of the presence of CNS-specific obstacles in their path. Since that time, a tremendous effort has been made to identify the characteristics of the PNS that allow regeneration, with the goal of converting the physiology of damaged CNS neurones to that of the PNS after injury, including regeneration and restored function. Unfortunately, this objective has not yet been achieved, because we still do not completely know the mechanisms of PNS regeneration. It is the goal of my work to better understand the regulation of this process.

1.1. Peripheral nervous system regeneration and its regulation

After peripheral nerve injury myelin debris is cleared by macrophages. Schwann cells de-differentiate, down-regulate the expression of myelin proteins and create a permissive environment for axonal regeneration. PNS axons can then regenerate between these permissive Schwann cells and the basal lamina (Fawcett and Keynes, 1990). Wallerian degeneration refers to the events that occur distal to the site of the injury. During the first week after axotomy axons fragment, are phagocytosed, and the myelin sheaths separate at incisures, breaking up into so-called ovoids. Over the next few weeks, these myelin ovoids are also phagocytosed in part by Schwann cells, but mainly by macrophages that invade the degenerating nerve. The clearance of myelin debris by macrophages promotes axonal regeneration (Dahlin, 1995). Schwann cells undergo extensive proliferation between the 3rd and 5th day post-axotomy (Jessen and Richardson, 2002). The basal lamina persists and surrounds the column of “denervated” Schwann cells.

Proximal to the lesion site, axons give rise to one or more sprouts, each of which is tipped by a growth cone (Ramon y Cajal, 1928). For axonal regeneration to be successful, growth cones must first reach the distal nerve stump, which they do even if they have to cross a small gap between the proximal and distal nerve stumps. Upon reaching the distal nerve stump, growth cones enter “Schwann tubes”, the Schwann cells and their basal laminae, which provide the sole pathway

for regenerating axons in the distal nerve stump (Ramon y Cajal, 1928). Eventually, Schwann cells establish a 1:1 relationships with each fiber, synthesise a new basal lamina and form myelin sheaths. A crucial role in this process is again assigned to macrophages, which, along with endoneurial fibroblasts, provide recycled lipoproteins for Schwann cell myelination. With time, remyelinated axons may enlarge to nearly normal diameters and the localisation of ion channels is re-established. However, the axo-glial junctions of remyelinated fibers are probably not as “tight” as those in unlesioned nerves, since regenerated nerves are more affected by potassium channel blockers (Jessen and Richardson, 2002).

Although many tissues and substrates will support axonal regeneration, none are as potent as a degenerating peripheral nerve, which appears to be uniquely adapted for this role (Ide et al., 1996). The degradation of myelin in lesioned nerves and denervated Schwann cells appear to be key factors that promote axonal regeneration (Jessen and Richardson, 2002). Schwann cells contribute several molecules that promote neurite outgrowth, including extracellular matrix molecules and cell adhesion molecules on their cell membrane (Wagner et al., 2002). They appear to be the main source of many trophic factors, including the mitogen(s) that stimulate their own proliferation, but fibroblasts and macrophages also contribute to the supply of neurotrophic factors. In addition to providing trophic (nurturing) factors, they supply tropic (guidance) factors that affect regenerating axons to guide their growth toward the distal nerve stump and enter Schwann tubes (Jessen and Richardson, 2002). Recently one particular family of neurotrophic molecules and its signalling have gained particular importance – the β -Neuregulin-1 family.

1.2. β -Neuregulin-1 and its signalling

The β -Neuregulins-1 (β -NRG-1) are a family of alternatively spliced, soluble and membrane bound proteins encoded by four known genes, three of which are expressed at high levels by PNS and CNS neurons during development (Buonanno and Fischbach, 2001). They are part of the epidermal growth factor (EGF) superfamily of growth factors that also includes the transforming growth factor alpha. Like all members of this family, β -NRG-1 mediates its effects by binding to and activating members of the erbB receptor tyrosine kinase family, which includes the EGF receptor (erbB1), erbB2, erbB3 and erbB4 (Tzahar et al., 1996). β -NRG-1 binds preferentially to the erbB3 and erbB4 receptors, which then form

heterodimers by recruiting either erbB1 or erbB2 co-receptors to propagate signalling (Wiley, 2003). In the PNS erbB2 is the preferred receptor tyrosine kinase. Homodimers of erbB4 are also signal competent (Murphy et al., 2002; Carpenter, 2003). Ligand-induced dimerization activates receptor kinase activity and the phosphorylation of tyrosine residues in the C-terminal tail. These phosphorylated tyrosines, as part of a consensus motif, serve as binding sites for signalling molecules containing Src homology or phosphotyrosine binding domains. Depending on the constituents of the erbB dimer, NRGs can activate several cellular signalling cascades, including Ras/Raf, Jak/STAT, PI3K and PLC γ cascades (Murphy et al., 2002; Citri et al., 2003). Cyclin D1 is a central effector of signalling by erbBs and has been implicated as the major player in the promotion of cell cycle progression by the neuregulin pathway (Citri et al., 2003).

The functions of β -NRG-1 include the regulation of the early cell fate determination, differentiation, migration, survival and maturation of satellite cells, Schwann cells and oligodendrocytes (Buonanno and Fischbach, 2001). Moreover, β -NRG-1 is a component of the “axon-associated mitogen” expressed by neonatal sensory neurons (Dong et al., 1999b). In the light of these developmental functions, it is possible that members of the neuregulin family, acting as axon-derived mitogen, promote Schwann cell proliferation also during Wallerian degeneration of adult peripheral nerves (Caroll et al., 1997). In the adult nervous system β -NRG-1 mRNA is detectable in neurons projecting into the sciatic nerve (lumbar DRG and spinal cord), as well as in brain and skeletal muscle (Buonanno and Fischbach, 2001). Schwann cells continue to express erbB2 and erbB3 throughout adulthood, although at reduced levels (Grinspan et al., 1996). Recent studies indeed suggest that β -NRG-1 may play a significant role during Wallerian degeneration, because the expression of various β -NRG-1 isoforms and of erbB2 and erbB3 by Schwann cells increases substantially during Wallerian degeneration (Caroll et al., 1997; Kim et al., 2002) and demyelination (Hall et al., 1997).

1.3. Molecular mechanisms of Wallerian degeneration

The molecular mechanisms that trigger Wallerian degeneration are unknown. Possibly, axotomy interrupts the supply of neuronal factors that maintain the myelinating phenotype of Schwann cells. On the other hand, a positive signal resulting from axonal degeneration may be involved (Fawcett and Keynes, 1990).

The main molecular features of Wallerian degeneration are the down-regulation of myelin-related genes and the up-regulation of nerve growth factor (NGF), the low affinity NFG receptor (NGFR/p75) and tenascin-C (Brown et al., 1991). Recently the early activation of another molecule, receptor tyrosine kinase erbB2, was shown. Within one hour following nerve transection, erbB2 is selectively phosphorylated as well as its downstream signals including Akt protein and the induction of the immediate early genes within the Schwann cell nuclei (Kim et al., 2002). The role of the increased expression of β -NRG-1 and the activation of erbB2 during Wallerian degeneration is speculative so far. The β -NRG-1 would be expected to promote the proliferation and survival of Schwann cells in the distal stump. However, the maximal expression of β -Neuregulin-1 and its receptors occurs later than the peak of Schwann cell proliferation and persists after proliferation has declined (Carroll et al., 1997). Thus, while β -NRG-1 receptor activation may potentiate Schwann cell proliferation, it is unlikely to initiate it. Nevertheless, recent studies using co-cultures of Schwann cells and neurons (Zanazzi et al., 2001) indicate that β -NRG-1 can itself promote Schwann cell de-differentiation and demyelination, even in the absence of nerve injury. These findings suggest that activation of signalling pathways by β -Neuregulin-1 may contribute to the ongoing demyelination that is associated with nerve injury.

Axon-Schwann cell interactions during regeneration are believed to be fundamentally similar to those that occur during development (Jessen and Richardson, 2002). Many transcriptional regulators implicated in the regulation of axonal regeneration (including Wallerian degeneration) are also assumed to regulate the metabolic state of developing neurons and Schwann cells. One candidate is the transcriptional activator retinoic acid which is an important morphogen in the development of many organ systems.

1.4. The retinoic acid signalling system and its contribution to nerve regeneration

Retinoids are vitamin A (retinol) derivatives. The most notable is retinoic acid, many biological effects of which are well known. It induces the differentiation of various populations of neurons and glia cells in embryonic spinal cord, cerebellum, dorsal root ganglia and sympathetic ganglia. As shown in this

thesis, it may also contribute to the signalling following nerve injury, when growth-related genes need to be reactivated.

Retinoic acid (RA) is a low molecular weight lipophilic molecule, whose synthesis is catalysed locally by aldehyde dehydrogenases (RALDH; Ross et al., 2000; Zhao et al., 1996) in the presence of cellular retinol binding protein I (CRBP-I; Napoli, 1996). Once RA enters the cell, it binds to cellular retinoic acid binding protein II (CRABP-II) and is transferred to the nucleus (Budhu and Noy, 2002). There it establishes or changes gene expression by binding to retinoic acid receptors (RARs) and retinoid X receptors (RXRs), which act as ligand-activated transcription factors. In mammals there are three RARs ($-\alpha$, $-\beta$, and $-\gamma$) and three RXRs ($-\alpha$, $-\beta$, and $-\gamma$), each of which has multiple isoforms. The RARs/RXRs act as heterodimers and recognise consensus sequences known as retinoic acid responsive elements (RARE) in the up-stream promoter sequences of RA-responsive genes (Bastie et al., 2001; Delva et al., 1999). In addition to the regulation of RARE-containing genes, retinoid receptors affect the transcriptional activity of AP-1 – another important transcriptional factor (Lin et al., 2002).

While the biological functions of retinoids in development have been studied in great detail, much less evidence exists so far that RA has physiological importance in the adult nervous system. Except for brain areas involved in bird song (Denisenko-Nehrbass et al., 2000) and the retina, where RA may be involved in circadian rhythms of gene expression (McCaffery et al., 1993) and gating of electrical synapses (Zhang and McMahon, 2001), no endogenous function for RA in the mature nervous system has been demonstrated either in healthy individuals or under pathological conditions.

Only recently some publications appeared about the possible role of retinoic acid during regeneration. In various cell cultures retinoids were found to interact with most cytokine signals that mediate cellular interactions after nerve lesion *in vivo* (Mey, 2001). RA induces the “super-regeneration” of organs that can already regenerate, such as the urodele amphibian limb by re-specifying positional information in the limb (Maden, 1998). In organs that cannot normally regenerate, such as the adult mammalian lung, RA induces the complete regeneration of alveoli that have been destroyed by various forms of noxious treatment (Belloni et al., 2000).

As for nervous system regeneration, RA induces neurite outgrowth in explant cultures of spinal cord from embryonic but not from adult mice. This decline in the regeneration-inducing potential of RA seems to be due to the loss of RAR β 2 (Corcoran et al., 2000; Corcoran et al., 2002), because after transfection to the adult spinal cord, RAR β 2 induced neurite outgrowth. This was observed even when no RA was added to the medium, implying an endogenous source of the needed ligand in the tissue. These data and experiments with retina and dorsal root ganglia demonstrate that retinoic acid is able to induce the regeneration of differentiated CNS neurons, when allowed to act on the genome through its normal pathway of receptors (Corcoran and Maden, 1999; Maden and Hind, 2003; Mey and Rombach, 1999).

1.5. Goals

The scientific goal of this project is to investigate the role of retinoic acid signal transduction following nerve injury of the peripheral nervous system. Abundance in PNS and changes in gene expression of (1) enzymes involved in retinoid metabolism, of (2) cellular retinoid binding proteins and of (3) nuclear receptors for retinoids will be monitored after nerve lesion. The physiological relevance of the altered retinoid signalling will be measured in the primary Schwann cell cultures by (4) monitoring the expression of β -Neuregulin-1 receptors erbB2 and erbB3.

2. Materials and Methods

2.1. Animal experimentation and surgical procedures

Since the project aims at strategies for medical intervention and since injury-related physiology differs fundamentally between vertebrate classes, the experiments require mammalian species. We chose rat sciatic nerve, because it is the largest peripheral nerve and there are established model systems for its regeneration. Mouse sciatic nerves were also studied since the *in situ* hybridisation probes were available only for this species.

A total of seventy adult male Sprague-Dawley rats weighing 150-300 g were used. Animals were kept under a 12-h light/dark cycle in the animal care facility. Animals had unlimited access to rat chow and water. Rats were deeply anaesthetised with intraperitoneal injection of 10% ketamine hydrochloride (Sanofi-Ceva); (0,1ml/100g body weight) and 0.1 ml Rompun (Bayer). Under aseptic conditions, the right sciatic nerve was exposed and a lesion was performed (**Fig. 2.1**). The nerve was crushed once with jeweller's' forceps for 10 s or completely transected and the distal stump sutured to the muscle. After a survival period of 30 minutes, 1, 2, 4, 7 or 14 days the animals were sacrificed (overdose of sodium-pentobarbital) to obtain a 1 cm nerve segment distal to the lesioned site. Contralateral nerves were used as controls. For quantitative RT-PCR analyses, L4-L6 dorsal root ganglia (DRG) ipsilateral and contralateral to the crushed sciatic nerves were also collected. The nerves and L4-L6 DRGs were washed in RNase-free 0.1 M phosphate buffered saline (PBS) and snap frozen in liquid nitrogen to maintain the RNA intact. Preparation instruments were cleaned with *RNase Away* (Molecular Bio Products) to eliminate RNase-contamination.

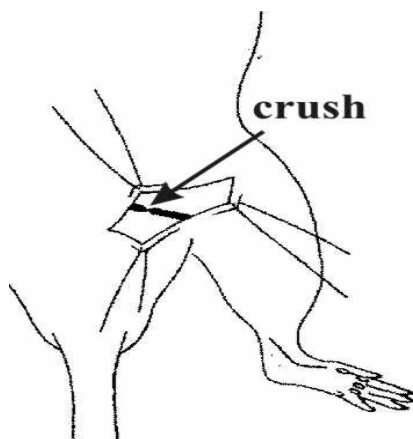


Fig. 2.1 Surgical procedure. A 10 s crush lesion was performed to induce regeneration of the right sciatic nerves of adult rats or mice. For the investigation of Wallerian degeneration the nerve was completely transected and the distal stump was sutured to the muscle.

Twenty adult female NMRI mice weighing 25-30 g were also used in this study. To induce regeneration, mice were anaesthetised with intraperitoneal injection of Avertin [1.25% 2,2,2-tribromoethanol (Sigma); 0.8% amyl alcohol (Sigma); 300µl/10g body weight] and the right sciatic nerve was exposed and crushed once as described for rat experiments (see **Fig. 2.1.**). After survival periods of 24 hours or 7 days the animals were sacrificed [inhalation of diethyl-ether (Merck)] to obtain a 0.5 cm nerve segment distal to the lesioned sites. Contralateral nerves were used as controls. Mouse nerves were used exclusively for *in situ* hybridisation experiments and therefore prepared in RNase free conditions as described above. All procedures were conducted in accordance with the animal protection laws and were approved by *Regierungspräsidium Köln, Tierversuchsgenehmigungen* AZ 23.203.2 AC 29, 8/97 and AZ 23.203.2 AC 29, 34/00.

2.2. Schwann cell cultures

In order to investigate physiological effects of retinoic acid, primary Schwann cell cultures from P0 Sprague-Dawley rats were established as previously described (Johann et al., 2003). Briefly, in one preparation 18 - 24 sciatic nerves were dissected, freed from blood vessels and fatty tissue, cut up in small pieces and digested for 1 h at 37°C in 10 ml Dulbecco's Modified Eagle's Medium (DMEM) containing 0.6% collagenase and 2.5% trypsin. To obtain single cells the nerve pieces were gently triturated (0.7 mm, then 0.4 mm gauge canulae). The cell suspension was plated out in uncoated culture flasks (25 cm²) with 5 ml DMEM containing 10 % heat-inactivated fetal calf serum (FCS). Fibroblast growth was reduced by the addition of 10 µM cytosine arabinoside to the medium for one day. To eliminate remaining fibroblasts, the cells were incubated 20 min with Thy 1.1 antibody (Sigma) at 37°C followed by treatment with baby rabbit complement (Linaris) for another 20 min. Schwann cells were then cultured in poly-L-lysine-coated flasks (25 cm²) in 4 ml DMEM supplemented with 10 % FCS, 2 µM forskolin (ICN) at 37 °C, 5 % CO₂ and 100 µg/ml bovine pituitary extract (Life Technologies; Johann et al., 2003). After the cells had reached confluence, complement lysis was repeated and cells were checked with microscopical inspection to ascertain the absence of fibroblasts. For retinoic acid treatment, cells were cultivated in medium without forskolin. Final concentrations of 0.1, 1, 10,

100 and 1000 nM all-*trans* RA (stock solutions 0.1 μ M - 1 mM in dimethyl sulfoxide, DMSO) were added and cells were kept in darkness for 24 or 40 hrs at 37 °C, 5 % CO₂. Control cells received an equal volume of DMSO. For further RNA isolation or immunoblotting cells were harvested by centrifugation and re-suspended in Trizol (for RNA isolation) or hypotonic buffer (for SDS-PAGE).

2.3. Immunocytochemistry

The avidin-biotin interaction provides a simple and sensitive method to localise antigens in tissues. It involves the application of biotin-conjugated secondary antibody followed by the addition of avidin-biotin-peroxidase complex. During formation of the complex, avidin acts as a bridge between biotin-labelled peroxidase molecules. Consequently, a “lattice” complex containing several peroxidase molecules is formed (Hsu et al., 1981). Binding of this complex to the biotin-labelled secondary antibodies results in a high staining intensity after the subsequent enzyme reaction.

The use of horseradish peroxidase (HRP) for enzyme-mediated immunodetection, commonly referred to as immunoperoxidase labelling, is a well-established cytochemical technique. The most widely used HRP substrate for these applications is diaminobenzidine (DAB), which generates a brown-coloured polymeric oxidation product. The DAB reaction product is discretely localised at HRP-labelled sites, providing high resolution images of sub-cellular antigen distribution. DAB staining can be visualised directly by bright-field light microscopy.

To perform this technique, intact sciatic nerves from adult rats were used. For this both sciatic nerves were sampled and washed in 0.1 M PBS. Following fixation of 1 hour in cold 96% ethanol (Roth), the nerves were cryoprotected in 25% sucrose (Sigma) for at least 12 hours. To obtain longitudinal sections, sets of three nerves were embedded in Tissue Tek (Sakura) and cut with a cryostat (Leica) in 20 μ m sections. The sections were collected with gelatin-coated (to achieve better adhesion) microscope slides. Series of eight slides were prepared for each nerve. Each slide was then processed for antibody staining. For this method the primary antibodies listed in **Tab. 2.1** and biotin-conjugated secondary antibodies goat anti-mouse and goat anti-rabbit were used.

The following schema was used for this method, all steps were performed at room temperature (RT) except where stated otherwise:

- Blocking of unspecific binding sites (0.4% Triton X-100 in 0.5 M Tris and 4% normal goat serum), 1h
- Washing [0.1 M tris buffered saline (TBS; 0.05 M Tris, 0.85% NaCl, pH 7.4)], 2x5 min
- Incubation with the primary antibody (0.04% Triton X-100 in 0.5 M Tris and 1% NGS; antibodies (AB) working dilutions are listed in **Tab. 2.1**), 12h, 4°C, moist
- Washing (0.1 M TBS), 2x5 min, 1x10 min
- Blocking of endogenous peroxidase activity (1% H₂O₂ in 0.5 M Tris), 3 min
- Washing (0.1 M TBS), 2x5 min, 1x 10 min
- Incubation with the secondary antibody (0.02% Triton X-100 in 0.5 M Tris; AB working dilutions are listed in **Tab. 2.1**), 2h, moist
- Washing (0.1 M TBS), 2x5 min, 1x 10 min
- Incubation with avidin-biotin-peroxidase complex [1% bovine serum albumin (BSA) in 0.5 M Tris with ABC Kit (Vector)], 1.5 h, moist
- Washing (first: 0.1 M TBS, 2x5 min; then: acetate-imidazole buffer (1M sodium acetate, pH 7.2; 0.2 M imidazole, 2x5 min)
- Chromogen reaction (100 mM NiSO₄; 125 mM sodium acetate; 10 mM imidazole; 0.03% 3,3-diaminobenzidine (DAB); 0.003% H₂O₂, pH 6.5) 15 – 30 min
- Washing (first: acetate-imidazole buffer, 2x5 min, 1x10 min; then: 0.1 M TBS, 2x5 min)
- Slides mounting with glycerol, visualisation of the precipitate

Table 2.1. Antibodies used for immunocytochemistry (IC) and Western blotting (WB).

Antibody	Manufacturer, catalogue Number	Working dilution, IC	Working dilution, WB
RAR α	Santa Cruz (sc-551)	1:200	1:500
RAR β	Santa Cruz (sc-552)	1:200	1:500
RAR γ	Santa Cruz (sc-7387)	1:200	1:500
RXR α	Santa Cruz (sc-553)	1:200	1:500
RXR β	Santa Cruz (sc-831)	1:200	1:500
RXR γ	Santa Cruz (sc-555)	1:200	1:500
CRABP-II	Gift from P.Chambon	--	1:1000
S-100	Sigma (S2644)	1:100	--
erbB3	Santa Cruz (sc-285)	--	1:200
GAM: goat anti-mouse Ig	Sigma (A3682) peroxidase-conjugated	--	1:5000
GAR: goat anti-rabbit Ig	Sigma (A6154) peroxidase-conjugated	--	1:10000
GAM: goat anti-mouse Ig	Vector (BA-2000) biotin-conjugated	1:1000	--
GAR: goat anti-rabbit Ig	Vector (BA-1000) biotin-conjugated	1:1000	--

2.4. *In situ* Hybridisation

In situ hybridisation (ISH) techniques allow specific nucleic acid sequences to be detected in morphologically preserved cells or tissue sections. Thus *in situ* hybridisation can relate microscopic topological information to gene activity at the mRNA level. *In situ* hybridisation requires a probe which contains a reporter molecule that can be detected by affinity cytochemistry. The reporter molecule is accessible to modified antibodies, which are then detectable in chromogen reaction.

2.4.1. Preparation of slides and fixation of the material

In order to detect the presence of retinaldehyde dehydrogenases (RALDHs) in sciatic nerves after the injury, the right sciatic nerves of 20 NMRI mice were crushed as described in 2.1. Since *in situ* hybridisation detects the molecules on mRNA level, all procedures were performed in RNase free conditions. This was achieved by cleaning all needed instruments with *RNase Away* (Molecular Bio Products). After washing in 0.1 M PBS, sciatic nerves were fixed in 4% paraformaldehyde (PFA) for 2 h at RT. Following fixation, the nerves were cryoprotected in 25% sucrose (Sigma) for at least 12 hours. To obtain longitudinal sections, each three nerves were embedded in Tissue Tek (Sakura) and cut with a cryostat (Leica) in 15 μ m sections. The sections were collected with gelatin-coated microscope slides. Series of eight slides were prepared from each nerve.

2.4.2. Synthesis of single-stranded digoxigenin-labelled RNA probes for ISH by *in vitro* transcription of double-stranded DNA templates

Double-stranded DNA templates which encode the gene of interest must be inserted into transcription vectors. The cloning procedure was performed in the laboratory of Dr. P. McCaffery (Harvard medical school, Boston). Vectors with inserted RALDH-1 and RALDH-2 partial sequences were provided.

Most transcription vectors are derived from plasmids and contain at least one of the three bacteriophage RNA polymerase promoters situated adjacent to a multiple cloning site. The designed restriction sites are presented at 5' and 3' of the insert. In this study pBluescript (Stratagene) phagemids were used. This vector has dual promoters - T3 and T7 – at either side of synthetic polylinker containing 23 unique restriction enzyme sites. These sites generate 5' and 3' sticky and blunt ends and generally allow the directional cloning of DNA inserts. pBluescript carries the regulatory sequences and amino terminus coding region of the *E. coli* β -galactosidase (lacZ) gene, which allows α -complementation when pBluescript is transformed into a bacterial host. The polylinker and both flanking promoters are situated within the lacZ coding region. Successful subcloning into the polylinker disrupts lacZ and results in a non-functional peptide. This allows screening by blue (non-recombinant) / white (recombinant) colony selection after induction of lacZ and incubation of bacteria on X-gal substrate.

After selection of the transfected colony, it was placed in the new vehicle and allowed to generate a liquid culture in LB medium (10% trypsin, 5% yeast extract, 10% NaCl) with 4% ampicillin by 37°C for 12 h. Afterwards plasmid DNA was isolated with Qiagen Plasmid Purification Kit (Qiagen) according to the manufacturers instructions.

Thereafter, *in vitro* transcription of insert DNA into complementary RNA was done according to the following scheme:

- Linearisation of DNA template with EcoR1 restriction enzymes, 2 h, 37°C
- Quality check: 0.8% agarose gel electrophoresis
- Evaporation (purification of DNA template from restriction enzymes and protein traces): 1) 3 M sodium acetate and 100% phenol/chloroform, centrifugation 12000 x *g* (10 min), DNA in the supernatant. 2) 95% ethanol, incubation at -80°C, 30 min followed by centrifugation 12000 x *g* (10 min), DNA is in pellet. RNase free conditions were followed from here on.
- Transcription of linear DNA with digoxigenin (DIG). DIG-labelled dNTP mix was used to generate RNA. For transcription of RALDH-1 – T3 RNA polymerase, for RALDH-2 – T7 RNA polymerase (MBI Fermentas) were used, 1 h, 37°C
- Removal of the rest DNA template with DNase I (Invitrogen), 15 min, RT; inactivation of the enzyme with 25 mM EDTA, 10 min, 65°C
- Purification and precipitation of the ready to use RNA-riboprobe with 4 M LiCl and 100% ethanol
- Dissolvement in diethyl-pyrocabonate (DEPC)-treated H₂O

The labelling method is based on a digoxigenin steroid isolated from digitalis plants (*Digitalis purpurea*), which are the only natural source of digoxigenin. Digoxigenin is linked to the C-5 position of uridine nucleotides via a spacer arm containing eleven carbon atoms. Hybridised DIG-labelled probes may be detected with high affinity anti-digoxigenin (anti-DIG) antibodies that are conjugated to alkaline phosphatase. In this case the visualisation is obtained with the colorimetric (NBT and BCIP) alkaline phosphatase substrates.

2.4.3. *In situ* hybridisation

My *in situ* hybridisation procedures followed this protocol:

- Pre-incubation washes. Serve to re-hydrate tissue sections, to enable the access of proteases to the tissue (ethanol series of 100%, 95%, 70%, 50% in 0.3 M NaCl and 0.03 M sodium citrate (2xSSC); each 1x1 min, RT)
- Protease treatment. Serves to increase target accessibility by digesting the proteins that surround the target nucleic acid (Protease K, 10 mg/ml, 3 min, 37°C)
- Washing, all at RT: 1) in DEPC-H₂O, briefly; 2) 0.1 M triethanolamine solution (TEA), pH 8.0, 3 min; 2.6% acetic anhydride in 0.1 M TEA, 10 min
- Dehydration of the slides in ethanol series: 2xSSC, 50%, 70%, 95%, 100%, each 1x1 min, RT
- Riboprobe preparation. The probes were denatured at 68°C, 10 min and added to the hybridisation mix [400 ng/ml in 50% formamide, 10% dextran sulfate, 1% Blocking reagent (Roche)]
- *In situ* hybridisation. Prepared probes were spread on the slides, which were then covered with a slide cover and the edges sealed with DPX. Incubation 12 h at 68°C
- Post-hybridisation washes with 1) 2xSSC, 55°C, 2x30 min; 2) 50% formamide, 55°C, 30 min; 3) 2xSSC, 37°C, 2x30 min
- RNase treatment. This step is done in order to eliminate traces of unbound riboprobes, which would otherwise enhance background staining (RNase A 100 mg/ml in 0.01 M Tris, 0.001M EDTA, 3% NaCl)
- Blocking of unspecific binding sites of the AB. 1) Incubation with 10% blocking buffer (2xSSC, 0.05% Triton X-100, 2% Blocking reagent (Roche), 2 h, RT; 2) 1x maleate buffer (0.58% maleic acid, 0.44% NaCl), 2x5 min, RT
- Incubation with anti-DIG antibody (Roche, 1093274). Antibody dilution of 1:1000 was prepared in an antibody buffer (1x Maleate buffer, 0.3% Triton X-100, 2% Blocking reagent), incubation for 24 h, 4°C, moist

- Post-incubation washes with 1) 1x Maleate buffer, 2x 10 min, RT; 2) Buffer 3 (100 mM Tris pH 9.5, 100 mM NaCl, 50 mM MgCl₂, 1 mM Levamisole), 10 min, RT
- Immunocytochemistry. Incubation with chromogen substrate (Buffer 3 with 0.34% nitro blue tetrazolium chloride (NBT), 0.17% 5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt (BCIP), 12 h, RT, in darkness
- Post-substrate wash with 1 mM EDTA, 0.9% NaCl, 0.01 M Tris-Cl, pH 7.5; 30 min, RT
- Mounting of slides with glycerol, visualisation of the precipitate

2.5. RNA isolation, reverse transcription and PCR

2.5.1. RNA isolation

Total RNA was isolated from sciatic nerves, DRGs and Schwann cells using Trizol (Invitrogen) based on the method of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). For Schwann cell RNA isolation two culture flasks were pooled to generate one sample. RNA isolation followed the outline listed below:

- Homogenisation. Incubation of tissues or cells with Trizol reagent (phenol-guanidine isothiocyanate), cells 5 min, nerves and DRGs 1 h; disruption of tissue residues with ultra-sound
- Phase separation. Addition of chloroform (Sigma), centrifugation in order to separate RNA from DNA and proteins, 15 min, 12000 x *g*
- RNA precipitation. Transfer of aqueous RNA phase to isopropyl alcohol in order to precipitate RNA, 1 h, -20°C. Since sciatic nerves are tissues with a low RNA yield, 1% glycogen was added to help pellet visualisation after centrifugation, 15 min, 12000 x *g*
- RNA wash. After removing of supernatant, RNA pellet was briefly washed with 75% ethanol
- Re-dissolving of RNA. After discarding of ethanol, RNA pellet was air dried and dissolved in DEPC-treated H₂O
- Estimation of RNA amount in the sample: photometrically the absorbance (A) of the RNA-water mixture at 260 nm was measured after the samples were diluted 1:100. The concentration of the total RNA was calculated as follows: $A_{260} \times 100 \text{ (1:100 dilution)} \times 40 \text{ ng/}\mu\text{l} = [\text{RNA}]$

2.5.2. DNase treatment

Samples of 2500 ng total RNA were treated with DNase I (Invitrogen) to eliminate traces of DNA. The following protocol was used for DNase treatment:

- Addition of 5 units of DNase I and DNase reaction buffer, 15 min, RT
- Inactivation of DNase I with 25 mM EDTA, 10 min, 65°C

2.5.3. RT-PCR

500 ng of DNase treated RNA was reverse transcribed with oligo-dT-primer and Omniscript reverse transcriptase (Qiagen) according to the manufacturer's instructions. The rest of RNA was used as a negative control for PCR reactions. Reverse transcription (RT) followed this protocol:

- Incubation of RNA with the following reagents: 1 µM oligo-dT₁₂₋₁₈-primer (Invitrogen; binds to poly-A tail of mRNA, used as priming for reverse transcriptase), 1x RT Buffer, 0.5 mM of each dNTP, 0.5 units/µl of RNase inhibitor RNaseOUT (Invitrogen), 0.2 units/µl of RT enzyme; 1 h, 37°C

2.5.4. PCR

Polymerase chain reaction (PCR) is an *in vitro* method for enzymatic amplification of defined sequences of DNA. The reaction uses two oligonucleotide primers that hybridise to opposite strands and flank the target DNA sequence that is to be amplified. The elongation of the primers is catalysed by a heat stable DNA polymerase. A repetitive series of cycles involving template denaturation, primer annealing, and extension of the annealed primers by the polymerase results in exponential accumulation of a specific DNA fragment. The ends of the fragment are defined by the 5' ends of the primers (position in the sequence). Because the primer extension products synthesised in a given cycle can serve as a template in the next cycle, the number of target DNA copies approximately doubles every cycle. Analysis of PCR products is done by separation in agarose gel electrophoresis according to their molecular weight. The visualisation of products in the gel is possible with ethidium bromide, which integrates in the structure of nucleic acid and after absorption of UV-light at 312 nm emits fluorescence at 590 nm.

Primer sequences for the amplification of the components of the retinoic acid system and erbBs are listed in **Tab. 2.2**. PCR primers from rat gene sequences were

generated using software of Whithead Institute for Biomedical research (Cambridge, MA; <http://www.genome.wi.mit.edu/cgi-bin/primer/primer3www.cgi>) and synthesised at MWG-Biotech.

Table 2.2. Primers used in PCR and quantitative “real-time” PCR.

Gene	Primer sequence (sense, antisense)	Position in sequence	Expected size of PCR fragment
CRBP-I	5'- ACGGGTACTGGAAGATGCTG -3' 5'- GGCCGCTCAGTGTACTTTCT -3'	111-130 489-508	398 bp
CRABP-I	5'- GACGAAGGACCAGAATGGAA -3' 5'- GGCCCATCACTTCTTTTGA -3'	58-77 447-466	409 bp
CRABP-II	5'- CTTCAAGATCGGGGAGGAAT -3' 5'- TGGTATGGAGCTGGGACTTC -3'	189-208 577-596	408 bp
RAR α	5'- CAGATGCACAACGCTGGC -3' 5'- CCGACTGTCCGCTTAGAG -3'	886-903 1263-1282	397 bp
RAR β	5'- GCGGAGAGATCATGTTTGAC -3' 5'- TGGCATCGATTCTTAGTGAC -3'	187-206 579-598	412 bp
RAR γ	5'- GTGGAGACCGAATGGACC -3' 5'- GACAGGGATGAACACAGG -3'	1328-1345 1829-1848	521 bp
RXR α	5'- CAATGGCGTCCTCAAGGTTC -3' 5'- ACTCCACCTCGTTCTCATTC -3'	531-550 837-856	326 bp
RXR β	5'- AGATCAACTCCACAGTGTCTG -3' 5'- TCTCCATCCCCGTCTTTGTC -3'	221-240 589-608	388 bp
RXR γ	5'- CTCAGCTCAATGTGGTCAAC -3' 5'- CACATTCTGCCTCACTCTCT -3'	58-77 425-444	387 bp
RALDH-1	5'- CTCAGCTCAATGTGGTCAAC -3' 5'- CACATTCTGCCTCACTCTCT -3'	1437-1456 1821-1840	404 bp
RALDH-2	5'- CTCAGCTCAATGTGGTCAAC -3' 5'- CACATTCTGCCTCACTCTCT -3'	1020-1039 1443-1462	443 bp
RALDH-3	5'- CTCAGCTCAATGTGGTCAAC -3' 5'- CACATTCTGCCTCACTCTCT -3'	1280-1399 1658-1679	400 bp
CYP26	5'- GTGCCAGTGATTGCTGAAGA -3' 5'- TCCTCTCTCCCATGAGTGCT -3'	480-499 850-869	390 bp
erbB2	5'- CCCATCAGAGTGATGTGTGG-3' 5'- TCATCTTCCAGCAGTGAACG-3'	2730-2749 3047-3066	337 bp
erbB3	5'- GAGGCTTGTCTGGATTCTGC-3' 5'- GGTGTGTATCTGGCATGACG-3'	3319-3338 3606-3625	307 bp
GAPDH	5'- AGAACATCATCCCTGCATCC -3' 5'- TTAATCCTTGGAGGCCATGT -3'	1454-1473 1832-1851	398 bp
β -actin	5'- GAGCTATGAGCTGCCTGACG -3' 5'- AGCACTTGCGGTCCACGATG -3'	636-655 1026-1045	410 bp

PCR was performed according to following protocol:

- 10 µl volume of a ReadyMix REDTaq solution (Sigma) contained: 0.08 mM dNTP (cATP, dCTP, dGTP, dTTP), 0.03 units/µl *REDTaq* polymerase, 4 mM Tris-HCl, 20 mM KCl, 0.6 mM MgCl₂, 0.0004% gelatin, stabilisers. To each ReadyMix aliquot 1 µM of sense and antisense primer (see **Tab. 2.2.**) were added as well as 25 ng of cDNA. Negative controls (reactions to prove that no DNA contamination is present in the samples) were carried with the same volume of DNase I treated RNA instead of cDNA
- Alternatively, Qiagen PCR Kit (Qiagen) was used. One reaction contained 1x PCR Buffer, 1x Q-solution, 5 mM MgCl₂, 0.1mM each dNTP, 0.6 µM of sense and antisense primer, 0.125 units/µl *Taq* DNA polymerase and 25 ng of cDNA
- Reaction parameters were: first denaturation 180 sec at 94°C, 30-35 cycles of annealing 60 sec at 51°C, extension 90 sec at 72°C, denaturation 60 sec at 94°C, followed by 60 sec annealing and last elongation 600 sec at 72°C. (Annealing temperatures of 7°C below T_m's of the primers were chosen for PCR).
- The PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide to verify their expected sizes

2.6. Quantitative “real-time” PCR

Analysing PCR products during amplification has become known as “real-time” PCR. The easiest way to monitor PCR is with fluorescence. The most frequently used fluorescence dye used for “real-time” PCR is SYBR Green I, which has similar properties as ethidium bromide. As PCR progresses, double stranded DNA is synthesised and the fluorescence of SYBR Green I increases. Thus the amount of synthesised PCR product may be monitored after each cycle by fluorescence. If fluorescence is plotted against cycle number, the accumulation of PCR products produces a growth curve (**Fig. 2.2.**) with an initial phase (below the sensitivity of fluorimeter), an exponential phase, and a final plateau phase. Monitoring fluorescence after each cycle is a very convenient way to quantify the initial number of template copies (which conventional qualitative PCR does not permit). Higher concentrations of template shift the growth curve parallel to the

abscissa and to the earlier cycles (to the left). This shift can be quantified as a fractional cycle number and is inversely proportional to the log of the initial template concentration.

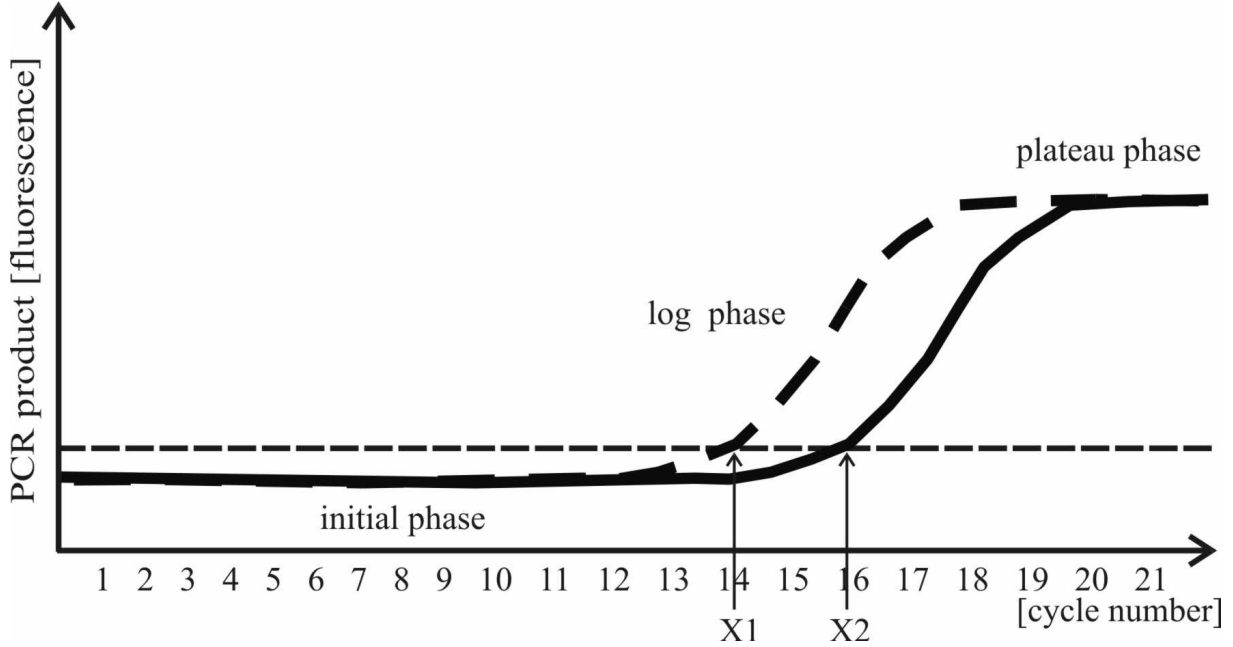


Fig. 2.2 Example of typical Light Cycler PCR curves. Crossing points (X1 and X2) can be determined by “real-time” monitoring.

The quantification was done using the following calculations:

During the log phase the amplification is described by this equation:

$$C_x = C_0 \times 2^x, \quad (1)$$

where C_x is the amount of target sequence at cycle x , C_0 is the initial amount of target and “2” is the efficiency of amplification. However the replication of PCR product is achieved only in the ideal case, thus

$$C_x = C_0 \times a^x \text{ where } a \leq 2 \quad (2)$$

In order to establish a (efficiency of amplification) a dilution series of cDNA were performed. For this three different cDNA concentrations ($C_1 = 12.5$ ng, $C_2 = 6.25$ ng and $C_3 = 3.125$ ng) were tested for each primer pair and in the same Light Cycler PCR run. The point of the log phase begin (crossing point) for each cDNA concentration was empirically determined and identified here as x . Since $C_1/C_2 = C_2/C_3 = 2$, the equation (1) can be modified as follows:

$$C_1/C_2 = a^{-\Delta x} \text{ where } \Delta x = X_1 - X_2 \quad (3)$$

$$\lg C_1/C_2 = -\Delta x \times \lg a \quad (3a)$$

$$\mathbf{a} = 10^{((\lg C_1/C_2)/-\Delta X)} \quad (3b)$$

$$C_1/C_2 = 2 \quad (4)$$

$$\mathbf{a} = 10^{(0,301/-\Delta X)} \quad (4a)$$

The factor **a** is now used to calculate the relative amount of initial template in the experiment sample $C_{\text{experiment}}$ compared to C_{control} :

$$C_{\text{experiment}} / C_{\text{control}} = a^{-\Delta X} \quad (5)$$

ΔX represents the curve shift (differences in the crossing points) between experimental and control samples:

$$\Delta X = X_{\text{experiment}} - X_{\text{control}} \quad (6)$$

These calculations were done for each primer pair investigated in this study: CRBP-I, CRABP-II, erbB2, erbB3, GAPDH and β -actin and the PCR-efficiencies **a**, were determined.

To establish whether the initial cDNA amount is the same in each sample, the expression of house-keeping genes GAPDH and β -actin was used as positive controls. Ideally, the point of beginning of the log phase by GAPDH or β -actin amplification is same for experimental and control samples:

$$\Delta X = 0 \quad (7)$$

$$(C_{\text{experiment}} / C_{\text{control}})_{\text{GAPDH}} = a^{-\Delta X} = 1 \quad (7a)$$

However, this is not the case in most of the experiments and the concentration differences are < 1 or > 1 , thus

$$(C_{\text{experiment}} / C_{\text{control}})_{\text{GAPDH}} = a^{-\Delta X} = \mathbf{Q} \quad (7b)$$

indicative of the differences of cDNA amounts in experimental and control samples. The quotient $1/\mathbf{Q}$ was used as a correction factor for the sample comparison. For this, all obtained concentration differences for CRBP-I, CRABP-II, erbB2 and erbB3 between experimental and control samples were multiplied by the correction factor $(1/\mathbf{Q})$.

The identification of PCR products was done by their melting temperature (T_m). It depends on the G:C content and the length of the synthesised product and can be calculated theoretically using this equation:

$$\mathbf{Tm} = 69.3 + 0.41 \times \text{GC\%} - 650/\text{sequence length} \quad (8)$$

To measure the relative change of mRNA expression quantitative PCRs were performed by means of the Roche Light Cycler System using the QuantiTect SYBR

Green PCR Kit (Qiagen) and Light Cycler Software, Version 3. Quantitative “real-time” PCR was performed according to the following protocol:

- 10 μ l volume of QuantiTect SYBR Green PCR Master Mix (Qiagen) contained: HotStarTaq DNA polymerase, Tris-HCl, KCl, $(\text{NH}_4)_2\text{SO}_4$, 2.5 mM MgCl_2 , SYBR green I, dNTPs. To each aliquot of this Master Mix 0.5 μ M of sense and antisense primer (see **Tab. 2.2.**) and 12.5 ng of cDNA were added. Negative controls were carried out with the same volume of DNase I treated RNA instead of cDNA (i.e. omission of the RT-PCR step).
- In order to determine primer and PCR efficiency three different concentrations of cDNA were tested for each primer pair: $C_1 = 12.5$ ng, $C_2 = 6.25$ ng and $C_3 = 3.125$ ng.
- The experimental protocol consisted of a first denaturation 15 min at 95°C, followed by 35-40 cycles of annealing 20 sec at 52°C (erbBs) or 58°C (CRBP-I, CRABP-II), amplification 20 sec at 72°C, fluorescence measurement 5 sec at 81°C and denaturation 15 sec at 94°C. The expression of glyceraldehyde phosphate dehydrogenase (GAPDH) or β -actin was used as a non-regulated control. (Annealing temperatures were consistent with the T_m 's of the primers; on additional fluorescence measurement step at 81°C was required to omit primer dimers built during amplification).
- The identity of PCR products was confirming with melting curve analysis and agarose gel electrophoresis.
- Logarithms of the mRNA ratios of RA different samples were calculated and corrected for differences in the extract concentrations based on the house-keeping gene analysis. After confirmation of their normal distribution (Shapiro-Wilk W test) the effect was tested with ANOVA. Differences between the mean quotients and zero were tested with a two-tailed t-test using Bonferroni-Holm correction or Dunnett's test.
- The amplified DNA fragments of CRBP-I and CRABP-II were cloned (TOPO-TA cloning, Invitrogen) and sequenced (ZEDA, *Frauenhofer Institut RWTH-Aachen*).

2.7. SDS-PAGE and Western blotting

2.7.1. Protein isolation and preparation

For immunoblotting nerves were isolated as described in part 2.1 and sonicated in hypotonic buffer with a mix of protease inhibitors. Schwann cells were harvested as described in 2.2. and re-dissolved in the same buffer as the nerves. Hypotonic buffer consisted of 20 mM HEPES, 1 mM PMSF, 1 μ M Leupeptin, 1% Aprotin, 1% Triton X-100. The protein concentration was measured using BCA test assay (Sigma). The principle of this test is based on the ability of proteins to reduce alkaline Cu(II) to Cu(I) in a concentration dependent manner. Bicinchoninic acid (BCA) is a highly specific chromogenic reagent for Cu(I) forming a purple complex with an absorbance maximum at 562 nm. The following protocol was used for the BCA test:

- Generating protein standards (bovine serum albumin): 0.1, 0.2, 0.4, 0.8, 1.4, 2, 3.2 μ g/ μ l
- Preparation of tested samples: dilution 1:10
- Addition of 1x 4% Cu₂SO₄ and 50x BCA to protein standards and test samples
- Incubation for 30 min. at 37°C, extinction measurement with ELISA-reader at 630 nM
- Calculating of the protein amount using calibration curve of known standards was done by linear regression

2.7.2. SDS-PAGE

SDS-PAGE (sodium dodecyl sulfate – polyacrylamide gel electrophoresis) is used to separate mixed protein samples according to size under denaturing conditions. Gel electrophoresis is a technique in which an electric current is used to move charged molecules through a gel-like matrix. Acrylamide is a 3-carbon amide which is polymerised to form long chains with cross-links between the chains. For SDS-PAGE, a discontinuous buffer system is used, where different buffer ions are present in the gel and electrode reservoirs. Using two different gel systems (stacking and resolving gels) enables the compression of the samples in a thin starting band followed by fine separation during electrophoresis. To achieve such separation proteins must first be denaturated by heating in buffer containing SDS and a thiol reducing agent 2-mercaptoethanol.

The protocol was performed according to the following protocol:

- Generating of 10% resolving gel (Acrylamide/Bis (30% T, 2.67% C), 10% SDS, 1.5 mM Tris-HCl pH 8.8, 10% APS, TEMED), polymerisation 2-3 h or overnight
- Generating of 5% stacking gel (Acrylamide/Bis (30% T, 2.67% C), 10% SDS, 0.5 mM Tris-HCl pH 6.8, 10% APS, TEMED), polymerisation 1 h
- Denaturation of proteins by heating with SDS and 2-mercaptoethanol and loading in the gel
- Electrophoresis by 15 mA/ 2 cm gel (in stacking gel) and 30 mA/ 6 cm gel (in resolving gel); 1.5 h at RT. Electrophoresis buffer contained 25 mM Tris, 192 mM glycine, 0.1% SDS and had pH 8.3

2.7.2. Western blotting

Separated by discontinuous SDS-PAGE proteins were transferred by blotting onto a nitrocellulose membrane. The transfer occurred in a gel cassette (Bio-Rad) and in the transfer buffer A (25 mM Tris, 192 mM glycine, 20% methanol, 0.1% SDS, pH 8.3) at 30 V and 90 mA for 16 h. Quantity check of the protein transfer was done by Ponceau-S-staining (3% Ponceau-S, 30% TCA, 30% Sulfosalicylic acid), incubation for 5 min at RT.

2.7.3. ECL

Enhanced chemiluminescence (ECL) is based on the light emission resulting from the dissipation of energy from a substance in an excited state. The excitation is the effect of chemical oxidation of luminol under alkaline conditions and in the presence of phenol by horseradish/hydrogen peroxidase. Immediately following oxidation, the luminol is in an excited state which then decays to ground state via a light emitting pathway. The maximum light emission is at a wavelength of 428 nm, which can be detected by a short exposure of an autoradiography film. For ECL the same antisera were used as for immunocytochemistry (see **Tab. 2.1**), however, detection of immunoreactivity was based on peroxidase-conjugated goat anti-rabbit and goat anti-mouse secondary antibodies (see **Tab. 2.1**) and ECL kit (Amersham). X-ray films (Hyperfilm, Amersham) were exposed 30 sec – 10 min to determine the linear range of the signal.

ECL was performed according to the following protocol, all procedures at room temperature on an orbital shaker:

- Blocking of non-specific binding sites by 10% bovine serum albumin (BSA) in Tris-buffered saline/Tween 20 buffer (TBS-T; 20 mM Tris, 0.8% NaCl, 0.1% Tween 20, pH 7.6); 10 min (polyclonal AB) or 1 h (monoclonal AB)
- Washing (TBS-T), 3x 15 sec
- Incubation with the primary antibody (for dilutions see **Tab. 2.1**), 1h
- Washing (TBS-T), 3x 15 sec, 1x 10 min
- Incubation with the secondary antibody (for dilutions see **Tab. 2.1**), 15 min
- Washing (TBS-T), 3x 15 sec, 1x 10 min
- Incubation with the detection solution (equal volumes of detection solution 1 with detection solution 2 from ECL kit) for 1 – 3 min
- Film exposure
- Evaluation with a digital image analysis system (LTF)

3. Results

The scientific goal of this project is to investigate the role of retinoic acid (RA) signal transduction after peripheral nerve injury. While a number of indirect pieces of evidence suggested RA as a regeneration-associated signal, not even the presence of this molecule in the PNS of adult mammals was known at the beginning of my project. Thus, the first part of my thesis was devoted to determining the expression of molecules involved in RA signalling in the PNS. For this the expression of the RA-signalling cascade was tested in sciatic nerves and Schwann cells *in vitro*. The activity of RA is generally inferred from the local distribution of retinaldehyde dehydrogenases. Therefore their expression was evaluated by RT-PCR and *in situ* hybridisation (3.1). Since cellular retinoid binding proteins also play important roles in retinoic acid signalling, the expression of CRBP-I and CRABP-II was evaluated and is described in (3.2). Transcriptional effects of retinoic acid are mediated via its receptors – ligand-activated transcriptional factors RARs and RXRs. Accordingly, their expression was measured during peripheral nerve regeneration (3.3). After establishing the fact that the retinoic acid system participates in the physiological reactions to nerve injury, the question about its physiological relevance arose. Taking into account the indirect evidence of RA-system involvement in Wallerian degeneration, the expression of β -Neuregulin-1 receptors erbB2 and erbB3 was measured in RA-treated Schwann cells *in vitro* (3.4), because these signals have been implicated as triggers of Wallerian degeneration.

3.1. RA signalling in the PNS

3.1.1. Abstract

Although the specific roles of Schwann cells, neurons and macrophages in injury-induced processes have been established, much less is known about the regulation of their functions. I propose that the transcriptional activator retinoic acid (RA) might control some of these functions. Since Schwann cells are major players in the physiological interactions following peripheral nerve injury I addressed the question whether the retinoic acid signalling system is present in sciatic nerves and in Schwann cells *in vitro*. With RT-PCR all necessary components of the RA signalling pathway were detected in the sciatic nerve of adult rats and in Schwann cell cultures. These are retinoic acid receptors, retinoid X receptors, the retinoic acid synthesising enzymes RALDH-1, -2, and -3, in addition, the cellular retinoid binding proteins CRBP-I, CRABP-I and -II. Even though the expression of RALDH-1 and RALDH-2 was not affected by the injury, the increase of retinoic acid responsive element (RARE) activity after crush suggests an involvement of RA in traumatic processes after peripheral nerve injury.

3.1.2. Introduction

The understanding of molecular processes that cause success or failure of neuronal regeneration has become one of the main problems in medical neurobiology. The physiological reactions after nerve injury in the peripheral nervous system (PNS) are regulated by interactions between damaged neurones, glia cells (Schwann cells) and hematogenous cells of the immune system. On the molecular level these interactions are mediated by signal transduction mechanisms between cells (via cytokines and cytokine receptors) and between cells and extracellular matrix (via ECM-glycoproteins and integrins; Jessen and Richardson, 2002).

Signal transduction processes after peripheral nerve lesions are well described (Gillen et al., 1997; Raivich et al., 1999), yet although injury-induced changes in gene expression of many cytokines have been described in this context, much less is known about their regulation. It has been suggested that the transcriptional activator retinoic acid (RA) might control the expression of signalling molecules in the adult nervous system (Mey, 2001). This hypothesis is supported by indirect evidence: several nuclear RA-receptors, RA-synthesising enzymes and cellular RA binding proteins have been located in the adult central nervous system (CNS; Dev et al., 1993), oligodendrocytes synthesise RA *in vitro* (Mey and Hammelmann, 2000) and in some cases RA promotes axonal regeneration *in vitro* (Corcoran and Maden, 1999; Corcoran et al., 2000; Mey and Rombach, 1999). In a variety of cell culture systems unrelated to the nervous system, RA regulated the expression of many intracellular signals which take part in the physiology of nerve regeneration (Chang et al., 2000; Choudhury et al., 2000; Mey, 2001). However, no experiments have so far been conducted to prove that functional retinoid signalling forms part of the traumatic reactions in the nervous system.

Pursuing the hypothesis that RA serves as a regulator of lesion-induced cytokine signalling I investigated the expression of the RA-signalling cascade in the PNS and in Schwann cell primary cultures. Taking into account the fact that the distribution of retinoic acid is determined by the activity of retinaldehyde dehydrogenases, their expression was measured after traumatic injury of rat and mouse sciatic nerves.

3.1.3. Materials and methods

Surgical procedures

Adult male Sprague-Dawley rats (for RT-PCR) and NMRI mice (for *in situ* hybridisation) were used in this study and handled as described in part 2.1.

Cell culture

Primary cultures of Schwann cells were prepared from new-born Sprague-Dawley rats and processed as described in 2.2.

Immunocytochemistry

Sciatic nerves from adult rats were fixed 1 h in cold ethanol, cryoprotected in sucrose and cut with a cryostat in 20 µm longitudinal sections. Immunocytochemistry procedures are described in the part 2.3.

RT-PCR

Total RNA was isolated using Trizol (Invitrogen), reverse transcribed, and cDNA was amplified as described in 2.5. The PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide to verify their expected sizes.

Quantitative PCR

To quantify the amounts of RALDH-I and RALDH-II mRNA after crush PCRs were performed by means of the Roche Light Cycler System using the QuantiTect SYBR Green PCR Kit (Qiagen) and Light Cycler Software, Version 3 (Roche) as described in 2.6.

***In situ* hybridisation**

In order to detect the presence of retinaldehyde dehydrogenases (RALDHs) in sciatic nerves of NMRI mice after the injury, *in situ* hybridisation (ISH) was performed according to the protocol described in 2.4. *In vitro* transcription of insert DNA into complementary DIG-labelled RNA riboprobe was done according to the scheme listed in 2.4.

3.1.4. Results

The retinoic acid signalling cascade is present in the rat sciatic nerve and in Schwann cells *in vitro*.

Signal transduction of retinoic acid requires the activation of nuclear receptors that act as transcription factors. I analysed their expression with RT-PCR. Genes of all known retinoic acid receptors ($RAR\alpha$, $-\beta$, $-\gamma$) and retinoid X receptors ($RXR\alpha$, $-\beta$, $-\gamma$) were found to be expressed in rat sciatic nerves (**Fig. 3.1.1**) and in Schwann cells of the primary culture (**Fig. 3.1.2**). In addition, I detected gene products of the cellular retinol binding protein CRBP-I and cellular retinoic acid binding proteins CRABP-I and CRABP-II, and of the retinoic acid catabolising enzyme CYP26 (it gave a weak but detectable signal). **Figs. 3.1.1** and **3.1.2** show the amplified fragments.

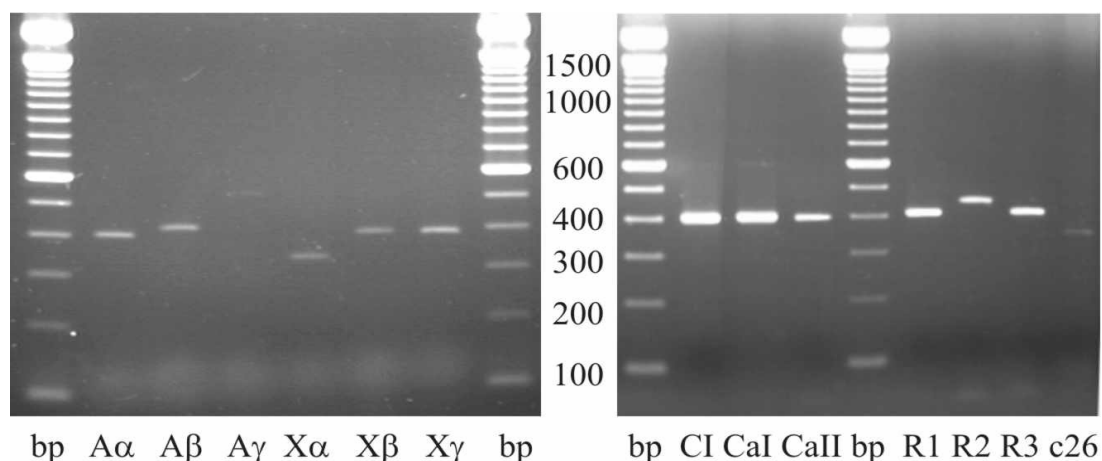


Fig. 3.1.1 The retinoic acid signalling system is present in the rat sciatic nerve. Gene expression of retinoid receptors ($A\alpha$: $RAR\alpha$, $X\alpha$: $RXR\alpha$ etc.), cellular retinoid binding proteins (CI : CRBP-I, CaI : CRABP-I, $CaII$: CRABP-II), aldehyde dehydrogenases ($R1$: RALDH-1, $R2$: RALDH-2, $R3$: RALDH-3) and RA degrading enzyme CYP26 ($c26$) are shown with RT-PCR. The molecular weights were consistent with the predicted sizes of all PCR fragments. bp : 100 bp maker, stronger band at 600 bp.

Traumatic injury of PNS does not induce changes in RALDH expression.

The temporal and spatial activity of retinoic acid is thought to be determined by the local distribution of RA synthesising aldehyde dehydrogenases (Ross et al., 2000). While in mammalian and avian embryos, three enzymes, RALDH-1, -2, and -3, are known to oxidise retinaldehyde to RA, only RALDH-1 and -2 have been detected after completion of development (Wagner et al., 2002). With RT-PCR I found tran-

scripts of RALDH-2 as well as of RALDH-1 and RALDH-3 in the intact sciatic nerves and 7 days after crush (**Fig. 3.1.3**).

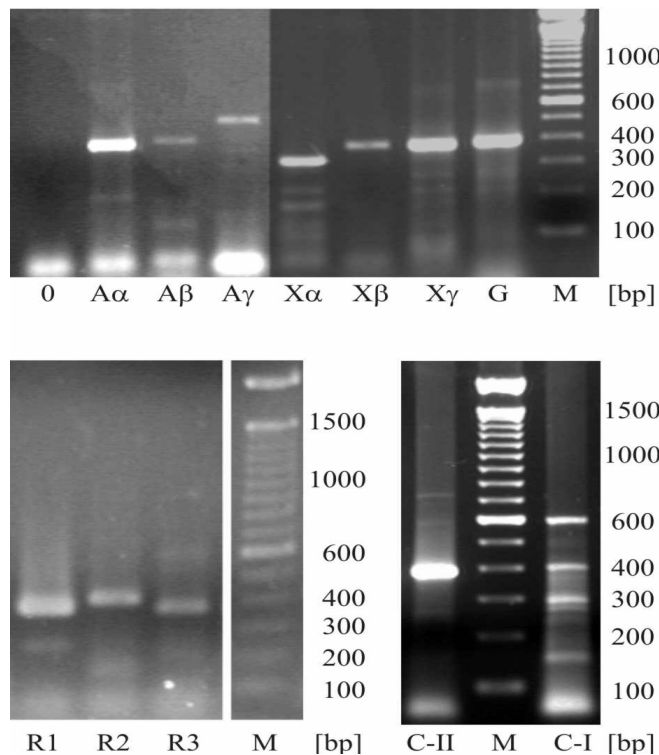


Fig. 3.1.2 The retinoic acid signalling system is present in the Schwann cell primary cultures. Gene expression of retinoid receptors ($A\alpha$: $RAR\alpha$, $X\alpha$: $RXR\alpha$ etc.), cellular retinoid binding proteins ($C-I$: $CRBP-I$, $C-II$: $CRABP-II$), aldehyde dehydrogenases ($R1$: $RALDH-1$, $R2$: $RALDH-2$, $R3$: $RALDH-3$) and of house-keeping gene $GAPDH$) is shown with RT-PCR. (0) – $RAR\alpha$ amplification of the negative control (w/o RT). The molecular weights were consistent with the predicted sizes of all PCR fragments.

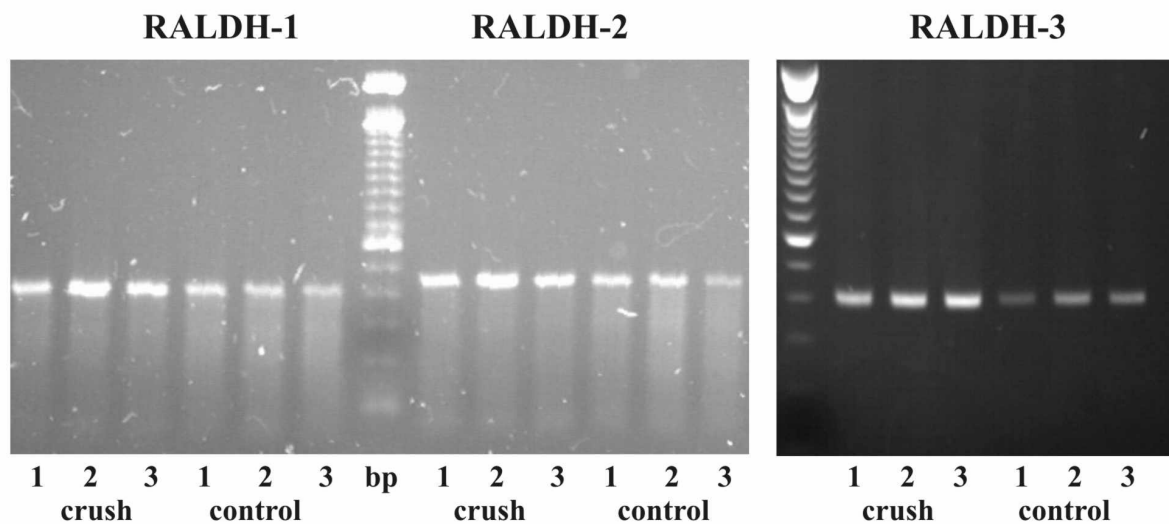


Fig. 3.1.3 RALDH-1, RALDH-2 and RALDH-3 are expressed in intact and lesioned sciatic nerves. Gene expression of RALDHs is shown with RT-PCR for crushed (cr) and control (c) sciatic nerves. The molecular weights were consistent with the predicted sizes of both PCR fragment: 404 bp for $RALDH-1$, 443 bp for $RALDH-2$ and 404 bp for $RALDH-3$. bp: 100 bp maker, stronger band at 600 bp.

The hypothesis of an altered expression of RALDHs after crush injury was tested using quantitative PCR on Light Cycler (Roche) and *in situ* hybridisation.

Even though the expression of RALDH-1, RALDH-2 and RALDH-3 was shown with conventional PCR (**Fig. 3.1.3**), I could not detect any expression of this transcripts with the Light Cycler. **Table 3.1.** shows all Light Cycler PCR parameters tested in this study. For RALDH-2 twelve different primer pairs, five $MgCl_2$ concentrations or annealing (AN) temperatures, as well as three different tissues were tested to optimise PCR reactions. In many cases irreproducible (*) results were obtained. In the case of oligodendrocytes (OLN-93 cell line) none of the tested parameters led to amplification, although in these cells the presence of RALDH-2 had been confirmed with Western blotting and enzyme assay (Mey and Hammelmann, 2000) .

Table 3.1. Light Cycler PCR parameters

tissue	AN temperature [°C]					MgCl ₂ [mM]					primer pairs	result (*/-)
	45	55	57	58	60	0	2	3	4	5		
Sciatic nerve		x	x	x	x	x	x	x	x	x	x	(*)
Sch. cells			x			x	x	x	x	x	x	(*)
OLN-93	x	x	x	x	x	x	x	x	x	x	x	(-)

The Light Cycler system is developed to use so called “rapid cycle PCR”, in which temperature changes occur according to a “kinetic” paradigm. This paradigm emphasises temperature transition, thus denaturation and annealing are reduced to only few seconds and the temperature may always be changing. Unfortunately, such a cycling pattern is not optimal for many genes, which seems to be the case for RALDHs. One of the alternative methods for evaluation of gene expression is *in situ* hybridisation (ISH). This technique allows specific nucleic acid sequences to be detected in morphologically preserved tissue sections. It requires a riboprobe which contains a reporter molecule that can be detected by affinity cytochemistry. The riboprobes for RALDH-1 and RALDH-2 used in this study were synthesised in co-operation with Dr. U. Commandeur (Institute of Biology VII, RWTH-Aachen). Since vectors with DNA inserts from *Mus musculus* genome were used, ISH was performed with crushed and intact sciatic nerves of NMRI mice. Fourteen pairs of crushed and control sciatic nerves were compared during three independent *in situ* hybridisation experiments. **Figure 3.1.4** shows representative nerve sections tested

for the presence of RALDH-2 in intact and lesioned tissue. For semi-quantification of RALDH-1 and RALDH-2 expression the following method was developed: Staining intensity of nerves and of the slide background was measured using *AxioVision* Software, Version 3 (Zeiss). From these values a quotient of staining intensities was calculated. These quotients were then statistically compared for intact and crushed sciatic nerves. For RALDH-1 23 sections of control nerves and 38 sections of the crushed (7 days) nerves were evaluated. For RALDH-2 24 sections of control nerves and 56 sections of crushed (7 days) nerves were evaluated. 12 mice were used to investigate the regeneration at this point of time. For the 24 h only 2 sections (2 mice) were evaluated for both genes.

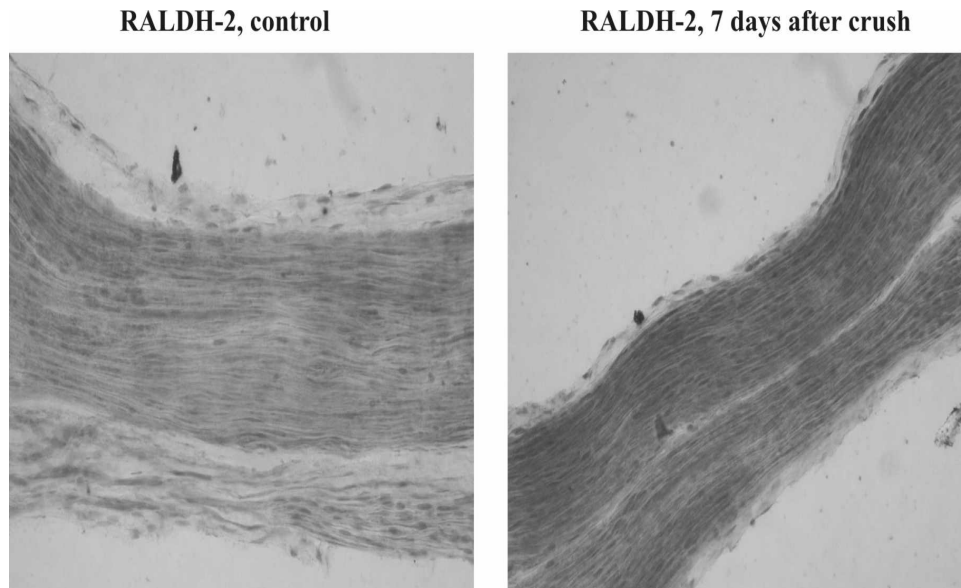


Fig. 3.1.4 ISH of RALDH-2 expression after sciatic nerve injury. *Riboprobe hybridisation of RALDH-2 in the intact sciatic nerve (control) and 7 days after lesion.*

Figure 3.1.5 shows the development of RALDH-1 and RALDH-2 transcript levels 24 h and 7 days after crush lesion. After confirming their normal distribution (Shapiro-Wilk W test) the effect of the lesion was tested with ANOVA. An apparent up-regulation of both transcripts was not significant either 24 h or 7 days after the injury.

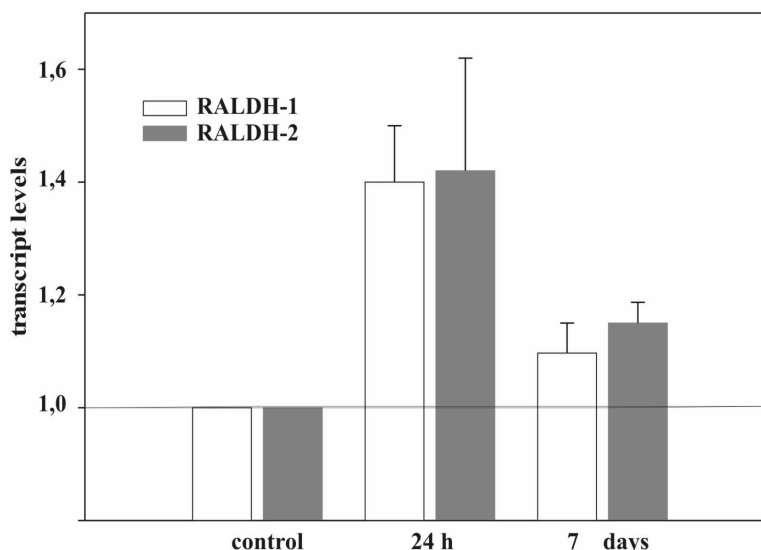


Fig. 3.1.5 Relative quantification of RALDH-2 mRNA expression. Staining intensity in non-injured (control) and lesioned (24 h or 7 days) nerves, black columns: RALDH-2, white columns: RALDH-1, error bars represent standard error of mean (SEM). Strength of the staining is calculated in relation to the contralateral (control) side. Lesion induced changes in immunoreactivity were not significant (ANOVA).

3.1.5. Discussion

The data obtained in this part of my thesis show the presence of all major retinoid signalling molecules in the intact PNS. With RT-PCR I detected the mRNA expression of all six retinoid receptors, as well as of retinoic acid synthesising enzymes, cellular retinoid binding proteins, retinoid receptors and the catabolising enzyme CYP26. Thus, RA can be synthesised in sciatic nerves (suggested by the presence of gene transcripts for RALDHs and CRBP-I), transported to the nucleus (CRABP-II) and activate gene transcription (RARs and RXRs). Moreover, the excess of RA can be compartmentalised (CRABP-I) and catabolised (CYP26). Even though the cells which express retinoid signalling in the sciatic nerve are unknown, the presence of all components of this system in primary Schwann cell cultures allows the possibility that these cells may be the source and/or putative targets of RA signalling.

These findings open a new field of the possible functions of RA: involvement in regeneration processes. Because of its role in neuronal and glial differentiation, RA has previously been suggested to regulate gene expression in regenerating nerves (Mey, 2001). Besides, as mentioned above, this hypothesis is supported by the recent finding of a RA-dependent down-regulation of the gene expression of

ciliary neurotrophic factor (CNTF) in Schwann cells (Johann et al., 2003), which is similarly happening after nerve injury (Sendtner et al., 1996).

The local distribution of RA synthesising aldehyde dehydrogenases determines the spatial and temporal activity of retinoic acid (Ross et al., 2000). To test whether increased expression of RALDH was responsible for the lesion-induced RA signal I evaluated the mRNA levels of RALDH-1 and RALDH-2 seven days after crush injury. Despite an apparent increase in the RALDH-1 and RALDH-2 signal after *in situ* hybridisation and RT-PCR, no statistically significant changes were observed at the mRNA level. To analyse the protein expression of RALDH-2, its immunoreactivity was tested with Western blot and shown to be present, however also at this level of expressing, no significant changes after crush (or transection) were observed (K. Schrage, Diploma thesis). Using a zymography bioassay RA-synthesising activity in cytosolic extracts from the nerves was previously investigated. With this test, enzyme activity of RALDH-2 but not of RALDH-1 and -3 was detected (Zhelyaznik et al., 2003). The pattern of RALDH-2 staining in cryostat sections suggested that this aldehyde dehydrogenase was present in the cytosol of Schwann cells because its distribution resembled that of S-100 protein, a marker for Schwann cells in the peripheral nerve (Zhelyaznik et al., 2003). Experiments with the Schwann cell cultures also showed the presence of RALDH-2 immunoreactivity in these cells (J.Mey and K.Schrage, unpublished observations). These findings suggested that retinoic acid may be synthesised in sciatic nerves and Schwann cells *in vitro*, but that retinoid signalling may not to be regulated at the level of RA-synthesis. This raised the question whether retinoic acid is transcriptionally active in sciatic nerve after injury. To monitor the local transcriptional activity of RA within the injured sciatic nerve a transgenic mouse strain where endogenous RA induces the local expression of β -galactosidase as a reporter gene (Rossant et al., 1991) was used. Colorimetric staining for the lacZ product indicated regions with local activation of RA response elements (RARE), indicative of all-*trans* RA signalling (Wagner et al., 2000). Whereas in normal, non-injured sciatic nerves no β -galactosidase activity was detected whatsoever, a crush lesion caused RARE-induced gene expression within 2 days. The staining increased with time, reached a maximum 7 days after injury and declined thereafter (Zhelyaznik et al., 2003). This activation of RARE-regulated gene expression after nerve lesion indicated the participation of RA in traumatic processes following PNS

injury, however the mechanisms of this process are still unclear. Obviously the expression of other components of RA-system must be studied in future, as well as possible biological functions of retinoic acid action after injury.

3.2. Activation of retinoic acid signalling after sciatic nerve injury: up-regulation of cellular retinoid binding proteins

3.2.1. Abstract

In mammalian peripheral nerves a crush lesion causes interactions between injured neurons, Schwann cells and hematogenous macrophages that can lead to successful axonal regeneration. I suggest that the transcriptional activator retinoic acid (RA) takes part in gene regulation after peripheral nerve injury and that RA signalling is activated via the cellular retinoic acid binding protein (CRABP)-II and cellular retinol binding protein (CRBP)-I. Sciatic nerve crush as well as transection resulted in a more than 10-fold up-regulation of CRBP-I, which is thought to facilitate the synthesis of RA. Both kinds of injury also caused a 15-fold increase in transcript and protein concentration of CRABP-II, a possible mediator of RA transfer to its nuclear receptors. Since the transcript and protein levels did not decline with time in degenerating nerves compared with regenerating tissue, I propose a function of RA during Wallerian degeneration.

3.2.2. Introduction

Retinoic acid (RA) is a transcriptional regulator of gene expression. Its intracellular processing is mediated by four binding proteins: cellular retinol binding protein (CRBP) types I and II and cellular retinoic acid binding proteins (CRABP) types I and II (Marill et al., 2003). After cellular uptake CRBP-I binds retinol and is involved in the storage as well as in oxidation of retinol via retinal to retinoic acid, reactions catalysed by alcohol dehydrogenases and RALDHs (McCaffery and Dräger, 2000; Ross et al., 2000). The role of CRBP-II, which is mainly found in the enterocytes of the gut, may be to handle retinoids after dietary uptake for further metabolism and transport to the liver (Napoli, 1996).

Both CRABP-I and CRABP-II have been shown to bind all-*trans* RA (Marill et al., 2003). However, the role of these two proteins is still not quite resolved. CRBP-I has been suggested to be involved in the regulation of RA availability for nuclear receptors by preventing RA from reaching the nucleus. Thus CRABP-I seems to protect cells from excess RA levels, since the CRABP-I/RA complex appears to favour RA-catabolism (Dong et al., 1999a). CRABP-II on the other hand facilitates the transport of RA to the nucleus, suggesting that it participates in the activation of nuclear receptors by RA (Budhu and Noy, 2002).

It was shown recently that the transcriptional activity of retinoic acid is enhanced when it was bound to CRABP-II (Delva et al., 1999; Dong et al., 1999a; Nikiforovich and Frieden, 2002), and that CRABP-II can directly co-activate RAR α /RXR α heterodimers, without engaging the ligand binding domain of CRABP-II (Bastie et al., 2001). While RA activates gene expression in the absence of CRABP-II (Lampron et al., 1995), the regulation of CRABP-II may be the trigger of retinoid signalling under certain circumstances, either by facilitating the nuclear transfer of RA or by direct co-activation of retinoid receptors. CRBP-I, being an indicator of cells which are able to synthesise RA, may also be important in nerve regeneration. By supporting enzymatic oxidation of retinol and retinal the elevated concentration of CRBP-I may as well lead to RA increase. Unbound CRBP-I (apo-CRBP) inhibits retinol acyltransferase, which esterifies retinol. Some pieces of evidence were collected that in the developing mouse central nervous system the CRBP-I-expressing cells synthesise and use RA in regulation of their own gene transcription (Napoli, 1996). Since some embryonic processes are

reactivated during regeneration in the adult, this observation may also apply for the regenerating peripheral nervous system.

Taking into account my previous studies, that show RA-system presence in the peripheral nervous system, I propose that retinoid signalling is activated by a peripheral nerve lesion via the up-regulation of the cellular retinoid binding proteins CRBP-I and CRABP-II.

3.2.3. Materials and methods

Surgical procedures

Adult male Sprague-Dawley rats were used in this study and handled as described in part 2.1.

RT-PCR

Total RNA was isolated using Trizol (Invitrogen), reverse transcribed and cDNA was amplified with PCR as described in 2.5. The PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide to verify their expected sizes.

Quantitative PCR

To quantify the amounts of CRBP-I and CRABP-II mRNA after crush or transection, PCRs were performed by means of the Roche Light Cycler System using the QuantiTect SYBR Green PCR Kit (Qiagen) and Light Cycler Software, Version 3 as described in 2.6. The PCR efficiencies (amplification per cycle) were for GAPDH 1.65; for β -actin, CRBP-I and CRABP-II 2.00. For every condition three to five independent Light Cycler experiments with pairs of crushed and control sciatic nerves and L4-L6 DRGs ipsilateral and contralateral to the lesion were performed and statistically analysed with Jmp software (SAS institute). For this, logarithms of the mRNA ratios of lesioned nerves to control nerves were calculated and corrected for differences in the extract concentrations based on the GAPDH or β -actin analysis as described in 2.6. After confirming their normal distribution (Shapiro-Wilk W test) data were tested with ANOVA and Dunnett's test. The identity of PCR products was confirmed with melting curve analysis and agarose gel electrophoresis. In addition, the amplified cDNA fragments of CRBP-I and CRABP-II were eluted from the agarose, cloned (TOPO-TA cloning; Invitrogen) and sequenced.

Western blotting

For immunoblotting, nerves were sonicated in hypotonic buffer with a mix of protease inhibitors as described in 2.7. Strength of immunoreactivity was always expressed in relation to the contralateral side, set at 100%. From immunoreactivity data the logarithms of the signal ratios of lesioned nerves to control nerves were calculated and tested with ANOVA and Dunnett's test.

3.2.4. Results

Nerve lesions cause up-regulation of CRBP-I and CRABP-II

Taking into account the rather stagnant level of RALDH-2, I surmised that the transcriptional effect of RA may be triggered through up-regulation of CRBP-I and CRABP-II. In order to investigate this, their mRNA concentrations were measured with quantitative RT-PCR (Light Cycler, Roche). The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β -actin was quantified as positive control in every experiment. Dilution series of cDNA extracts were used to determine PCR efficiencies for each primer pair.

Fig. 3.2.1a shows representative amplification curves where 7 days after nerve crush the expression of CRBP-I as well as of CRABP-II was increased compared to GAPDH expression. At this point in time, axonal regeneration occurred within the nerve segment. In order to control the identity of PCR amplicates, melting curve analysis and agarose gel electrophoresis of PCR products were performed after every experiment (**Fig. 3.2.1b, c**). In addition, amplified fragments were cloned and sequenced, and the obtained sequences were identical to the predicted sequences of the target genes. **Fig. 3.2.2** shows the development of CRBP-I and CRABP-II transcript levels up to 14 days after sciatic nerve lesion. Gene expression was investigated in the nerve segment 1 cm distal from the lesion site.

The up-regulation of CRBP-I and CRABP-II gene expression started during the first 24 hrs after a nerve crush and remained above control levels for at least 2 weeks (ANOVA, $p < 0.05$). For both genes, a more than 10-fold increase was seen 2 days after a crush lesion (first maximum; Dunnett's test, CRBP-I $p < 0.05$, CRABP-II $p < 0.01$), CRABP-II reached a second maximum again after 7 days ($p < 0.01$). Then, at two weeks after a crush, gene expression decreased in comparison with the situation after the first week when axonal regeneration occurred in the nerve segment. After 14 days the mRNA levels of CRBP-I were not significantly higher than in non-injured control nerves.

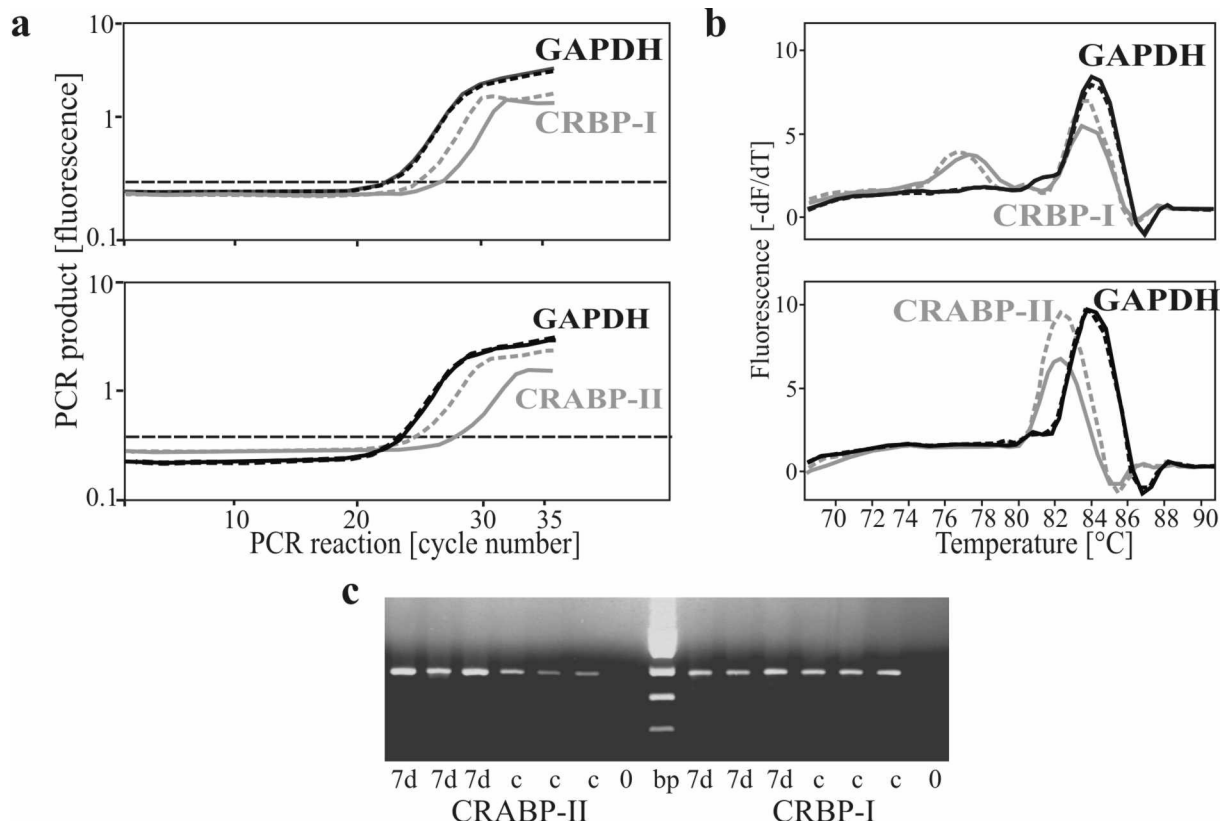


Fig. 3.2.1 Up-regulation of CRBP-I and CRABP-II mRNA after sciatic nerve injury, quantitative RT-PCR. *a* Representative amplification curves with primers for CRBP-I and CRABP-II. Broken lines represent results for sciatic nerves 7 days after nerve crush, continuous lines for non-injured nerves. The horizontal dashed line indicates background level of SYBR Green fluorescence which was used for quantification. While no lesion-induced change is observed for the concentration of GAPDH, the left shift of crossing points after crush injury indicates higher transcript concentrations of CRBP-I and CRABP-II. After all quantitative RT-PCR runs the amplified fragments were characterised *b* by their melting curves, and *c* with agarose gel electrophoresis. Expected fragment sizes were 408 bp (CRABP-II) and 409 bp (CRBP-I). Three independent experiments are shown with amplified fragments from nerves 7 days after crush (7d) and the contralateral controls (c), PCR amplification without reverse transcription is marked 0. The three distinct marker bands have 400, 300 and 200 bp.

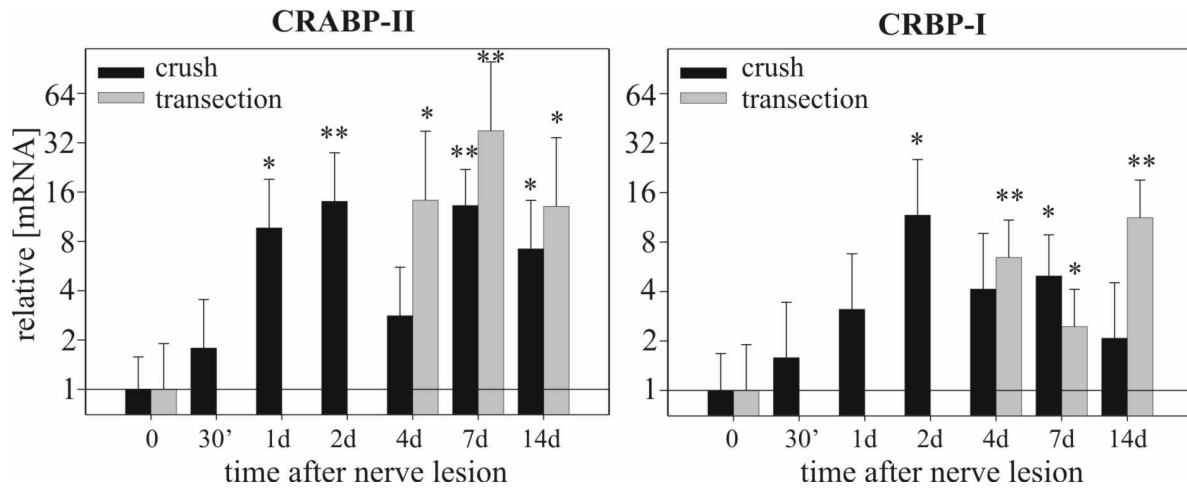


Fig 3.2.2 Relative quantification of mRNA concentrations. Data for the first 2 weeks after sciatic nerve lesions, normalised to transcript levels in the contralateral, non-injured nerves. Analyses were performed after sciatic nerve crush (black columns) and after complete transections (grey columns). Error bars indicate 95% confidence intervals. Analysis of variance showed a significant effect of both kinds of lesion on CRBP-I and CRABP-II expression ($p < 0.05$). Asterisks indicate significant differences compared to the non-lesioned control ($n = 5$ for the point in time 7 days, $n = 3$ for all others; Dunnett's test, * $p < 0.05$, ** $p < 0.01$).

Sciatic nerve crush causes a transient and nerve transection a lasting increase of cellular retinoid binding proteins

In addition to crush lesions I also performed nerve transections to cause Wallerian degeneration, but prevented axonal regeneration into the distal nerve segment. While in these experiments the expression of CRBP-I and CRABP-II rose similarly to the regeneration paradigm, they did not decline during the second week in the nerve segment distal to the lesion, such that after 14 days transcript levels remained higher than when regeneration occurred (**Fig. 3.2.2**). Same RT-PCR experiments were performed for L4-L6 dorsal root ganglia (DRG) ipsilateral and contralateral to the crushed nerve. During the first week after injury I found a 2-fold to 3-fold increase in gene expression in DRG, yet these effects were not significant. The maximum mRNA increases compared to the contralateral side were for CRBP-I: 2.2 ($n = 4$, $SD = 2.1$) and for CRABP-II: 3.2 ($n = 4$, $SD = 2.4$).

For the assessment of CRABP-II immunoreactivity in the sciatic nerves I used a monoclonal antibody kindly provided by Pierre Chambon (see **Tab. 2.1**). Consistent with results on gene expression, the protein levels of the retinoic acid binding protein increased 2 days after a crush lesion, reached a maximum after 7

days and were lower again at 14 days, when axonal regeneration had occurred in the nerve segment (**Fig. 3.2.3a**). In case of nerve transection I also found CRABP-II increase in the distal nerve stump, which initially paralleled the development in the crush model. As axonal regeneration was not possible in the distal stump, CRABP-II remained high for at least two weeks after the lesion (**Fig. 3.2.3b**). Injury-induced changes in protein concentration were significant in both paradigms (ANOVA $p < 0.05$). In order to investigate local CRABP-II distribution, 11 pieces of sciatic nerve 2.5 mm each were obtained. CRABP-II immunoreactivity reached its highest level around and distal to the site of the lesion (**Fig. 3.2.4**).

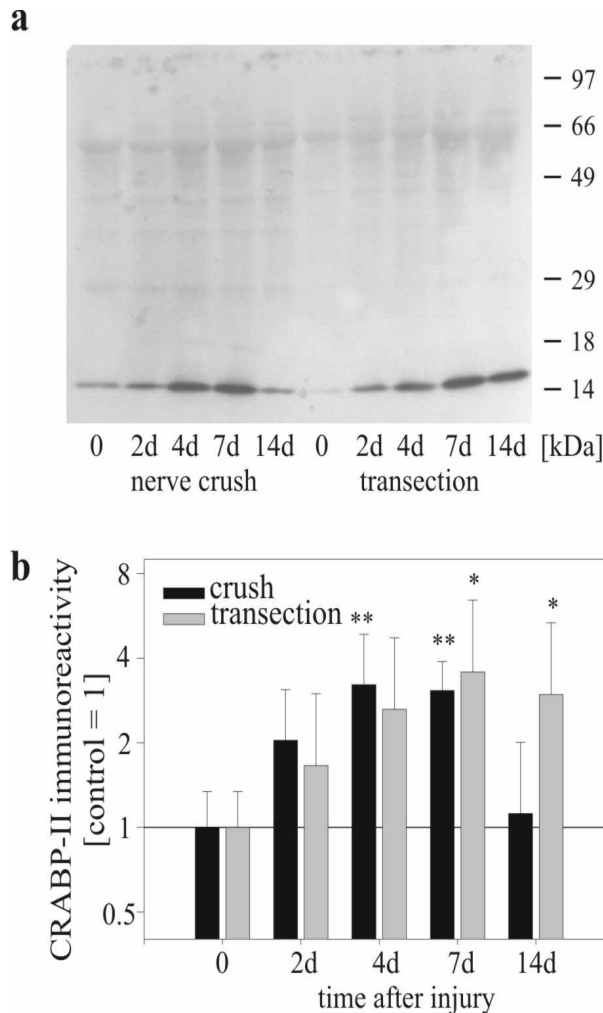


Fig. 3.2.3 Increase of CRABP-II immunoreactivity after sciatic nerve crush and transection. **a** CRABP-II signalling the intact sciatic nerve and distal to the site of crush injury and transection, immunoblots, 10 μ g protein per lane. Indicated are time intervals after injury when nerves were prepared and proteins extracted. **b** Quantification of the immunoreactivity signal. Effects of nerve crush and transection on CRABP-II immunoreactivity were significant ($n=3$, ANOVA $p < 0.05$), and signal strength were compared with non-injured controls using Dunnett's test (error bars show 95% confidence interval, $*p < 0.05$; $**p < 0.01$).

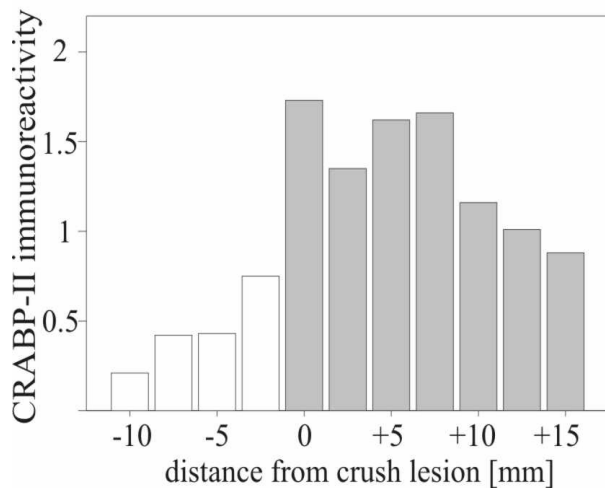


Fig. 3.2.4 CRABP-II signal in 2.5 mm nerve sections. Proximal and distal parts to the site of crush injury, 7 days after the lesion, $n=1$. X-axis indicates distance from the lesion (white columns: proximal, grey columns: distal), y-axis shows relative strength of the immunoreactive signal in Western blots.

3.2.5. Discussion

Although RALDH transcripts and immunoreactivity in my previous study were present in the intact PNS, transcriptional RARE activation was apparent in the injured nerves. *In vitro* experiments using RA-sensitive reporter cells indicate that rat sciatic nerves do not release RA, suggesting that the activation of RARE-regulated genes is confined to the nerve itself (Zhelyaznik et al., 2003). Additional mechanisms of control may therefore be at work. These could include RA degrading enzymes (Mey, 2001; Swindell et al., 1999; Mey et al., 2001), orphan receptors like COUP-TFII that interfere with DNA-binding of the retinoid receptors (McCaffery et al., 1999), retinoid receptors themselves or cellular retinoid binding proteins.

In this part of my thesis I showed that sciatic nerve lesions induced up-regulation of cellular retinol binding protein (CRBP)-I and cellular retinoic acid binding protein (CRABP)-II. Relative quantification of obtained gene expression of CRABP-II is high already 24 hours after lesion and reaches its maximum on the second day. This was also observed for CRBP-I. Then gene expression decreased when the regeneration has occurred. When no regeneration was allowed (after sciatic nerve transection) the expression of CRBP-I and CRABP-II rose similar to regeneration paradigm, but did not decline during the second week.

In response to nerve injury it is therefore possible that RA is synthesised due to RALDH-2 activity in the presence of increased CRBP-I. The up-regulation of CRABP-II will then facilitate RARE-regulated gene activation. Many genes related

to the regulation of Wallerian degeneration and peripheral nerve regeneration count among the targets of RA. They include neurotrophin receptors, neuropoietic cytokines, TGF β 1-3 and various transcription factors (Mey, 2001). In contrast to the drastic increase of CRABP-II and CRBP-I expression in injured sciatic nerves, there was only a slight and non-significant up-regulation of the two binding proteins in L4-L6 DRGs, suggesting that the nerve cells are possibly neither the origin of RA signalling nor its target. However one can not exclude that RA, enzymes or binding proteins in injured nerves may be retrogradely transported to the neuronal cell bodies.

Gene expression analysis as well as the data on protein immunoreactivity suggest that CRBP-I and CRABP-II activity decreases subsequent to axonal regeneration. Their up-regulation was however observed in degenerating nerves severed from the central neuronal cell bodies, and here transcript and protein expression remained higher even after 2 weeks. This observation suggests the involvement of retinoic acid signalling in Wallerian degeneration – the process which initiates axonal regeneration (Fawcett and Keynes, 1990). Additional evidence supporting this hypothesis was recently obtained in our laboratory: retinoic acid induced the down-regulation of ciliary neurotrophic factor (CNTF) in Schwann cells from the primary culture, similar to the development during Wallerian degeneration (Sendtner et al., 1996; Curtis et al., 1993).

My present data, showing strong and significant up-regulation of CRBP-I and CRABP-II after sciatic nerve lesions in connection with the activation of an RARE-reporter gene *in vivo* (Zhelyaznik et al., 2003), support the hypothesis of retinoic acid involvement in gene regulation after nerve injury. However, additional levels of RA-signalling regulation can not be excluded. Thus the cellular levels of retinoid receptors must be studied next.

3.3. Retinoic acid receptors and retinoid X receptors are activated after sciatic nerve injury

3.3.1. Abstract

As it becomes clear from 3.1 and 3.2, the retinoic acid system is present in peripheral nervous system. There it is possibly taking part in regeneration since all necessary components of RA signalling pathway are detectable in sciatic nerves, and the expression and protein levels of cellular retinoid binding proteins CRBP-I and CRABP-II are strongly up-regulated by the lesion. Since the effect of RA is mediated via the ligand-activated transcription factors retinoic acid receptors (RAR) and retinoid X receptors (RXR), the levels of these were measured in the present study with RT-PCR and immunoblotting. It was shown that the immunoreactivity of RAR α was about 4 times, of RXR α about 2 times and of RXR β about 1.5 times higher than in unlesioned tissue. Thus, retinoid receptors too are affected by the injury. In addition, Schwann cells were identified as possible targets of RA transcriptional effects in sciatic nerves because they express all six retinoid receptors, which was shown with immunocytochemistry analysis and, according to RT-PCR data, the expression of RAR β and RAR γ was found to be up-regulated after RA treatment.

3.3.2. Introduction

The retinoid signal is transduced by two families of nuclear receptors, the retinoic acid receptor (RAR) family comprising 3 genes: RAR α , RAR β , RAR γ , and the retinoid X receptor (RXR) family also including 3 genes, RXR α , RXR β and RXR γ (Mangelsdorf and Evans, 1992; Chambon, 1996). Due to alternative splicing and differential promoter usage there are also several isoforms of each family member. RARs and RXRs belong to the superfamily of nuclear hormone receptors and act as ligand activated transcriptional factors. The natural ligands for the RARs are all-*trans*-retinoic acid and its stereo isomer 9-*cis*-RA, whereas RXRs are activated by the natural ligand 9-*cis*-RA only (Mangelsdorf and Evans, 1992; Marill et al., 2003; Chambon, 1996). Five types of retinoic acid response elements (RARE) were found on RA-regulated genes, they consist of inverted (palindrome) base repetitions (direct repeats, DR) with 1-5 nucleotides as a spacer. The most frequent are DR-5 of the type, which are present in RAR β 2 and RAR α 2 genes. Other types are less common, for instance DR-1 and DR-2 were found in CRABP-II and CRBP-I genes respectively (Marill et al., 2003; Chambon, 1996). While RAR must bind to RXR and thus can act only as heterodimer, RXR can act as homodimer via the retinoid X response element (RXRE, DR-1) and also as heterodimer with several nuclear receptors, e.g. thyroid hormone receptor, vitamin D receptor, or orphan receptor COUP (Mangelsdorf and Evans, 1992; Marill et al., 2003; Chambon, 1996), indicating a second retinoid signalling pathway. This ability of RXR to interact with receptors responsive to a variety of ligands establishes a central role for RXR in modulating multiple hormone pathways.

The activity of RARs may be modulated by the nuclear transcription factor AP-1, the complex formed by the proto-oncoproteins Jun and Fos, whose activity is stimulated by tumour promoters and growth factors. Agents which stimulate AP-1 synthesis block the action of RA. Reciprocally, RA treatment is capable of influencing the action of AP-1. Possibly, a cross-coupling mechanism is involved, by which retinoid and other nuclear receptors can regulate the function of other transcription factors through both DNA-binding and DNA-independent protein-protein interactions (Mangelsdorf and Evans, 1992).

The examination of homozygous mutant mice generated by targeted disruption of RAR or RXR genes in embryonic stem cells showed that RAR α ^{-/-} mice die early postnatally due to testis degeneration. RXR α ^{-/-} mutant mice die as embryos

because these receptors are required for heart development. RAR β null-mutants appear completely normal, and RAR γ , RXR β and RXR γ have subtle defects mimicking a mild form of the fetal vitamin A deficiency syndrome (Ross et al., 2000). These results suggest a high degree of functional redundancy among retinoid receptors. However, double mutants carrying two RAR defects die either in utero or shortly after birth, showing most symptoms of severe fetal vitamin A deficiency (Roos et al., 1998). These studies demonstrate that RARs are essential for vertebrate development.

The pleiotropic effects of retinoids might be explained in part by the patterns of expression of various RAR and RXR genes. In adult mammals RAR α and RXR β are widespread, RAR β is specifically expressed in muscle and prostate, RAR γ in skin and lung, RXR α in liver, skin and kidney, RXR γ in muscle and heart (Mangelsdorf and Evans, 1992; Sugawara et al., 1997). Less is known about the adult nervous system. The functions of the RA-system were studied in adult CNS (Denisenko-Nehrbass et al., 2000; Weiler et al., 2000; Zhang and McMahon, 2001) and the expression of retinoid receptors was shown for different brain regions (Cullingford et al., 1998; Zetterström et al., 1999). The aim of this study was therefore to investigate the expression of RARs and RXRs in the PNS and compare their expression after peripheral nerve lesion.

3.3.3. Materials and methods

Cell culture

Primary cultures of Schwann cells were prepared from new-born Sprague-Dawley rats and processed as described in 2.2.

Surgical procedures

Adult male Sprague-Dawley rats were used in this study and handled as described in part 2.1.

Immunocytochemistry

Sciatic nerves were fixed in ethanol and treated as described in 2.3. Primary antisera against S-100, RAR α , - β , - γ and RXR α , - β , - γ were detected with peroxidase-conjugated secondary antibodies and the avidin-biotin-peroxidase method as described in 3.3.

RT-PCR

Total RNA was isolated using Trizol (Invitrogen) and reverse transcribed to obtain cDNA, which was amplified with PCR as described in 2.5. The PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide to verify their expected sizes. From fluorescence intensity data the logarithms of the signal ratios of lesioned nerves to control nerves were calculated and tested with ANOVA and Dunnett's test.

Western blotting

For immunoblotting, nerves were sonicated in hypotonic buffer with a mix of protease inhibitors as described in 2.7. Strength of immunoreactivity was always expressed in relation to the contralateral side, set at 100%. From immunoreactivity data the logarithms of the signal ratios of lesioned nerves to control nerves were calculated and tested with ANOVA and Dunnett's test.

3.3.4. Results

Retinoid receptors are present in PNS and are probably localised to Schwann cells

As shown in part 3.1, sciatic nerves express all known retinoic acid receptors ($RAR\alpha$, $-\beta$, $-\gamma$) and retinoid X receptors ($RXR\alpha$, $-\beta$, $-\gamma$; **Fig. 3.1.1**), however, their cellular location has not yet been determined. In this study, immunocytochemistry analysis was performed and the cellular localisation was investigated. All antibodies and working dilutions used are listed in **Tab. 2.1**. The positive staining for $RAR\alpha$, $-\beta$, $-\gamma$ (**Fig. 3.3.1**) and $RXR\alpha$, $-\beta$, $-\gamma$ in cryostat sections suggested that they were present in sciatic nerves, however their localisation was not determined due to the low resolution of the light microscopy method. The positive control was done using antibody against S-100 protein, a marker for Schwann cells in the peripheral nerve.

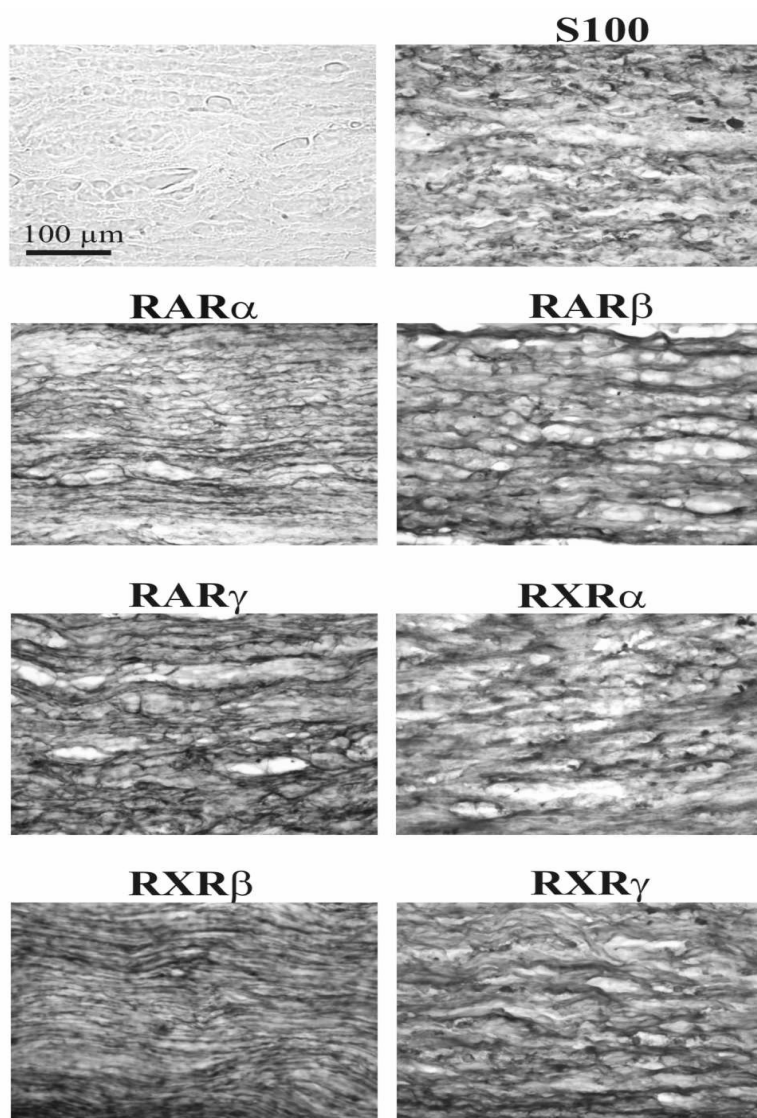


Fig. 3.3.1 Immunostaining of retinoid receptors distribution in rat sciatic nerve.

Longitudinal cryostat sections of nerves: negative control, S-100, $RAR\alpha$, $RAR\beta$, $RAR\gamma$, $RXR\alpha$, $RXR\beta$, $RXR\gamma$. The immunostaining of retinoid receptors resembled that of S-100 – a marker of Schwann cells in the peripheral nerve, scale bar 100μm.

Ligand stimulated transcriptional factors retinoic acid and retinoid X receptors are activated after the injury

The activation of retinoic acid receptors after the lesion may be another key regulatory factor of retinoic acid action. In order to investigate their expression I performed semi-quantitative Western blotting and PCR analyses. For the assessment of RAR α , - β , - γ and RXR α , - β , - γ immunoreactivity I used commercial antibodies, which are listed in **Tab. 2.1**. The loading of proteins in each gel slot was checked by evaluating Ponceau-S staining of the blot (**Fig. 3.3.2b**). For gene expression the levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were tested as a positive control for the same cDNA concentration (**Fig. 3.3.4g**). Comparing crushed (7 days post lesion) and intact sciatic nerves I found a strong and highly significant up-regulation of RAR α immunoreactivity and the more moderate but still significant up-regulation of RXR α and RXR β (**Fig. 3.3.2a, c**).

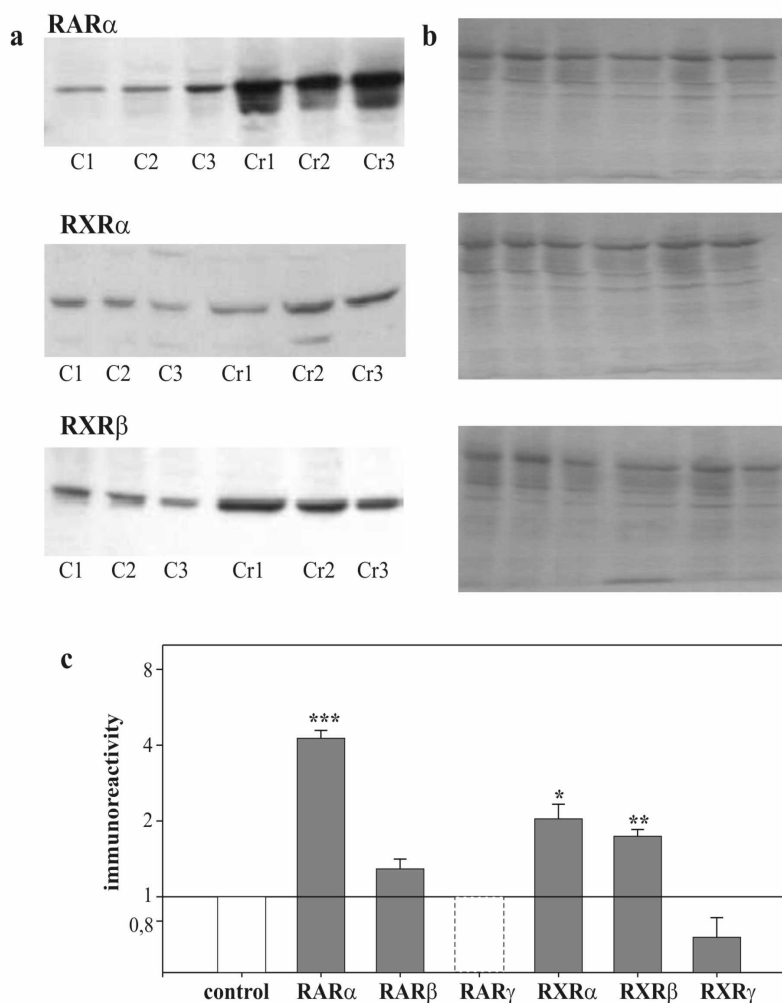


Fig. 3.3.2 Immunoreactivity of RAR α , RXR α and RXR β after sciatic nerve injury. **a** Western blots, 15 μ g protein per lane. Immunoreactivity in the intact nerves (C1-3) and after a crush lesion (Cr1-3). **b** The equal loading of all slotst was verified with Ponceau-S staining. **c** Relative quantification of immunoreactivity of all retinoid receptors (see also **Fig. 3.3.3**). Strength of immunoreactivity is expressed in relation to the contralateral side (control) Error bars indicate standard error of mean. Asterisks indicate significant differences compared to the nonlesioned control ($n=3$, ANOVA $p<0.05$; Dunnett's test * $p<0.05$; ** $p<0.01$; *** $p<0.001$)

The quantification of the immunoblots is presented in **Fig. 3.3.2c**. Immunoreactivity levels of RAR β (**Fig. 3.3.3a**) and RXR γ (**Fig. 3.3.3c**) were not statistically different from control, while the staining of RAR γ (**Fig. 3.3.3b**) was present at very low levels, which was below the sensitivity of the image detection system.

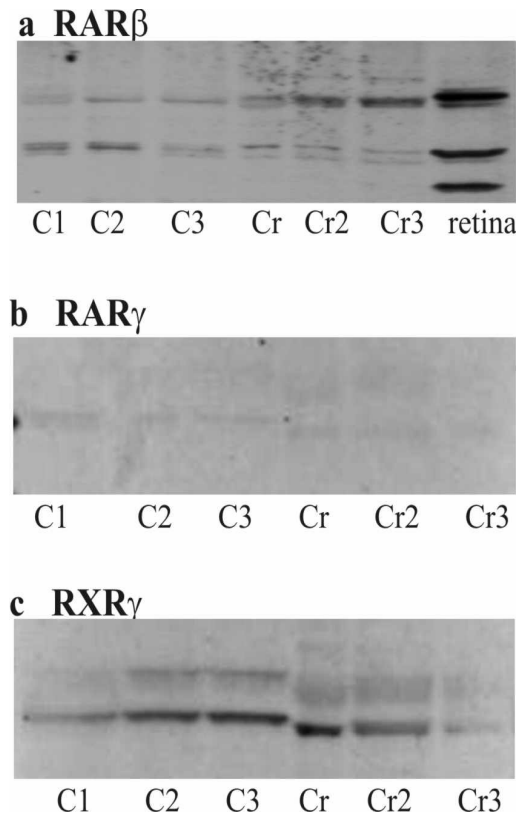


Fig. 3.3.3
Immunoreactivity of RAR β , RAR γ and RXR γ after sciatic nerve injury.
a RAR β , b RAR γ , c RXR γ
Western blots, 15 μ g protein pro lane. Immunoreactivity in the intact nerves (C1-3) and 7 days after crush lesion (Cr1-3), n=3. Proteins extracted from adult retina were used as a positive control for ECL. Band's shift in c is due to the experimental artefact.

On the mRNA levels, the increase of gene transcripts was also apparent (**Fig. 3.3.4**). In these experiments semi-quantification was performed using the same analysing software as for immunoreactivity, but this time with ethidium bromide signals in agarose gels, run subsequently to conventional PCR. Seven days after sciatic nerve crush lesion elevated levels of gene expression of RAR α , RAR β , RAR γ and RXR α were detected (ANOVA, $p < 0.05$). For RXR β and RXR γ no significant changes were observed. Thus at least two functional heterodimers (concluded from up-regulated immunoreactivity) of RAR α /RXR α and RAR α /RXR β may be triggered by the injury. The up-regulation of RAR β was not as prominent and significant only on the level of the gene transcript.

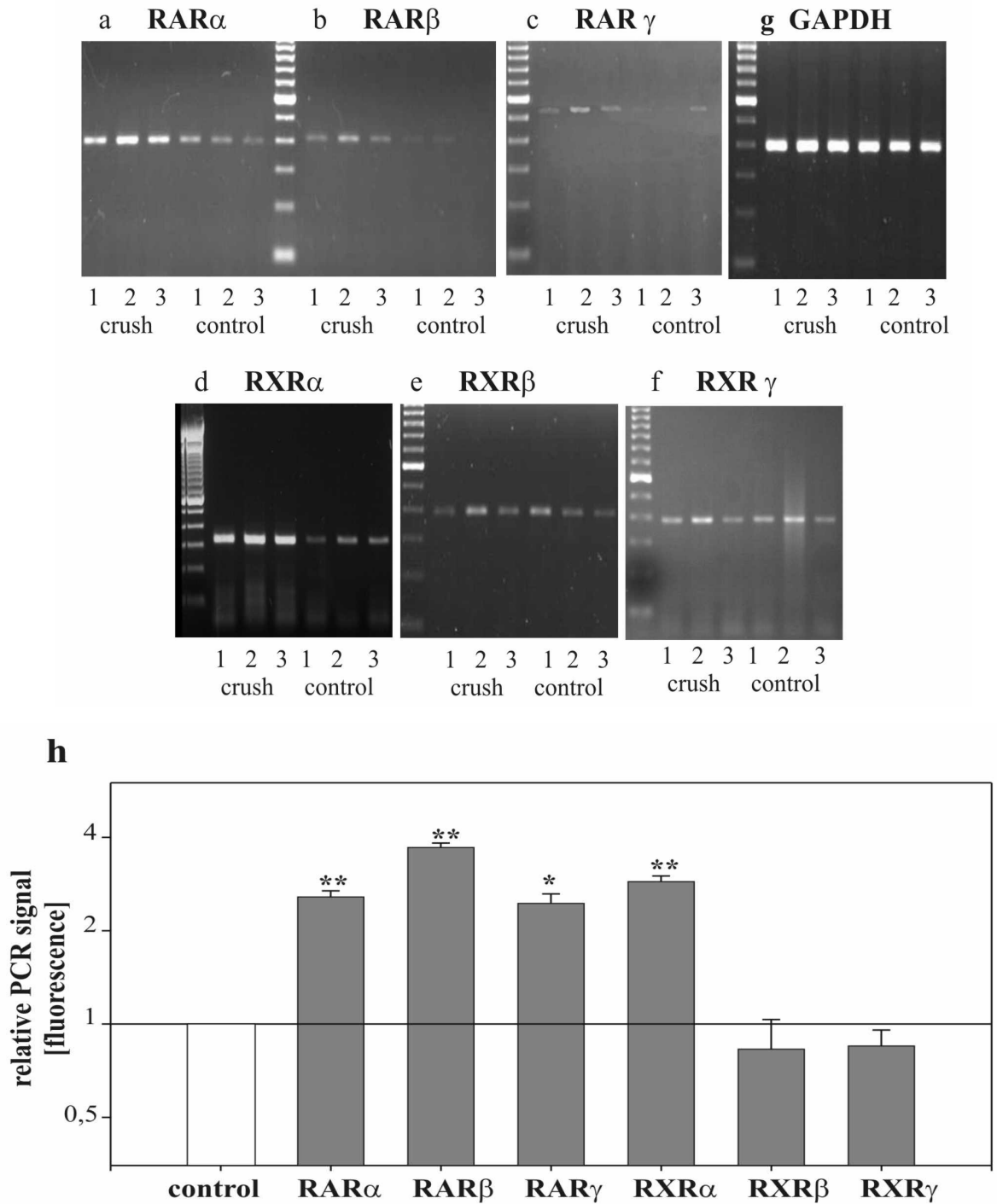


Fig. 3.3.4 Sciatic nerve lesion induces activation of retinoid receptors. **a** RAR α , **b** RAR β , **c** RAR γ , **d** RXR α , **e** RXR β , **f** RXR γ , **g** GAPDH. Three independent experiments are shown for each receptor. Molecular weight marker at 100 bp (bottom band), 200 bp, 300 bp etc. Stronger band at 600 bp. The molecular weights were consistent with the predicted sizes of all PCR fragments. **h** Relative quantification of mRNA concentrations. Strength of fluorescence is expressed in relation to the contralateral side (control) and corrected for the expression of GAPDH for each sample. Error bars indicate standard error of mean. Asterisks indicate significant differences compared to the non-lesioned control (n=3, ANOVA $p < 0.05$, Dunnett's test * $p < 0.05$; ** $p < 0.01$)

Retinoic acid autoregulates mRNA expression of its own receptors in Schwann cells *in vitro*.

One mechanism by which RA may co-ordinate complex gene expression is through the direct autoregulation of the retinoid receptors themselves (Mangelsdorf and Evans, 1992). To test whether it is the case in PNS, Schwann cell primary cultures were used.

Previously I have shown the expression of all six retinoid receptors in Schwann cells *in vitro* (**Fig 3.1.2**). To investigate dose-dependent effects of RA, the cultures were exposed to 1, 10 and 100 nM all-*trans* RA for 24 hrs, and the mRNA expression of RAR α , - β , - γ and RXR α , - β , - γ was analysed with PCR. Control cells were treated with DMSO.

While the expression of GAPDH was constant for RA-treated and control cells, the expression of RAR β and RAR γ was induced in RA-treated cells. The expression of RAR α , RXR α , RXR β and RXR γ was not changed even by the highest dose of retinoic acid (**Fig 3.3.5**). This experiment was repeated only once.

3.3.5 Discussion

This part of my work was devoted to the question whether retinoic acid receptors and retinoid X receptors are regulated by sciatic nerve injury. In part 3.1. of my thesis I showed the presence of all six receptors in sciatic nerve, however, their cellular localisation remained unclear. In this study I performed immunocytochemistry analysis in order to show the possible localisation of the receptors. However, the differential interference contrast light microscopy method can not provide the resolution to qualify the staining obtained. Obviously, further experiments using electron microscopy must be performed. My previous experiments suggest, however, that the staining is localised to the Schwann cells, since Schwann cells express retinoid receptors (see part 3.1.) and are immunopositive for all RARs and RXRs (J. Mey and K.Schrage, unpublished observations) *in vitro*.

To evaluate the possible changes in gene and protein levels, crush lesions were again performed to the sciatic nerves of adult rats and the expressions measured with PCR and Western blot. Discrepancies at these two expression levels are not infrequent, which is apparent in my data as well.

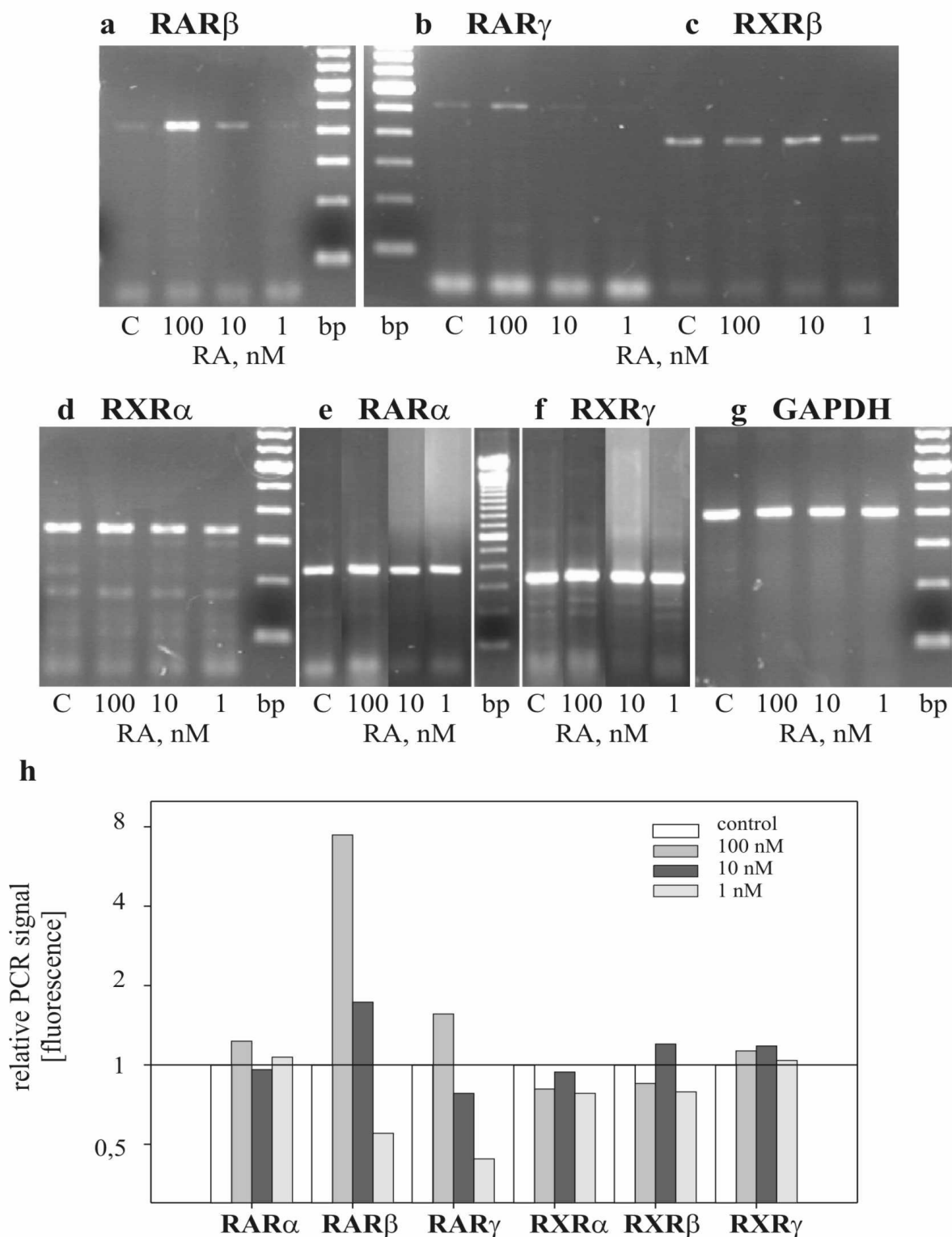


Fig. 3.3.5 RA-dependent activation of retinoid receptors. **a** RAR β , **b** RAR γ , **c** RXR β , **d** RXR α , **e** RAR α , **f** RXR γ , **g** GAPDH. Cells were treated 24 h with all-trans RA, concentrations in nM; bp: molecular weight marker at 100 bp (bottom band), 200 bp, 300 bp etc. The molecular weights were consistent with the predicted sizes of all PCR fragments. **h** Relative quantification of PCR results. Strength of fluorescence is expressed in relation to the control (DMSO-treated cells) and corrected for the expression of GAPDH for each sample, $n=1$.

At the mRNA level all three RAR receptors and RXR α are significantly up-regulated, while the immunoreactivity of only RAR α , but of two RXRs: RXR α and RXR β is positively regulated. These findings suggest that not all elevated genes are translated into comparable elevated levels of protein. However the up-regulated levels of at least two functional heterodimers: RAR α /RXR α and RAR α /RXR β may be generated in cells of regenerating sciatic nerves, enabling more retinoic acid to propagate its transcriptional effects.

One mechanism by which RA may regulate gene expression is through the direct autoregulation of the retinoid receptors (Mangelsdorf and Evans, 1992). According to my data, the expression of RAR β and RAR γ is up-regulated by retinoic acid treatment in Schwann cells. This effect of RA could be both direct and via additional intermediate gene activation, because only RAR β of these two contains RA response elements in its promoter (Mangelsdorf and Evans, 1992). The effect of RA on its nuclear receptors is dose-dependent, the highest dose of 100 nM induced the most potent expression of RAR β and RAR γ . As for the biological meaning of such regulation, RA may mediate its own positive feedback.

The implication of the RA-system in regeneration was discussed in the previous parts (3.1-3.2). RA is able to induce regeneration in the developing CNS and in the CNS *in vitro* (Corcoran and Maden, 1999; Corcoran et al., 2000; Corcoran et al., 2002; Mey and Rombach, 1999) and activate genes in cell cultures unrelated to the nervous system (Chang et al., 2000; Choudhury et al., 2000; Mey, 2001). However, when Corcoran and Maden (1999) introduced RA into the lesioned adult CNS no regeneration was induced. A possible explanation of this phenomenon is that during transition from embryo to adulthood some or all of the transducers of the RA signal may be permanently shut down by transcriptional inactivation. The mechanism of retinoid receptor transcriptional inactivation is known. It involves histone deacetylase (HDAC)-containing complexes that are tethered through co-repressors or a silencing mediator to retinoid and thyroid hormone receptors thereby inhibiting their functions (Marill et al., 2003).

On the contrary, axons in the adult PNS are able to regenerate and all components of retinoid signalling are present in this tissue. When at first proposing a role of retinoic acid in PNS-regeneration, the RA synthesising enzymes seemed to be the proper candidates for the regulation of RA availability. However, an up-regulation of RALDH was not detected after sciatic nerve crush injury (Zhelyaznik

et al., 2003). What is more, the application of excess RA (Maden and Hind, 2003), which should have overcome the requirement of the RA-synthesising enzymes, did not induce neurite outgrowth. Thus RALDH is not the most important key regulator in either CNS or PNS regeneration. Other possible key molecules in RA signalling activation are cellular binding proteins. CRBP-I is crucial for the RA synthesis (Napoli, 1996) and CRABP-II, being able to bring RA to the nuclear receptors, is the co-activator of transcription (Budhu and Noy, 2002). Their silencing in adult CNS might be one of the explanations of regenerative failure. As shown in part 3.2, I showed indeed a strong and significant up-regulation of CRBP-I and CRABP-II following peripheral nerve injury (Zhelyaznik et al., 2003). Even though there was a report that CRABP-II alone is able to activate RAR α /RXR α heterodimers (Bastie, 2001), it was proved to be insufficient to elicit a transcriptional effect of RA, even when excessive RA was available for the cells (Budhu and Noy, 2002). Thus the regulation of the nuclear receptors, which are necessary for RA transcriptional activity, seems to be another key process. Performing several *in vitro* studies, Corcoran and Maden (2002) have shown that one of the retinoic acid receptors – RAR β 2 – is the essential transducer of the RA signal in cultured spinal cords. After RAR β 2 vector introduction many neurites extended from the explanted adult spinal cords in both mouse and rat *in vivo*. No neurites were observed in several control experiments where the cords were transfected with either LacZ or the RAR β 4 isoform, which confirmed the authors' hypothesis of a specific RAR β 2 involvement. In addition, an RAR β agonist rather than an RAR α or RAR γ agonist specifically induced neurite outgrowth from embryonic neurons (Corcoran and Maden, 1999; Corcoran et al., 2000). Even though, one can not exclude that some other of the receptors and their splicing isoforms also participate in regeneration. In the regenerating PNS the expression of RAR α , RXR α and RXR β is up-regulated, while the levels of the other three receptors, including RAR β are unaltered. However, high levels of RAR β transcript and protein were expressed at all times, and I can not completely rule out that the expression of RAR β isoform 2 is regulated.

Nevertheless, the elevated expression of retinoid receptors in the present study can be considered as a specific physiological response of the PNS to injury. This additionally supports the proposed role of retinoic acid in regenerating peripheral nerves. Further, my finding of a different pattern of receptor expression in the PNS

compared to the report of Corcoran and Maden (Corcoran et al., 2000) suggests that retinoic acid may take part in regeneration of CNS and PNS via different nuclear receptors, which will probably activate different downstream signalling.

In the future, more detailed comparison of retinoid signalling in PNS and CNS must be performed. For instance, the comparison of transcriptional activity of RA within the injured sciatic nerve and spinal cord can be done. The expression of cellular retinoid binding proteins should also be evaluated, as well as the cellular localisation of the RA-system. Further experiments with CRABP-II-/-, RAR β -/- and RXR β -/- mutant mice will clear the possible physiological roles of these three molecules in the regeneration processes. The experiments in this direction are already being performed in our laboratory. Their results will enable the more concrete speculations about the functional relevance of retinoic acid in these two systems. Further on, the biological functions of retinoids – the activation of downstream gene signalling – must be studied.

3.4. Retinoic acid reinforces the expression of erbB3 in Schwann cells

3.4.1. Abstract

In the peripheral nervous system neuregulins regulate Schwann cell proliferation, Wallerian degeneration and development of neuromuscular junctions. Retinoic acid (RA) is a possible regulator of neuregulin receptors because it interacts with the neuregulin pathway in breast cancer models. It was discovered that the retinoid signalling system is activated after sciatic nerve injury. In light of these data I investigated the effect of retinoic acid on the expression of neuregulin receptors in Schwann cell primary cultures. Transcripts of the erbB2 tyrosine kinase and the erbB3 receptor were detected. In order to investigate dose-dependent effects of RA, cultures were exposed to 0.1, 1, 10, 100 and 1000 nM all-*trans* RA for 24 hrs, and the mRNA expression of erbB2 and erbB3 was analyzed with quantitative RT-PCR. While RA exerted only a non-significant effect on erbB2 expression, concentrations of 0.1 to 10 nM RA induced a 4- to 5-fold significant up-regulation of erbB3. The transcriptional effect on this receptor was confirmed on the protein level with Western blotting experiments. These results indicating a possible interaction of RA and β -NRG-1/erbB signalling in the peripheral nervous system suggest a biological function of retinoic acid signalling after peripheral nerve injury.

3.4.2. Introduction

Neuregulins (NRGs) belong to the family of multipotent growth-promoting peptides known to be important in neural and mesenchymal tissue development. In the peripheral nervous system (PNS) they are crucial for a number of processes including Schwann cell proliferation (Jessen and Mirsky, 1999; Cheng et al., 1998), Wallerian degeneration (Kim et al., 2002) and development of neuromuscular junctions (Noll and Miller, 1994). So far, four genes are known to encode members of the neuregulin family: NRG-1, NRG-2, NRG-3 and NRG-4 (Noll and Miller, 1994; Falls, 2003). All of them bind preferentially to the receptors erbB3 and erbB4. ErbB3 has a high ligand-binding affinity but only impaired kinase activity due to substitutions in critical residues of its catalytic domain (Citri et al., 2003). ErbB3 and erbB4 receptors form heterodimers by recruiting either erbB1 or erbB2 tyrosine kinase co-receptors to propagate signalling (Wiley, 2003).

Among the four neuregulins, β -Neuregulin-1 (β -NRG-1) was initially found to function in the regulation of nicotinic acetylcholine receptor transcription at the neuromuscular junction (Falls, 2003; Fischbach, 1997), hence it is also referred to as ARIA (acetylcholine receptor inducing activity). The functions of β -NRG-1 also include regulation of early cell fate determination, differentiation, migration and survival of satellite cells, Schwann cells and oligodendrocytes (Buonanno and Fischbach, 2001).

Recent data indicate a functional role of β -NRG-1 after peripheral nerve injury. At the neuromuscular junction, β -NRG-1 regulates the expression of acetylcholine receptors, thereby controlling the specificity of reinnervation after nerve injury (Noll and Miller, 1994). Along the nerve, β -NRG-1, released from injured axons promotes Schwann cell proliferation during regeneration (Carroll et al., 1997). This cytokine, as well as the expression of its receptors quickly respond to the injury. Within one hour after nerve transection, the NRG receptor erbB2 is selectively activated at the site of injury as are its downstream signals, including immediate-early genes. Based on these data Kim and co-workers (Kim et al., 2002) suggested that neuregulin signalling triggers Wallerian degeneration in the adult PNS. In addition, the expression of erbB2 and erbB3 genes and protein increases strongly after sciatic nerve injury (Carroll et al., 1997). This now raises the question about the causes of altered β -NRG-1 signalling and of the regulation of erbB2/erbB3 expression.

One possible regulator is the transcriptional activator retinoic acid (RA) because it interacts with the neuregulin pathway in breast cancer models (Offterdinger et al., 2003; Offterdinger et al., 1998; Tari et al., 2002; Martin-Subero et al., 2001). RA has therapeutic value for the treatment of breast cancer. It is known to inhibit chemically induced cancerogenesis *in vivo* and is anti-proliferative *in vitro*, where RA can act as a repressor of neuregulin/erbB expression (Offterdinger et al., 1998; Tari et al., 2002; Martin-Subero et al., 2001).

Since the discovery of the retinoid signalling system activation after sciatic nerve injury, the question of its biological meaning has remained unanswered. The pattern of up-regulation of cellular retinoid binding proteins suggests their involvement in Wallerian degeneration (see part 3.2). Since Schwann cells were found to express all retinoic acid receptors they are considered possible candidates for RA-dependent gene regulation in the sciatic nerve (see part 3.1). At the same time, Schwann cells are the prime targets of the neuregulin signal via erbB2/erbB3 (Caroll et al., 1997). In the light of these data and the recent findings of immediate erbB signalling after nerve injury (Kim et al., 2002) I have investigated the effect of retinoic acid on expression of neuregulin receptors erbB2 and erbB3 in Schwann cell primary cultures.

3.4.3. Materials and Methods

Cell culture

Primary cultures of Schwann cells were prepared from new-born Sprague-Dawley rats and processed as described in 2.2.

RT-PCR

Total RNA was isolated using Trizol as described in 2.5. Samples of 500 ng total RNA were treated with DNase I (Invitrogen), reverse transcribed and cDNA was tested for the presence of erbB2 and erbB3 transcripts. For detailed protocols and primer sequences see 2.5.

Quantitative PCR

To measure the relative change of erbB2 and erbB3 mRNA expression after RA treatment, quantitative PCRs were performed by means of the Roche Light Cycler System (Roche) using the QuantiTect SYBR Green PCR Kit (Qiagen). The detailed description of cycler programming and quantification principle is described in 2.6. The PCR efficiencies (amplification per cycle) were for GAPDH 1.65, for erbB2 and erbB3 2.00. For every RA concentration three to eight independent cell culture experiments with pairs of RA and DMSO treated Schwann cells were performed and statistically analysed with Jmp software (SAS institute).

Western blotting

For immunoblotting, Schwann cells were triturated in hypotonic buffer with protease inhibitors 1 mM PMSF, 1 μ M leupeptin, 1 % aprotinin. Protein extracts were separated with discontinuous SDS-PAGE (10% T, 3.3% C) and transferred by semidry blotting onto a nitrocellulose membrane. Detection of immunoreactivity was based on a peroxidase-conjugated goat anti rabbit secondary antibody and the enhanced chemoluminescence method. Detailed descriptions are in part 2.7.

3.4.4. Results

Schwann cell primary cultures

In the peripheral nervous system erbB2 and erbB3 are expressed by Schwann cells and, among other functions, serve as a putative triggers of Wallerian degeneration (Kim et al., 2002). As described above (3.1, 3.3), I found that Schwann cell primary cultures prepared from rat sciatic nerves express RA receptors and retinoid X receptors, as well as retinaldehyde dehydrogenases, cellular retinoid binding proteins and CYP26 (**Fig. 3.1.2**). Thus these cells may be both: the source and the sink of retinoic acid action. Further on I investigated erbB2- and erbB3 regulation by retinoic acid in Schwann cells. In addition to the expression of retinoid receptors, cultured Schwann cells expressed the neuregulin receptor erbB3 and the erbB2 tyrosine kinase (**Fig. 3.4.1a**). With an antibody against erbB3, the presence of this protein was demonstrated with Western blotting (**Fig. 3.4.1b**).

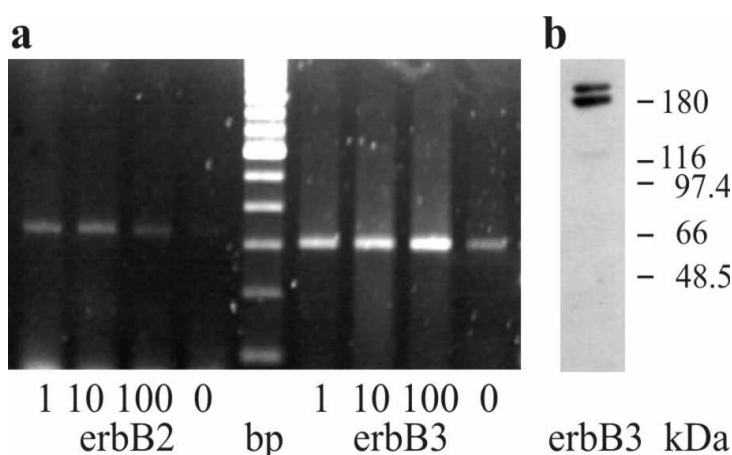


Fig. 3.4.1. Expression of erbB2 and erbB3 in Schwann cell primary cultures from rat sciatic nerves. **a** Expression of erbB2 and erbB3 transcripts (RT-PCR) and **b** erbB3 protein (Western blot). Cells were treated 24 hrs with all-trans RA, concentrations in nM; bp: molecular weight markers at 100 bp (bottom band), 200 bp, 300 bp etc.; kDa: position of protein molecular weight markers. The expected molecular weight of erbB3 is 185 kDa (Carroll et al., 1997).

Effect of retinoic acid on erbB2 and erbB3 transcript concentrations

With Light Cycler RT-PCR I successfully amplified transcripts of erbB2 and erbB3 in Schwann cells (**Fig. 3.4.2a**). PCR-fragments, separated in an agarose gel stained with ethidium bromide, had the expected sizes (erbB2: 337 bp, erbB3: 307 bp, **Fig. 3.4.1a**). In order to investigate dose-dependent effects of RA, cultures were then exposed to 0.1, 1, 10, 100 and 1000 nM all-*trans* RA for 24 hrs, and the mRNA expression of erbB2 and erbB3 was analysed with quantitative RT-PCR. The validity of these experiments was controlled by melting curve analysis (**Fig. 3.4.2b**) and gel electrophoresis of PCR products amplified with the Light Cycler (**Fig. 3.4.2c**). An example of two experiments comparing RA-treated samples with the controls is shown in **Figure 3.4.2a**. The dotted horizontal line in the semi-logarithmic graphs shows the level of background fluorescence used to determine the crossing points.

With identical mRNA concentrations of GAPDH there was a slight increase of erbB2 transcript (**Fig. 3.4.2a**, upper panels, curve shifted 1.0 cycle to the left) and a 5.3-fold up-regulation of erbB3 (**Fig. 3.4.2a**, lower panels, curve shift 2.4 cycles to the left). **Figures 3.4.3a, b** show the relative changes in transcript concentrations of erbB2 and erbB3 in response to five different doses of RA. While the effect on erbB2 was not significant, RA caused a highly significant increase in erbB3 expression (ANOVA $p < 0.001$). Concentrations of 0.1 nM to 10 nM RA induced a 4- to 5-fold up-regulation of erbB3 (* $p < 0.05$, ** $p < 0.01$, corrected t-tests). Higher concentrations of 100 nM and 1 μ M RA had no significant effects.

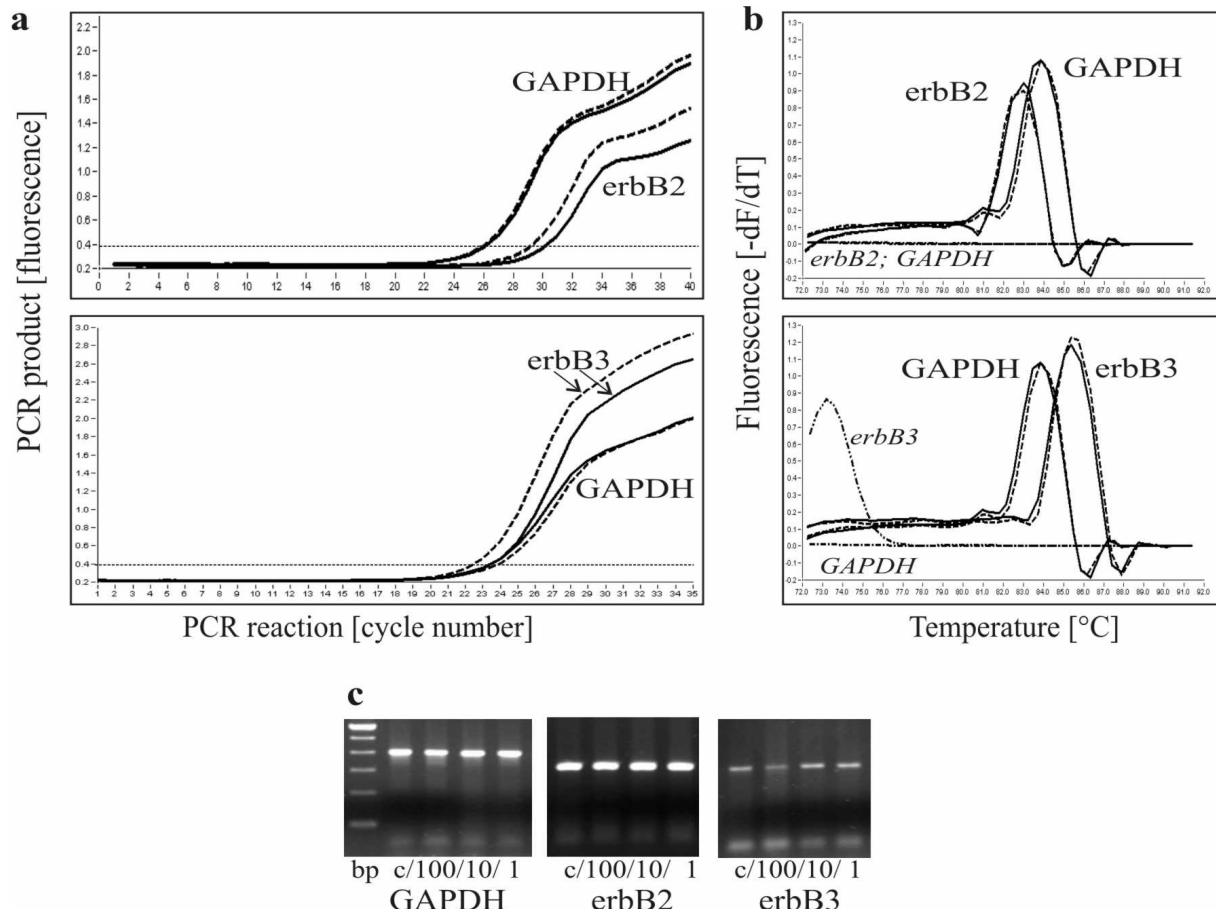


Fig. 3.4.2 Changes in erbB2 and erbB3 mRNA expression of Schwann cells after retinoic acid treatment, quantitative RT-PCR. **a** Representative amplification curves with primers for erbB2 and erbB3. Broken lines represent results from Schwann cells, which were treated 24 hrs with 10 nM all-trans RA, continuous lines from DMSO-treated cells. While no RA-induced change is observed for the concentration of GAPDH, the left shift of crossing points after treatment indicates higher transcript concentrations of erbB2 and erbB3 in RA-treated cells. After all quantitative RT-PCR runs the amplified fragments were characterised **b** by their melting curves (italicised erbB and GAPDH symbolise negative controls without RT), and **c** with agarose gel electrophoresis. Expected fragment sizes were 337 bp (erbB2) and 307 bp (erbB3). Three different RA concentrations: 1, 10 and 100 nM and DMSO treated cells (C) are shown. The six distinct marker bands have 600 (stronger band), 500, 400, 300, 200 and 100 bp.

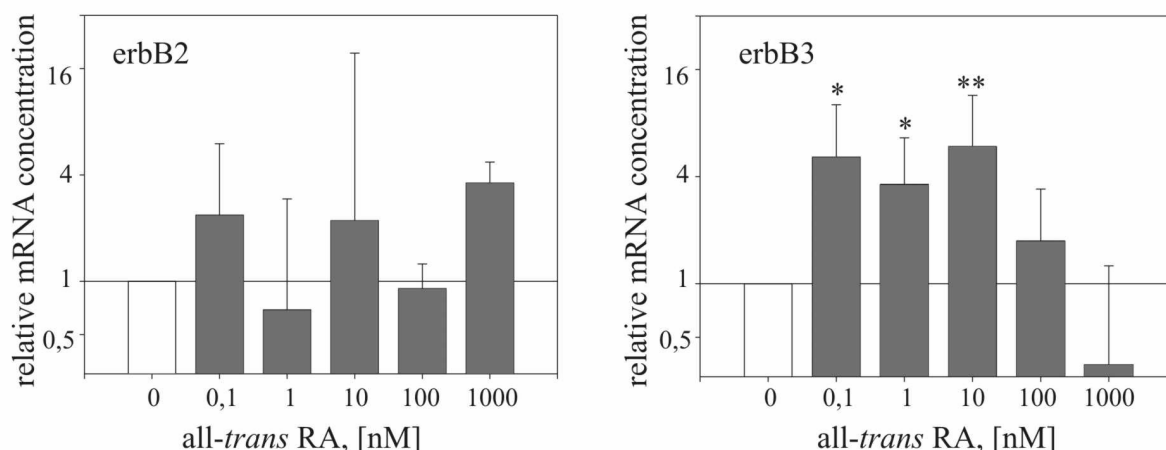


Fig. 3.4.3 Relative quantification of mRNA concentrations 24 hrs after RA treatments. Data are normalised to transcript levels of the DMSO-treated Schwann cells. Error bars indicate standard error of mean. The effect of RA on *erbB3* expression was highly significant (ANOVA, $p < 0.001$). A posteriori comparisons showed a significant up-regulation at 0.1 ($n=5$), 1 ($n=6$) and 10 nM ($n=5$) RA (corrected *t*-test, $*p < 0.05$, $**p < 0.01$). The concentrations of 100 ($n=5$) and 1000 ($n=3$) nM of RA showed no significant effects.

Effect of retinoic acid on *erbB3* protein expression

Since discrepancies in transcript expression and protein synthesis are not infrequent, I evaluated *erbB3* immunoreactivity in Schwann cell protein extracts with semiquantitative Western blotting experiments. To induce protein synthesis Schwann cells were treated with 0.1, 1, 10 and 100 nM all-*trans* RA for 40 hrs as described above. After SDS-PAGE, immunoreactive signals were evaluated with enhanced chemoluminescence and an *Image Analyser*. Consistent with the results on gene expression, the protein signals of *erbB3* were raised (ANOVA, $p < 0.005$; **Fig. 3.4.4a, b**). In these experiments, lower doses of RA (0.1 and 1 nM) were not significant, while 10 and 100 nM produced significant increases (Dunnett's test).

On the both expression levels, 10 nM t-RA produced the highest effect on *erbB3* levels.

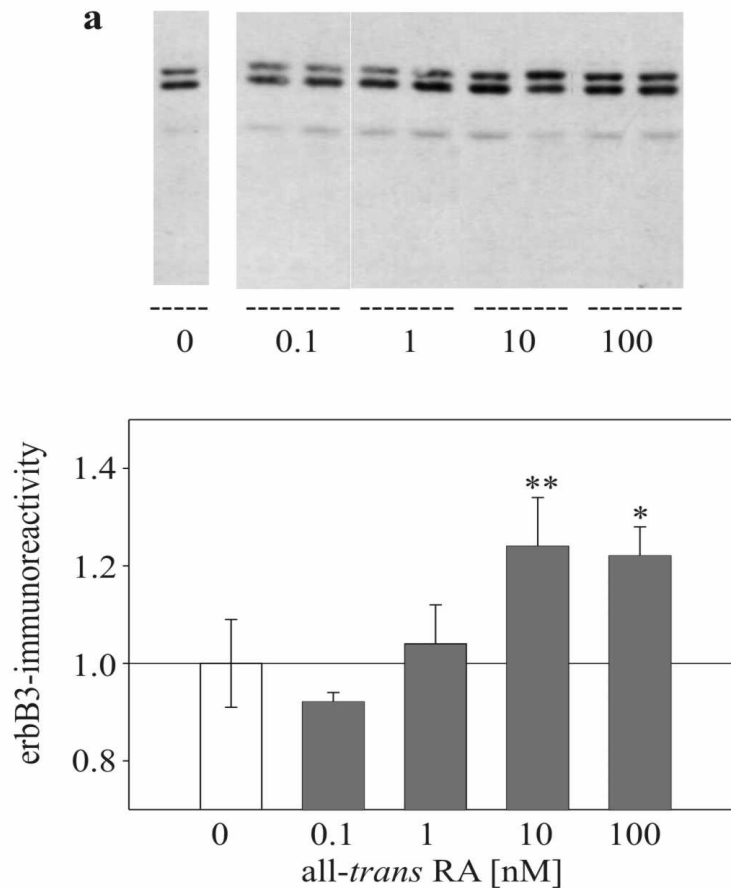


Fig. 3.4.4 Increase of erbB3 immunoreactivity after retinoic acid treatment. a Western blot of Schwann cell protein extracts using the erbB3 antiserum; 10 μ g protein per lane. Cells were treated 40 hrs with DMSO (“0”, no RA) or all-trans RA (0.1, 1, 10 or 100 nM). Two strong bands represent erbB3 at around 185 kDa. **b** Quantification of the immunoreactive signal (both erbB3 positive bands combined; ECL, linear range of X-ray film). The RA effect was small but highly significant ($n=3$, ANOVA, $p<0.001$; post hoc comparisons with control $*p<0.05$, $**p<0.01$). Three independent experiments were performed, error bars indicate standard deviation.

3.4.5. Discussion

Recent experiments indicate that retinoic acid may act as a regulator of traumatic reactions after peripheral nerve injury (Zhelyaznik et al., 2003). The expression of CRABP-II was most altered during Wallerian degeneration and did not decline during at least two weeks after nerve transection, unless regeneration was allowed (part 3.2.2). This prompted me to suggest an involvement of RA in the process of Wallerian degeneration. Another piece of evidence backing this assumption is the fact that RA down-regulates the gene expression of CNTF in primary Schwann cell cultures (Johann et al., 2003), which was also noticed during

Wallerian degeneration *in vivo* (Sendtner et al., 1996). With the possible exception of this negative effect on ciliary neurotrophic factor, the physiological functions of retinoic acid during PNS regeneration are completely unknown. Using Schwann cell primary cultures from rat sciatic nerves I showed that retinoic acid exerts a significant regulatory effect on the expression of the neuregulin receptor erbB3. The dose-dependent effect of RA was obvious on mRNA and protein levels.

β -NRG-1 and its receptors erbB2 and erbB3 are essential during Schwann cell differentiation (Jessen and Mirsky, 2002; Grinspan et al., 1996). In the late stages of nerve regeneration the proposed function of β -NRG-1 is to control development of neuromuscular reinnervation (Noll and Miller, 1994). While the function of β -NRG-1 in the early stages of regeneration has not been studied in detail, several lines of evidence indicate that this pathway plays an important role during Wallerian degeneration as well. An increased expression of erbB receptors is observed immediately after nerve injury (Kim et al., 2002). It has been suggested that the immediate expression of β -NRG-1 in Schwann cells after nerve injury may prevent their apoptotic death (Grinspan et al., 1996), stimulate their proliferation and migration and may influence the production of chemotactic cues for macrophages which will clear myelin debris (Tofaris et al., 2002; Carroll et al., 1997). The results of the present study point to retinoic acid as a possible regulator of these physiological responses.

What is the molecular mechanism of the observed RA-dependent regulation of erbB3? Up to now, cancer research is the only field where studies concerning the molecular interaction of retinoids and neuregulins have been performed (Offterdinger et al., 1998; Tari et al., 2002; Schneider et al., 1999). In this context retinoid signalling has been identified as a key regulatory element at least for a subset of breast cancer types, where the application of retinoic acid down-regulates the expression of erbBs (Zou et al., 1994; Huang et al., 1988). None of the three erbB promoters contain classical retinoid response elements, whereas potential AP1 sites are present in the erbB2 and erbB3 promoters (Offterdinger et al., 1998). Several molecular events are involved in the activation of AP-1: the transactivating potential of AP-1 depends on induction and phosphorylation of AP-1 components (*c-fos* and *c-jun*) by mitogen-activated protein kinase (MAPK). The expression of *c-fos* is regulated by ternary complex factors whose activity is regulated by the extracellular signal-regulated kinase p38 MAP kinase and c-Jun N-terminal kinase

(JNK). Expression of *c-jun* is regulated by ATF-2, which is phosphorylated by JNK and/or p38 MAPK. As said above, post-translational activation of AP-1 is also regulated by MAPK-mediated phosphorylation (Konta et al., 2001). RA is able to mediate both positive and negative regulatory effects on *c-jun/c-fos* depending on the cell type (Li et al., 1999). As for the negative mechanism, RAR upon ligand binding is able to disrupt *c-jun/c-fos* dimerization *in vivo*, which would preclude the formation of the DNA-binding component AP-1 complex. Another suggested mechanism is competition for the common co-activator cAMP-responsive element binding protein (CBP); (Zhou et al., 1999).

The positive effect of RA on AP-1 is possibly mediated via the protein kinase C (PKC) protein (Desai and Niles, 1997). PKC, an intermediate signal of the PLC γ signalling cascade, is thought to regulate gene expression by inducing the activity of the AP-1. RA increases the activity of PKC δ (Kambhampati et al., 2003), while on the other hand it prevents activation of PKC α (Radomska-Pandaya et al., 2000). RA-induced differentiation of certain tumour cells is accompanied by an increase in PKC expression (Desai and Niles, 1997). Another suggested mechanism of the up-regulation of AP-1 activity due to RA treatment is the activation of MAPK pathway (Huang et al., 2003). Thus PKC and MAPK activity may correlate with the positive effect of RA on AP-1. One cannot exclude that the effect of RA on the β -NRG-1/erbB signalling requires activation of additional intermediates. It is known that the rapid increase of β -NRG-1 mRNA *in vitro* (Loeb et al., 2002) and at the neuromuscular junctions *in vivo* is induced by a number of regulatory factors, such as NT-3, NT-4, BDNF and GDNF (Loeb and Fischbach, 1997), which are also the targets of the transcriptional activity of RA in the variety of cell cultures (Mey, 2001). Therefore a number of molecular mechanisms could account for the effect of RA on β -NRG-1/erbB signalling.

While this remains to be studied, the observation that all-*trans*-RA doses as low as 0.1 and 1 nM potentiated erbB3 gene expression in Schwann cells suggests interactions of RA and β -NRG-1/erbB signalling in the PNS. This constitutes one possible biological function of retinoid signalling triggered by peripheral nerve injury.

4. General Discussion

Physiological functions of retinoic acid after nerve injury are almost unstudied, even though the potential of this molecule is enormous. During embryonic development RA regulates differentiation of brain cells (Noll and Miller, 1994; Wuarin et al., 1990; Henion and Weston, 1994) and in some cases promotes axonal regeneration *in vitro* (Corcoran and Maden, 1999; Corcoran et al., 2000; Mey and Rombach, 1999). In some cell cultures unrelated to the nervous system, RA regulated the expression of many intracellular signals which take part in the physiology of nerve regeneration (Chang et al., 2000; Choudhury et al., 2000; Mey, 2001). However, no experiments have so far been conducted to prove that functional retinoid signalling forms part of the traumatic reactions in the nervous system.

In my work the presence of the retinoic acid signalling system was shown for the adult PNS (part 3.1), and the activation of RARE-regulated gene expression in a transgenic reporter mouse specifically after nerve lesion indicates the involvement of RA in traumatic processes following nerve injury. The possible scheme of retinoic acid signalling in the PNS is summarised in **Fig. 4.1**.

Since the local distribution of RA synthesising aldehyde dehydrogenases is considered to determine the spatial and temporal activity of retinoic acid (Ross et al., 2000), their molecular levels and RA-synthesising activity were measured in sciatic nerves. While the enzyme activity of RALDH-2 was detected, no altered expression of either RALDH-2 or RALDH-1 and -3 was noticed (part 3.1, Zhelyaznik et al., 2003). Moreover, gene expression and immunoreactivity of CRBP-I (part 3.2), which is an indicator of cells that are able to synthesise RA and supports the enzymatic oxidation of retinol and retinal to retinoic acid, was found to be up-regulated. This corroborates the hypothesis of retinoic acid synthesis in sciatic nerves and its elevated expression after injuries of the PNS.

The control of retinoic acid action after PNS injury may involve different mechanisms. They could include negative regulation by 1) RA-degrading enzymes, such as CYP26 (Mey, 2001; Swindell et al., 1999), 2) compartmentalisation of RA by CRABP-I (Dong et al., 1999a) and 3) competition of orphan receptors like COUP-TFII that interfere with DNA-binding of the retinoid receptors (McCaffery et al., 1999). The mechanisms of positive regulation may include changes in expression of 1) CRABP-II, which transports retinoic acid to its nuclear receptors

(Delva et al., 1999; Budhu and Noy, 2002) or 2) retinoid nuclear receptors, which activate gene transcription upon ligand (RA) binding.

Thus, elevated concentrations of CRABP-II and RARs/RXRs may lead to enhanced transcriptional activity. Taking into account their physiological functions, molecular levels of these molecules were evaluated and shown to be strongly increased. The observation of a drastic up-regulation of CRBP-I and CRABP-II (part 3.2) after nerve transection (in degrading nerves) and an early raise of their expression after crush lesion or transection, suggests the involvement of retinoic acid signalling in Wallerian degeneration – the process which initiates axonal regeneration (Fawcett and Keynes, 1990). However, I cannot exclude that retinoic acid also mediates axonal regeneration directly, since the elevated levels of RAR α , RXR α and RXR β (part 3.3) were observed 7 days after crush lesion, when regenerating axons are most apparent.

What exactly could have caused the increased expression of CRBP-I, CRABP-II, RAR α , RXR α and RXR β after axotomy remains to be studied. We also do not know the cellular source of these molecules *in vivo*. At this point of time I can only speculate about the possible triggers. Wallerian degeneration and axonal regeneration are based on a corresponding sequence of cellular and molecular interactions, which in turn reflect the differential expression of specific genes with functions in nerve degeneration and repair. Comparing the gene expression and the time periods of action for different genes (Gillen et al., 1997) with the time course of expression of RA-system molecules, several possible candidates are obvious. They may include neuropoietic cytokines: interleukin (IL)-6, ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF); neurotrophins: nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) and their receptors NGFR-p75, trkB and trkC; neuregulins; and transforming growth factor TGF β .

Within 24 hours after rat sciatic injury, LIF protein increases in Schwann cells close to the lesion site. It is then retrogradely transported in the axons and then functions as a neurotrophic factor for sensory and motoneurons (Mey, 2001). Although the expression of CNTF is down-regulated due to the injury, cell damage causes release of CNTF from those cells. CNTF, which is also taken up and retrogradely transported, prevents the degradation of motoneurons (Sendtner et al., 1992). IL-6 is also induced by the nerve injury and supports macrophage activity like myelin breakdown, which in turn stimulates Schwann cell proliferation and

axonal regeneration (Gillen et al., 1997). The expression of the different members of the TGF β family reach their maxima two or four days after injury and, apart from their important anti-inflammatory functions, stimulate Schwann cell proliferation (Mey, 2001). Thus the expression of these factors may influence the up-regulation of CRBP-I and CRABP-II in the first days after injury.

Triggered by a peripheral nerve injury, the expression of NGF, BDNF and their receptors goes up within a few hours, although another maximum of NGF expression is reached 3 days after the lesion, and in degenerating nerves its increased expression persists up to four weeks after nerve transection (Zhao and Schwartz, 1998). A second peak of BDNF expression is also observed. Two weeks after the injury this neurotrophin is expressed in Schwann cells, while the initial up-regulated expression in neurones returns almost to normal (Gillen et al., 1997). This delayed activation of NGF and BDNF may therefore influence the second expression peaks of CRABP-II and CRBP-I, as well as the elevated expression of retinoid receptors.

Most probably, the cocktail of these cytokines could influence the expression of the retinoic acid signalling system. E.g., first CNTF and/or LIF, then NGF, BDNF and/or TGF β . Obviously, a set of additional experiments is required to investigate these interactions. Specific silencing of different cytokines with siRNAs will allow to determine the contribution of each of them in the regulation of elevated expression of CRBP-I, CRABP-II, RAR α , RXR α and RXR β after axotomy.

Even though my experiments point to the Schwann cells as a possible cellular source of retinoid receptors, additional experiments are also required here. What is/are the source of retinoic acid? Are Schwann cells the only targets of RA transcriptional action? My experiments with dorsal root ganglia showed the presence of CRBP-I and CRABP-II in the sensory nerve cells, however, their expression was not affected by a degenerating or regenerating injury. Thus, nerve cells are possibly neither the source, nor the target of retinoic acid action during PNS injury. Fibroblasts and macrophages contribute to the supply of neurotrophic and neurotropic factors in the injured nerves. Recent experiments with spinal cord lesions (P. McCaffery, unpublished observations) point to the possible involvement of fibroblasts in retinoid acid synthesis. These cells are immuno-positive for RALDH-2, which enzymatically synthesises RA. There is no direct evidence of

macrophage involvement in the activation of the RA-system, however, the peak of their infiltration happens at the second day after lesion and remains high up to four weeks post axotomy, coincident with the elevated expression of CRBP-I and CRABP-II. Thus the investigation of these two cell types is required in the future.

Here I would like to propose a possible scheme of the retinoic acid system signalling response to the nerve injury (**Fig. 4.1**): after injury, RA is synthesised due to RALDH-2 activity in the presence of increased CRBP-I. The up-regulation of CRABP-II will then facilitate RARE- and/or AP-1-regulated gene activation during Wallerian degeneration by trafficking RA to the nuclear receptors, the levels of which are also increased. The proposed scheme represents the normal signalling pathway of retinoic acid during development (McCaffery et al., 1999). Since no direct comparing of the levels of the molecules involved in retinoid signalling in regenerating PNS and during embryonic development has ever been done, I can only speculate about the similarities. The activation of members of the pathway shown in my study allows to suggest that the similar signalling cascades are taking part in regenerating sciatic nerve and during embryogenesis. However the involvement of CRABP-I, CYP26, and the regulation of AP-1 in sciatic nerves remains unknown.

Many genes related to the regulation of Wallerian degeneration count among the targets of RA. They include neurotrophin receptors and cytokines as well as different transcription factors. Up to now only three possible physiological effects of RA are observed in the PNS. All of them were derived, however, from experiments *in vitro*. RA autoregulates the gene expression of its own nuclear receptors (part 3.3), exerts a negative effect on CNTF expression (Johann et al., 2003) and up-regulates the expression of β -NRG-1 receptor erbB3 (part 3.4), which is essential during Schwann cell differentiation (Grinspan et al., 1996; Jessen and Mirsky, 2002), and takes part in Wallerian degeneration (Caroll et al., 1997; Kim et al., 2002). The regulation of erbB3 expression by retinoic acid is another piece of evidence for the RA involvement in biological processes of Wallerian degeneration and confirms the hypothesis that retinoid signalling is triggered by peripheral nerve injury.

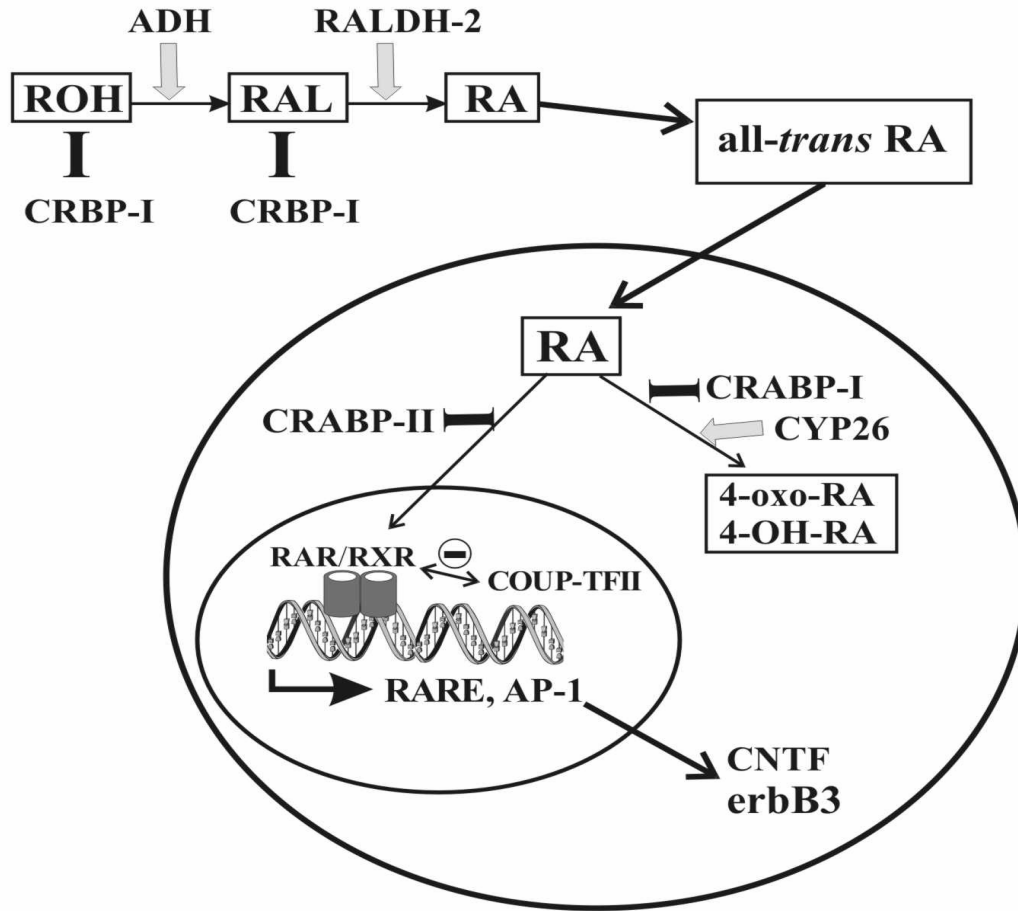


Fig. 4.1. Possible scheme of retinoic system response to the nerve injury.

Retinol is oxidised in retinoic acid (RA) via retinaldehyde (RAL) intermediate. This process is catalysed by retinaldehyde dehydrogenases (RALDH) in the presence of cellular retinol binding protein (CRBP)-I. RA is then either stored after binding to the cellular retinoic acid binding protein (CRABP)-I and further catabolised by cytochrome (CYP)-26 to 4-oxo-RA or 4-OH-RA, or transferred to the nucleus due to the CRABP-II activity. There it can influence gene expression after binding to the retinoic acid receptors (RAR) or retinoid X receptors (RXR), which activate retinoic acid response elements (RARE) or transcription factors like activating protein (AP)-1. As a response to this gene activation, ciliary neurotrophic factor (CNTF) and/or β -Neuregulin-1 receptor erbB3 expression is mediated. Alternatively, RA signalling can be silenced by the orphan receptors COUP-TFII, which interfere with DNA-binding of the retinoid receptors.

The molecular mechanisms described in this work extend our understanding of the processes following peripheral nerve injury. According to my data, one more signalling cascade – the retinoic acid system – takes part in these processes. However, my findings raise several open questions, some of which were already mentioned above. The most important of them concerns the exact role of retinoic acid after axotomy. The indirect pieces of evidence of RA involvement in Wallerian

degeneration guide further possible experiments to prove its role there. Such experiments may include the investigation of the physical features of Wallerian degeneration (Schwann cell de-differentiation, monocyte invagination, myelin phagocytosis, etc.) or molecular levels of the key molecules involved in these processes (TGF β , p75, β -NRG-1, etc.). To investigate the role of RA, either the systemic injections of retinoic acid or the use of the transgenic animals with a manipulated RA-system are required.

The role of retinoic acid in regeneration is to be another topic of subsequent investigations. Since tropic functions of RA are known from developmental studies, does it provide guidance cues during regeneration of adult PNS? Does it influence the production of trophic factors essential for regeneration? Does it influence myelination or the re-innervation of muscle?

Answers to these questions will enable us to develop more effective pharmacological strategies for neurodegenerative diseases and traumatic injuries.

5. Summary

Although the problem of nerve regeneration has been studied since the beginning of the 20th century, the molecular mechanisms of this process are still largely unknown. Recently, the involvement of retinoic acid (RA) signalling was also proposed to regulate PNS regeneration based on its regulation of a variety of intercellular signals, which take part in the physiology of nerve regeneration. However, no experiments have so far been conducted to prove that functional retinoid signalling forms part of the traumatic reactions in PNS. The results of my thesis allow to confirm this hypothesis: With RT-PCR and immunoblotting all necessary components of the RA signalling pathway were detected in the sciatic nerve of adult rats. These are retinoic acid receptors, retinoid X receptors, the retinoic acid synthesising enzymes RALDH-1, RALDH-2 and RALDH-3, and the cellular retinoid binding proteins CRBP-I, CRABP-I and CRABP-II. Although the transcript levels of RALDHs were not significantly altered by a sciatic nerve injury, sciatic nerve crush and transection resulted in a more than 10-fold up-regulation of CRBP-I mRNA, a protein which is thought to facilitate the synthesis of RA. Transcript and protein levels of CRABP-II were elevated 15-fold. This is a possible mediator of RA transfer to its nuclear receptors. The expression of retinoid binding proteins remained elevated during and two weeks after nerve transection, thus the involvement of these proteins in the first phase of axonal regeneration – Wallerian degeneration – may be assumed. Other key molecules of RA regulation are its nuclear receptors, the expression and immunoreactivity of which was measured in the next part of my work. Seven days after crush, the protein levels of RAR α , RXR α and RXR β were significantly up-regulated (4-, 2- and 1.5-fold respectively). On the mRNA level, all three RARs and RXR α were elevated as well. In order to investigate putative biological effects of RA, Schwann cell were investigated. These cells were found to express all components of the retinoic acid signalling cascade and thus can be the source of RA and the target of its functions. I discovered that retinoic acid autoregulates the gene expression of RAR β and RAR γ in Schwann cells, as well as the expression of erbB3, an important signalling intermediate during Wallerian degeneration. These findings confirm our hypothesis of an involvement of RA in Wallerian degeneration. However, they rise a number of open questions about the cellular source of RA signalling, the trigger of its activation and the downstream mechanisms of RA action. The answers to these questions in the future will enable the better understanding of the molecular processes of PNS regeneration.

6. Bibliography

1. Abe K, Namikawa K, Honma M, Iwata T, Matsuoka I, Watabe K, Kiyama H (2001) Inhibition of Ras extracellular-signal-regulated kinase (RK) mediated signaling promotes ciliary neurotrophic factor (CNTF) expression in Schwann cells. *J Neurochem* 77: 700-703.
2. Bastie J, Despouy G, Balitrand N, Rochette-Egly C, Chomienne C, Delva L (2001) The novel co-activator CRABP II binds to RAR α and RXR α via two nuclear receptor interacting domains and does not require the AF-2 "core". *FEBS Letters* 507: 67-73.
3. Belloni PN, Garvin L, Mao CP, Bailey-Healy I, Leaffer D (2000) Effect of all-trans retinoic acid in promoting alveolar repair. *Chest* 117: 235S-241S.
4. Brown MC, Lunn ER, Perry VH, Gordon S, Heumann R (1991) Macrophage dependence of peripheral sensory nerve regeneration: possible involvement of nerve growth factor. *Neuron* 6: 359-370.
5. Budhu A, Noy N (2002) Direct channelling of retinoic acid between cellular retinoic acid-binding protein II and retinoic acid receptor sensitises mammary carcinoma cells to retinoic acid-induced growth arrest. *Mol Cell Biology* 22: 2632-2641.
6. Buonanno A, Fischbach G (2001) Neuregulin and ErbB receptor signalling pathways in the nervous system. *Current Opinion Neuro Biol* 11: 287-296.
7. Carroll SL, Miller ML, Frohnert PW, Kim SS, Corbett JA (1997) Expression of neuregulins and their putative receptors, ErbB2 and ErbB3, is induced during Wallerian degeneration. *J Neurosci* 17: 1642-1659.
8. Carpenter G (2003) ErbB-4: mechanism of action and biology. *Exp Cell Res* 284: 66-77.
9. Chambon P (1996) A decade of molecular biology of retinoic acid receptors. *FASEB J* 10: 940-954.
10. Chandrasekaran V, Zhai Y, Wagner M, Kaplan PL, Napoli JL, Higgins D (2000) Retinoic acid regulates the morphological development of sympathetic neurons. *J Neurobiol* 42: 383-393.
11. Chang MM, Harper R, Hyde DM, Wu R (2000) A novel mechanism of retinoic acid-enhanced interleukin-8 gene expression in airway epithelium. *Am J Respir Cell Mol Biol* 22: 502-510.
12. Cheng L, Esch FS, Marchionni MA, Mudge AW (1998) Control of Schwann cell survival and proliferation: autocrine factors and neuregulins. *Mol Cell Neurosci* 12: 141-156.
13. Chomczynski P, Sacchi N (1987) Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162: 156-159.

14. Choudhury A, Singh RK, Moniaux N, El-Metwally TH, Aubert JP, Batra SK (2000) Retinoic acid dependent transforming growth factor-beta2-mediated induction of MUC4 mucin expression in human pancreatic tumor cells follows retinoic acid receptor-alpha signalling pathway. *J Biol Chem* in press.
15. Citri A, Skaria KB, Yarden Y (2003) The deaf and the dumb: the biology of ErbB-2 and ErbB-3. *Exp Cell Res* 284: 54-65.
16. Corcoran J, Maden M (1999) Nerve growth factor acts via retinoic acid synthesis to stimulate neurite outgrowth. *Nat Neurosci* 2: 307-308.
17. Corcoran J, Shroot B, Pizzey J, Maden M (2000) The role of retinoic acid receptors in neurite outgrowth from different populations of embryonic mouse dorsal root ganglia. *J Cell Sci* 113: 2567-2574.
18. Corcoran J, So P-L, Barber RD, Vincent KJ, Mazarakis ND, Mitrophanous KA, Kingsman SM, Maden M (2002) Retinoic acid receptor β 2 and neurite outgrowth in the adult mouse spinal cord in vitro. *J Cell Sci* 115: 3779-3786.
19. Cullingford TE, Bhakoo K, Peuchen S, Dolphin CT, Patel R, Clark JB (1998) Distribution of mRNAs encoding the peroxisome proliferator-activated receptor α , β and γ and the retinoid X receptor α , β and γ in rat central nervous system. *J Neurochem* 70: 1366-1375.
20. Curtis R, Adryan K, Zhu Y, Harkness P, Lindsay R, DiStefano P (1993) Retrograde axonal transport of ciliary neurotrophic factor is increased by peripheral nerve injury. *Nature* 365: 253-255.
21. Dahlin LB (1995) Prevention of macrophage invasion impairs regeneration in nerve grafts. *Brain Research* 679: 274-280.
22. Delva L, Bastie J, Rochette-Egly C, Kraiba R, Balitrand N, Despouy G, Chambon P, Chomienne C (1999) Physical and functional interactions between cellular retinoic acid binding protein II and the retinoic acid-dependent nuclear complex. *Mol Cell Biology* 19: 7158-7167.
23. Denisenko-Nehrbass N, Jarvis E, Scharf C, Nottebohm F, Mello C (2000) Site-specific retinoic acid production in the brain of adult songbirds. *Neuron* 27: 359-370.
24. Desai SH, Niles RM (1997) Characterization of retinoic acid-induced AP-1 activity in B16 mouse melanoma cells. *J Biol Chem* 272: 12809-12815.
25. Dev S, Adler AJ, Edwards RB (1993) Adult rabbit brain synthesises retinoic acid. *Brain Res* 632: 325-328.
26. Dong D, Ruuska SE, Levinthal DJ, Noy N (1999a) Distinct roles for cellular retinoic acid-binding proteins I and II in regulating signalling by retinoic acid. *J Biol Chem* 274: 23695-23698.

27. Dong Z, Sinanan A, Parkinson D, Parmantier E, Mirsky R, Jessen KR (1999b) Schwann cell development in embryonic mouse nerves. *J Neurosci Res* 56: 334-348.
28. Falls DL (2003) Neuregulins: functions, forms, and signalling strategies. *Exp Cell Res* 284: 14-30.
29. Fawcett JW, Keynes RJ (1990) Peripheral nerve regeneration. *Annu Rev Neurosci* 13: 43-60.
30. Fischbach GD (1997) ARIA: a neuromuscular junction neuregulin. *Ann Rev Neurosci* 20: 429-458.
31. Gillen C, Korfhage C, Müller HW (1997) Gene expression in nerve regeneration. *Neuroscientist* 3: 112-122.
32. Grinspan JB, Marchionni MA, Reeves M, Coulaloglou M, Scherer SS (1996) Axonal interactions regulate Schwann cell apoptosis in developing peripheral nerve: neuregulin receptors and the role of neuregulins. *J Neurosci* 16: 6107-6118.
33. Hall SM, Li H, Kent AP (1997) Schwann cells responding to primary demyelination in vivo express p75NTR and c-erbB receptors: a light and electron immunohistochemical study. *J Neurocytol* 26: 269-290.
34. Haskell GT, Maynard TM, Shatzmiller RA, Lamantia A-S (2002) Retinoic acid signalling at sites of plasticity in the mature central nervous system. *J Comp Neurol* 452: 228-241.
35. Henion PS, Weston JA (1994) Retinoic acid selectively promotes the survival and proliferation of neurogenic precursors in cultured neural crest cell populations. *Dev Biol* 161: 243-250.
36. Holst Av, Rodríguez-Tébar A, Michaille J-J, Dhouailly D, Bäckström A, Ebendal T, Rohrer H (1995) Retinoic acid-mediated increase in TrkA expression is sufficient to elicit NGF-dependent survival of sympathetic neurons. *Mol Cell Neurosci* 6: 185-198.
37. Hsu SM, Raine L, Fanger H (1981) Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *J Histochem Cytochem* 29: 577-580.
38. Huang ME, Ye YC, Chen SR, Chai JR, Lu JX, Zhao L, Gu LJ, Wang ZY (1988) Use of all-trans retinoic acid in the treatment of acute promyelocytic leukemia. *Blood* 72: 567-572.
39. Huang Y, Boskovic G, Niles RM (2003) Retinoic acid - induced AP-1 transcriptional activity regulates B16 mouse melanoma growth inhibition and differentiation. *J Cell Physiol* 194: 162-170.
40. Ide C, Osawa T, Tohyama K (1996) Peripheral nerve regeneration. *Neuroscience Research* 25: 101-121.

41. Jessen KR, Mirsky R (1999) Schwann cells and their precursors emerge as major regulators of nerve development. *Trends Neurosci* 22: 402-410.
42. Jessen KR, Mirsky R (2002) Signals that determine Schwann cell identity. *J Anat* 200: 367-376.
43. Jessen KR, Richardson WD (2002) *Glia cell development*. Oxford: Oxford University Press.
44. Johann V, Jeliashnik N, Schrage K, Mey J (2003) Retinoic acid down-regulates the expression of ciliary neurotrophic factor in rat Schwann cells. *Neuroscience Letters* 339: 13-16.
45. Kambhampati S, Verma A, Sassano A, Marjchrzak B, Deb DK, Parmar S, Giafis N, Kalvakolanu DV, Rahman A, Uddin S, Minucci S, Tallman MS, Fish EN, Plataniias LC (2003) Activation of protein kinase C δ by all-trans retinoic acid. *J Biol Chem* 278: 32544-32551.
46. Kim HA, Zhang D, Stiles CD (2002) Signal requirement for Wallerian degeneration revealed in a neuron - Schwann cell co-culture system. *Soc Neurosci Abstr* 819.13.
47. Konta T, Xu Q, Furusu A, Nakayama K, Kitamura M (2001) Selective roles of retinoic acid receptor and retinoid X receptor in the suppression of apoptosis by all-trans-retinoic acid. *J Biol Chem* 276: 12697-12701.
48. Lampron C, Rochette-Egly C, Gorry P, Dolle P, Mark M, Lufkin T, LeMeur M, Chambon P (1995) Mice deficient in cellular retinoic acid binding protein II (CRABPII) or in both CRABPI and CRABPII are essentially normal. *Development* 121: 539-548.
49. Li Y, Hashimoto Y, Agadir A, Kagechika H, Zhang X (1999) Identification of a novel class of retinoic acid receptor beta -selective retinoid antagonists and their inhibitory effects on AP-1 activity and retinoic acid-induced apoptosis in human breast cancer cells. *J Biol Chem* 274: 15360-15366.
50. Lin F, Kolluri SK, Chen G, Zhang X (2002) Regulation of retinoic acid-induced inhibition of AP-1 activity by orphan receptor chicken ovalbumin upstream promoter-transcription factor. *J Biol Chem* 277: 21414-21422.
51. Loeb JA, Fischbach G (1997) Neurotrophic factors increase neuregulin expression in embryonic ventral spinal cord neurons. *J Neurosci* 17: 1416-1424.
52. Loeb JA, Hmadcha A, Fischbach GD, Land SJ, Zakarian VL (2002) Neuregulin expression at neuromuscular synapses is modulated by synaptic activity and neurotrophic factors. *J Neurosci* 22: 2206-2214.
53. Maden M (1998) Retinoids as endogenous components of the regenerating limb and tail. *Wound Rep Reg* 6: 358-365.
54. Maden M, Gale E, Zile M (1998) The role of vitamin A in the development of the CNS. *J Nutr* 128: 471S-475S.

55. Maden M, Hind M (2003) Retinoic acid, a regeneration-inducing molecule. *Dev Dyn* 226: 237-244.
56. Mangelsdorf DJ, Evans RM (1992) Retinoid receptors as transcription factors. *Transcript Reg* 42: 1137-1167.
57. Marill J, Idres N, Capron CC, Nguyen E, Chabot GG (2003) Retinoic acid metabolism and mechanism of action: a review. *Curr Drug Met* 4: 1-10.
58. Martin-Subero JI, Harder L, Gesk S, Schoch R, Novo FJ, Grote W, Calasanz MJ, Schlegelberger B, Siebert R (2001) Amplification of ERBB2, RARA, and TOP2A genes in a myelodysplastic syndrome transforming to acute myeloid leukemia. *Cancer Genetics and Cytogenetics* 127: 174-176.
59. McCaffery P, Dräger UC (2000) Regulation of retinoic acid signalling in the embryonic nervous system: a master differentiation factor. *Cyt & Growth Fact Reviews* 11: 233-249.
60. McCaffery P, Mey J, Dräger UC (1996) Light-mediated retinoic acid production. *Proc Natl Acad Sci USA* 93: 12570-12574.
61. McCaffery P, Posch K, Napoli J, Gudas L, Dräger UC (1993) Changing patterns of the retinoic acid system in the developing retina. *Developmental Biology* 158: 390-399.
62. McCaffery P, Wagner E, O'Neil J, Petkovich M, Dräger UC (1999) Dorsal and ventral retinal territories defined by retinoic acid synthesis, break-down and nuclear receptor expression. *Mechanisms of Development* 85: 203-214.
63. Mey J (2001) Retinoic acid as a regulator of cytokine signalling after nerve injury. *Z Naturforsch* 56c: 163-176.
64. Mey J, Hammelmann S (2000) OLN-93 oligodendrocytes synthesise all-*trans*-retinoic acid in vitro. *Cell Tissue Res* 302: 49-58.
65. Mey J, McCaffery P, Klemeit M (2001) Sources and sink of retinoic acid in the embryonic chick retina: distribution of aldehyde dehydrogenase activities, CRABP-I, and sites of retinoic acid inactivation. *Dev Brain Res* 127: 135-148.
66. Mey J, Rombach N (1999) Retinoic acid increases BDNF-dependent regeneration of chick retinal ganglion cells in vitro. *Neuroreport* 10: 3573-3577.
67. Murphy S, Krainock R, Tham M (2002) Neuregulin signalling via ErbB receptor assemblies in the nervous system. *Mol Neurobiol* 25: 49-59.
68. Napoli J (1996) Retinoic acid biosynthesis and metabolism. *FASEB J* 10: 993-1001.
69. Nikiforovich GV, Frieden C (2002) The search for local native-like nucleation centers in the unfolded state of β -sheet proteins. *PNAS* 99: 10388-10393.

70. Noll E, Miller RH (1994) Regulation of oligodendrocyte differentiation: a role for retinoic acid in the spinal cord. *Development* 120: 649-660.
71. Offterdinger M, Schneider SM, Grunt TW (2003) Heregulin and retinoids synergistically induce branching morphogenesis of breast cancer cells cultivated in 3D collagen gels. *J Cell Physiol* 195: 260-275.
72. Offterdinger M, Schneider SM, Huber H, Grunt TW (1998) Retinoids control the expression of c-ErbB receptors in breast cancer cells. *Biochem Biophys Res Com* 251: 907-913.
73. Radomska-Pandaya A, Chen G, Czernik PJ, Little JM, Samokyszyn VM, Carter CA, Nowak G (2000) Direct interaction of all-trans-retinoic acid with protein kinase C (PKC). *J Biol Chem* 275: 22324-22330.
74. Raivich G, Bohatschek M, Kloss CUA, Werner A, Jones L, Kreutzberg GW (1999) The neuroglial activation repertoire in the injured brain: molecular mechanisms and cues to physiological functions. *Brain Res Review* 30: 77-105.
75. Ramon y Cajal S (1928) Degeneration and regeneration of the nervous system. Oxford: Oxford University Press.
76. Roos TC, Jugert FK, Merk HF, Bickers DR (1998) Retinol metabolism in the skin. *Pharmacol Review* 50: 315-333.
77. Ross S, McCaffery P, Dräger UC, De Luca LM (2000b) Retinoids in embryonal development. *Physiol Rev* 80: 1021-1054.
78. Rossant J, Zirngibl R, Cado D, Shago M, Giguere V (1991) Expression of a retinoic acid response element *hsplac* Z. transgene defines specific domains of transcriptional activity during mouse embryogenesis. *Genes Dev* 5: 1333-1344.
79. Schneider SM, Offterdinger M, Huber H, Grunt TW (1999) Involvement of nuclear steroid/thyroid/retinoid receptors and of protein kinases in the regulation of growth and of c-erbB and retinoic acid receptor expression in MCF-7 breast cancer cells. *Breast Cancer Res Treat* 58: 171-181.
80. Sendtner M, Götz R, Holtmann B, Escary JL, Masu Y, Carroll P, Wolf E, Brem G, Brulet P, Thoenen H (1996) Cryptic physiological trophic support of motoneurons by LIF revealed by double gene targeting of CNTF and LIF. *Curr Biol* 6: 686-694.
81. Sugawara A, Sanno N, Takahashi N, Osamura RY, Abe K (1997) Retinoid X receptors in the kidney: their protein expression and functional significance. *Endocrinol* 138: 3175-3180.
82. Swindell E, Thaller C, Sockanathan S, Petkovich M, Jessel T, Eichele G (1999) Complementary domains of retinoic acid production and degradation in the early chick embryo. *Developmental Biology* 216: 282-296.

83. Tari AM, Lim S-J, Hung M-C, Esteva FJ, Lopez-Berestein G (2002) Her2/neu induces all-*trans* retinoic acid (ATRA) resistance in breast cancer cells. *Oncogene* 21: 5224-5232.
84. Tofaris GK, Patterson PH, Jessen KR, Mirsky R (2002) Denervated Schwann cells attract macrophages by secretion of leukemia inhibitory factor (LIF) and monocyte chemoattractant protein-1 in a process regulated by interleukin-6 and LIF. *J Neurosci* 22: 6696-6703.
85. Tzahar E, Waterman H, Chen X, Levkowitz G, Karunakaran D, Lavi S, Ratzkin BJ, Yarden Y (1996) A hierarchical network of interreceptor interactions determines signal transduction by Neu differentiation factor/neuregulin and epidermal growth factor. *Mol Cell Biol* 16: 5276-5287.
86. Vartanian T, Goodearl AD, Lefebvre S, Park SK, Fischbach G (2000) Neuregulin induces the rapid association of focal adhesion kinase with the erbB2-erbB3 receptor complex in Schwann cells. *Biochemical and Biophysical Research Communications* 271: 414-417.
87. Wagner E, Luo T, Dräger UC (2002) Retinoic acid synthesis in the postnatal mouse brain marks distinct developmental stages and functional systems. *Cerebral Cortex* 12: 1244-1253.
88. Wagner E, McCaffery P, Dräger UC (2000) Retinoic acid in the formation of the dorso-ventral retina and its central projections. *Developmental Biology* 222: 460-470.
89. Weiler R, Schulz K, Pottek M, Tieding S, Janssen-Bienhold U (2000) Retinoic acid has light-adaptive effects on horizontal cells in the retina. *Proc Natl Acad Sci* 95: 7139-7144.
90. Wiley HS (2003) Trafficking of the ErbB receptors and its influence on signaling. *Exp Cell Res* 284: 78-88.
91. Wuarin L, Sidell N, De Vellis J (1990) Retinoids increase perinatal spinal cord neuronal survival and astroglial differentiation. *Int J Devl Neuroscience* 8: 317-326.
92. Zanazzi G, Einheber S, Hannocks MJ, Westreich R, Marchionni MA, Salzer JL (2001) Effects of GGF/ β -neuregulin-1 on myelination: evidence that mitogen activation results in demyelination. *J Cell Biol* 152: in press.
93. Zetterström R, Lindqvist E, Mata de Urquiza A, Tomac A, Eriksson U (1999) Role of retinoids in the CNS: differential expression of retinoid binding proteins and receptors and evidence for presence of retinoic acid. *Eur J Neuroscience* 11: 407-416.
94. Zhang D-Q, McMahon DG (2000) Direct gating by retinoic acid of retinal electrical synapses. *Proc Natl Acad Sci USA* 97: 14754-14759.
95. Zhang D, McMahon D (2001) Direct gating by retinoic acid of retinal electrical synapses. *PNAS* 97: 14754-14759.

96. Zhang D, Sliwkowski MX, Mark M, Frantz G, Akita R, Sun Y, Hillan K, Crowley C, Brush J, Godowski PJ (1997) Neuregulin-3 (NRG3): A novel neural tissue-enriched protein that binds and activates ErbB4. *Proceedings of the National Academy of Sciences* 94: 9562-9567.
97. Zhao B, Schwartz JP (1998) Involvement of cytokines in normal CNS development and neurological diseases: recent progress and perspectives. *J Neurosci Res* 52: 7-16.
98. Zhao D, McCaffery P, Ivins K, Neve R, Hogan P, Chin W, Dräger UC (1996) Molecular identification of a major retinoic-acid-synthesising enzyme, a retinaldehyde-specific dehydrogenase. *Eur J Biochem* 240: 15-22.
99. Zhelyaznik N, Schrage K, McCaffery P, Mey J (2003) Activation of retinoic signalling after sciatic nerve injury: up-regulation of cellular retinoid binding proteins. *Eur J Neurosci* 18: 1033-1040.
100. Zhou XF, Shen XQ, Shemshedini L (1999) Ligand-activated retinoic acid receptor inhibits AP-1 transactivation by disrupting c-Jun/c-Fos dimerization. *Mol Endocrinol* 13: 276-285.
101. Zou CP, Clifford JL, Xu XC, Sacks PG, Chambon P, Hong WK, Lotan R (1994) Modulation by retinoic acid (RA) of squamous cell differentiation, cellular RA-binding proteins, and nuclear RA receptors in human head and neck squamous cell carcinoma cell lines. *Cancer Res* 54: 5479-5487.

5. Attachments

Abbreviations

AP-1	– activator protein - 1
APS	– ammonium persulfate
ARIA	– acetylcholine receptor-inducing activity
ATF-2	– activating transcription factor - 2
BCA	– bicinchoninic acid
BCIP	– 5-bromo-4-chloro-3-indolyl phosphate, toluidine salt
BDNF	– brain derived neurotrophic factor
BSA	– bovine serum albumine
CNS	– central nervous system
CNTF	– ciliary neurotrophic factor
COUP-TFII	– chicken ovalbumine upstream promoter-transcription factor II
CRABP	– cellular retinoic acid binding protein
CRBP	– cellular retinol binding protein
CYP 26	– cytochrome P 450-oxidase, number 26
DAB	– diamino-benzidine
DEPC	– diethyl-pyrocaborate
DIG	– digoxigenin
DMEM	– Dulbecco's modified Eagle's medium
DMSO	– dimethyl sulfoxide
DNA	– deoxyribonucleic acid
DPX	– p-Xylene-bis(N-pyridinium bromide)
DR	– direct repeat
DRG	– dorsal root ganglion
ECL	– enhanced chemiluminescence
ECM	– extracellular matrix
EcoR1	– <i>Escherichia coli</i> restriction endonuclease I
EDTA	– ethylenediamine - tetra acetic acid
EGF	– epidermal growth factor
ELISA	– Enzyme-Linked Immunosorbent Assay
erbB	– erythroblastosis gene B
ERK	– extracellular signal-regulated kinase
FCS	– fetal calf serum
GAM Ig	– goat anti mouse immunoglobulin
GAPDH	– glyceraldehyde phosphate dehydrogenase
GAR Ig	– goat anti rabbit immunoglobulin
GDNF	– growth derived neurotrophic factor
HEPES	– N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HDAC	– histone deacetylase
HRP	– horseradish peroxidase
ISH	– <i>in situ</i> hybridisation
IL	– interleukin
Jak	– Janus kinase
lacZ	– <i>E. coli</i> β -galactosidase (reporter) gene
LB	– Luria-Bertani
MAPK	– mitogen activated protein kinase

NBT	– nitro blue tetrazolium chloride
NGF	– nerve growth factor
NGS	– normal goat serum
NMRI	– Naval Medical Research Institute
NRG	– Neuregulin
NT	– neurotrophin
dNTP	– 2'-Deoxyribonucleoside-5'-tri-phosphates
OLN-93	– oligodendrocyte cell line 93
p75	– protein 75
PAGE	– polyacrylamide gel electrophoresis
PBS	– phosphate buffered saline
PCR	– polymerase chain reaction
PFA	– paraformaldehyde
PI3P	– phosphatidylinositol 3-phosphate
PKC	– protein kinase C
PLC γ	– phospholipase C γ
PMSF	– phenylmethanesulfonylfluorid
RA	– retinoic acid
RALDH	– retinaldehyde dehydrogenase
RAR	– retinoic acid receptor
RARE	– retinoic acid response element
Raf	– <i>Ras</i> associated factor
Ras	– rat sarcoma viral oncogene homolog
RNA	– ribonucleic acid
RT	– reverse transcription
RXR	– retinoid X receptor
RXRE	– retinoid X response element
SEM	– standard error of mean
SD	– standard deviation
SDS	– sodium-dodecylsulfate
SSC	– sodium citrate
TBS	– Tris buffered saline
TBS-T	– <i>TBS</i> -Tween 20
TEMED	– N,N,N',N'-tetramethylethylenediamine
TGF β	– transforming growth factor β
Tris	– tris-(hydroxymethyl)aminomethane
Trk	– tropomyosin related kinase

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Publications

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2. Ashmarin IP, Samonina GE, Zhelyaznik NIa, Bakaeva ZV. Protective effect of peptide PGP on gastric mucosa. *Dokl Akad Nauk*. 368; 5 (1999): 709-10. *Russian*
3. Zhelyaznik NIa, Samonina GE, Sergeev VI, Ashmarin IP. Increased blood flow in the stomach as a possible mechanism of the anti-ulcer effect of Pro-Gly-Pro. *Biull Eksp Biol Med*. 129; 2 (2000):133-4. *Russian*
4. G. Samonina, L. Lyapina, N. Jeliarnik und I. Ashmarin. Protection of gastric mucosal integrity by gelatin and simple proline-containing peptides. *Pathophysiology* 7 (2000): 69-73
5. V. Johann, N. Jeliarnik, K. Schrage und J. Mey. Retinoic acid downregulates the expression of ciliary neurotrophic factor (CNTF) in rat Schwann cells. *Neurosci. Lett.* 339 (2003): 13-16.
6. N. Zhelyaznik, K. Schrage, P. McCaffery and J. Mey. Activation of retinoic acid signalling after sciatic nerve injury: up-regulation of cellular retinoid binding proteins. *Eur J Neurosci*. 18 (2003): 1033-1040

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