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Differential Patterns of Local Gene Regulation in Crush Lesions of the Rat Optic and Sciatic Nerve: Relevance to Posttraumatic Regeneration

Philipp Zickler*, Patrick Küry*, Michael Gliem, Hans-Peter Hartung and Sebastian Jander

Department of Neurology, Heinrich-Heine-University, Düsseldorf, *These authors contributed equally to this study

Key Words
Nerve crush • Axon • Inflammation • Aquaporin 4 • Plasminogen activator

Abstract
Axon regrowth after nerve injury can occur in the peripheral but fails in the central nervous system. Cellular reactions at the lesion site affect axonal regrowth. We compared gene regulation in optic nerve (ON) and sciatic nerve (SN) crush lesions in adult rats by cDNA array analysis, quantitative RT-PCR and immunohistochemistry, focusing on the primary lesion site rather than the proximal or distal nerve stump. Four days after injury, identical gene regulation in ON and SN lesions was found for 19/1185 genes (15 up, 4 down). In contrast, tissue-specific regulations were identified for 48 genes in ON and 50 genes in SN crush lesions. Among these, in the ON many genes were downregulated (23 up, 25 down) whereas upregulation predominated in SN lesions (43 up, 7 down), especially for signaling, metabolism, and translation/transcription-related genes. In ON lesions aquaporin 4 downregulation corresponded to a transient loss of astrocytes. Tissue-type plasminogen activator was upregulated in the lesion and distal stump of SN while the urokinase-type plasminogen activator was upregulated only in the ON lesion indicating differences in local proteolysis between the systems. Typical neuronal genes were regulated at the crush site comprising neurotransmitter genes in ON and actin cytoskeleton-related genes in the SN. The differential orchestration of local gene regulation has implications for axonal regeneration in central nervous system lesions.

Introduction

Axonal damage is a common consequence of traumatic and inflammatory damage to the nervous system causing severe and persistent disability in diseases such as spinal cord injury [1, 2] and multiple sclerosis [3, 4]. The central and peripheral nervous systems differ fundamentally in their response to axonal injury. Transected axons spontaneously regrow beyond the site of injury in the peripheral nervous system (PNS) and achieve at least partial functional recovery. Contrastingly, in the central nervous system (CNS) abortive sprouting

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Dr. Patrick Küry or Dr. Sebastian Jander
Department of Neurology, Heinrich-Heine-University
Moorenstr. 5, 40225 Düsseldorf (Germany)
Tel. +49-2118117822 (PK) or +49-2118118978 (SJ), Fax +49-(0)211-81-18485
E-Mail kuery@uni-duesseldorf.de or jander@uni-duesseldorf.de

does not result in successful innervation of distal parts of injured fiber tracts.

Apart from the intrinsic neuronal growth capacity [5] the extent of axon regeneration depends on a permissive environment [6, 7]. Differences in the glial response, inflammation, cytokine and growth factor release, clearance of myelin debris, and scar formation may contribute to the failure of the CNS to support axonal regeneration after injury [8, 9]. Transcriptional profiling in models of axotomy has identified candidate genes which may contribute to axon regeneration. However, a direct comparison of local gene responses at PNS vs. CNS lesion sites has not been performed yet. We therefore compared local molecular responses in peripheral and central nerves at the site of crush injury with the aim to identify targets for novel therapies. Crush lesions of the optic nerve (ON) and sciatic nerve (SN) are well-defined models to compare axotomy-induced changes in the CNS and PNS, respectively. Furthermore, ON crush injury is of clinical relevance for ophthalmological diseases such as glaucoma and optic neuritis. We compared lesion-associated local transcriptional changes four days after ON and SN crush injury in adult Wistar rats by means of cDNA array hybridization analysis. This time point was chosen because macrophage recruitment as a critical prerequisite for tissue remodeling after injury is most pronounced at this stage. For selected genes, spatiotemporal patterns of gene regulation were further studied by real-time quantitative RT-PCR (Q-PCR) and immunohistochemistry.

Materials and Methods

Animal experiments

All animal experiments were performed in 8 to 10 weeks old male Wistar rats (250-300 g body weight) deeply anesthetized with enflurane in a 2:1 N_2O/O_2 atmosphere. The experimental protocol was in accordance with international guidelines of handling laboratory animals and had been approved by the institutional animal experimentation review committee. For SN crush, the right SN was exposed at mid-thigh level and crushed with a sterile forceps (Dumont #2) for 60 sec with maximal strength. After wound suturing, the animals were held at standard laboratory conditions. For intraorbital crush injury of the right ON the animals' head was positioned in a stereotactic frame. An incision was made through the temporal palpebral tissue and the lacrimal gland was largely removed. After mobilization of the eyeball the ON was exposed and crushed 2 mm posterior to the eyeball with a sterile forceps (Dumont #5) for 30 sec with maximal strength, always leaving the ophthalmic artery intact. All procedures were performed under visual control using a binocular dissection microscope. After the nerve crush the eyeball was relocated and the wound was sutured. In both paradigms, ON or SN of control animals were exposed in parallel for a sham operation without applying the crush lesion.

For mRNA isolation and tissue preparation, rats were killed by an overdose of ether at days 2, 4, 7, 14 and 30 after operation. The crush site and the entire accessible distal nerve stump were prepared. The prepared nerve segments were chopped into pieces of approximately 2 mm length, and immediately stored in RNA later™ solution (Ambion). For the array analysis, the primary lesion site of ON and SN was isolated from n=20 rats per paradigm. Uncrushed controls were generated from n=20 ON and n=25 SN from sham-operated animals. For analysis by Q-PCR, tissue samples at days 2, 4, 7 and 14 post crush were collected from n=5 animals for each paradigm from both, the crush site and the distal nerve stump. For the array and Q-PCR analysis samples of n=5 animals were pooled, respectively, and total RNA was isolated using the TRIzol™ reagent (Life Sciences, Gaithersburg, MD, USA) according to the manufacturer's instructions and quantified spectrophotometrically. For immunohistochemistry, crushed ON and SN from n=3 animals per condition and time point were prepared on days 2, 4, 7, 14, 30 after crush injury, longitudinally embedded in Tissue-Tek mounting medium, snap frozen in isopentane precooled to -50°C on dry ice, and stored at -80°C.

cDNA array hybridization and analysis

The array analyses were performed on 6 µg pooled total RNA using Atlas rat 1.2 cDNA arrays (BD Biosciences/ Clontech, Heidelberg, Germany) essentially according to previously established procedures [10, 11]. Total RNA was reverse transcribed with the array-specific rat 1.2 CDS primer mix in the presence of [α^{32}P]-dATP and the MMLV reverse transcriptase (BD Biosciences/Clontech). The hybridization procedure and washing steps were performed according to the manufacturer's protocol. Hybridized arrays were exposed to phosphoimaging screens, scanned [BAS-1500 reader (Fujifilm); Raytest, Straubenhardt, Germany] and digitalized for analysis and quantification with the image analysis software AIDA Array Metrix 3.0 (Raytest). Signals were normalized using the average signal intensity of all expressed genes as reference. For the ON crush cohort 4 individual datasets from lesioned and sham operated ONs were collected. In the SN crush paradigm n=4 datasets from lesioned nerves were compared to n=5 array hybridizations from sham operated nerves. Accordingly, 16 ON and 20 SN cross-comparison datasets were obtained for gene expression analysis. Only genes showing a significant regulation, i.e. at least twofold altered expression levels, in more than two-thirds of the comparison datasets (11/ 16 for ON and 14/20 for SN comparisons) were considered to be regulated and consecutively used to calculate the mean regulation factor for each individual gene. For further control, array filter hybridization dots were visually inspected in order to confirm the results.

Quantitative real time PCR (Q-PCR)

Real time Q-PCR was performed for validation of the array results on the same mRNA samples as used for the array analysis

FUNCTION	GenBank number	GENE NAME	OPTIC NERVE average regul. up	down	datasets max. 16	SCIATIC NERVE average regul. up	down	datasets max. 20
growth / differentiation	L26110	TGF-beta receptor type 1 precursor	5,0		13	5,6		20
	X52498	TGF-beta 1	2,5		16	5,6		15
trafficking / metabolism	U13253	fatty acid -binding protein (epidermal)	4,9		16	4,9		16
	J04526	hexokinase type I		-3,1	13		-2,6	17
ion channel	M26161	voltage -gated potassium channel KV1.1		-4,9	15		-4,1	17
inflammation / chem otaxis	U54791	CXCR4	7,5		16	15,9		20
	AB003042	C5a anaphylatoxin chemotactic receptor	7,1		16	4,6		17
	M57276	leukocyte antigen CD53 (MRC -OX44)	4,7		16	2,8		15
	U04740	platelet -activating factor receptor	3,5		16	4,6		18
	U77776	IL-18	2,9		13	2,7		16
	U38376	cytosolic phospholipase A2	2,3		12	3,3		17
proteolysis	X82396	cathepsin B	7,6		16	3,4		16
	X54467	cathepsin D	7,0		16	7,1		20
	Y00697	cathepsin L	3,3		15	3,1		16
	M58593	proteasome component C8	2,7		14	4,6		16
transcription	Z46372	DNA topoisomerase II alpha	4,5		16	26,0		20
	D84418	high mobility group protein 2	3,6		11	3,1		19
myelination	U31367	myelin protein MVP17		-6,4	16		-13,7	20
	M25889	myelin basic protein S (MBP S)		-2,9	12		-8,5	20

Table 1. Genes with a common regulation pattern in both lesion types 4 days after nerve crush injury were predominantly upregulated (15 out of 19 genes). In the array analysis significant gene regulation was defined by more than twofold expression changes in more than two-thirds out of 16 optic or 20 sciatic nerve crush datasets cross-compared with sham-operated nerves. For each gene the average up- or downregulation and number of cross-comparison datasets with significant regulation were listed for the optic and sciatic nerve, respectively.

at day 4 and for time course analysis on additionally isolated mRNA from days 2, 4, 7 and 14 post crush. Total RNA was reverse transcribed using Superscript II enzyme (Invitrogen, Carlsbad, CA) and dT19(A/C/G) oligonucleotide primers as described previously [12]. According to the manufacturer's instructions cDNA samples were analyzed on an ABI 5700 Sequence Detection System (Applied Biosystems, Darmstadt, Germany) using the Sybr Green Universal Master Mix (Applied Biosystems). Glyceraldehyde 3-phosphate dehydrogenase was used as reference gene. Primer sequences were designed using the PrimerExpress 2.0 software (Applied Biosystems) and tested for the generation of specific amplicons. The forward and reverse primer sequences used were: aquaporin 4 (TGC TGG CAG GTG CAC TTT AC; TGA GCT CCA CGT CAG GAC AG), glyceraldehyde 3-phosphate dehydrogenase (GAA CGG GAA GCT CAC TGG C; GCA TGT CAG ATC CAC AAC GG), glial cell line-derived neurotrophic factor receptor alpha (CTC AGC AGC ATT GCC TTC TG; TGT CAT TCA CAC TAG GCT GCG), insulin-like growth factor I (IGF) (CAC GTC ACC GCA AGA TCC TT; TTC CGA TGT TTT GCA GGT TG), IGF-binding protein 3 (GGT GCG TGG ACA AGT ACG G; TCC CCT TGG TGT CAT AGC CT), neuropeptide Y (NPY) (GAA ACC AGT CTG CCT GTC CC; GGA ATC CAG CCT GGT GGT G), tissue-type plasminogen activator (tPA) (GCC CCC TAA AAC CCT TGA AA; TGA GGA TTG TGG GAG GAT GG), urokinase-type plasminogen activator (uPA) (CAC TCA TCC CCA CGC TGA C; ACA TGA TGG AGA TGA CCC TGC), thymosin-beta 15 (GCT GCG GAC AGA ATT GCT G; CTT GAC TGC TCG TTC CAA AGC), thyrotropin-releasing hormone (TRH) (TCT GTA ATC TGC CCC ATG TGG; ATG CGC TGA AGC TAT ACC AGG). Relative gene expression levels were determined according to the manufacturer's ΔΔCt method. Each sample was analyzed in two independent Q-PCR reactions and the results were calculated as mean values and standard error of the mean.

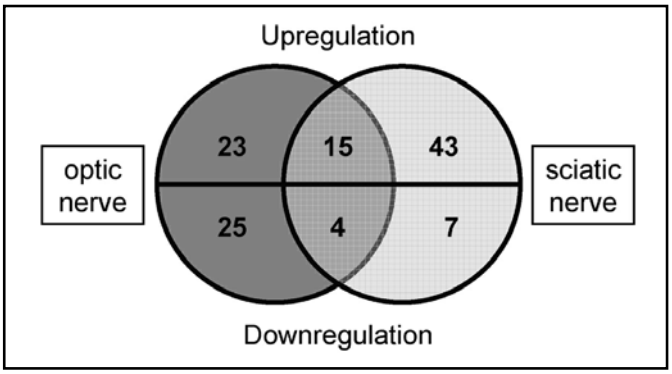

Fig. 1. Overall distribution of significantly regulated genes in the array analysis 4 days after nerve crush. Upregulation clearly predominated for genes regulated selectively in the sciatic nerve lesion (right) and in both tissue-types (overlap in the middle) in contrast to the optic nerve (left) where downregulated genes closely outnumbered upregulation.

Immunohistochemistry

Serial adjacent cryostat sections (10 μm) of crushed ON and SN were fixed in 4% paraformaldehyde in phosphate-

FUNCTION	OPTIC NERVE GenBank number	GENE NAME	average regul.	datasets max. 16	SCIATIC NERVE GenBank number	GENE NAME	average regul.	datasets max. 20
growth / differentiation	M15480	insulin like growth factor I (IGF)	6,4	13	U97142	GDNF receptor alpha	9,4	20
	M13750	prolactine like protein A (rPLP-A)	-3,8	12	M89791	IGF binding protein 1 precursor	7,2	17
					M31837	IGF binding protein 3 precursor	3,2	20
					M18416	NGF-induced protein 1 (NGFI-A)	3,2	14
trafficking / metabolism	D13871	fructose (glucose) transporter	3,0	15	D10874	vacuolar ATP synthase (16-kDa)	6,3	20
	D10041	long-chain fatty acid-CoA ligase	-11,0	14	D10952	cytochrome c oxidase (COX5B)	6,2	19
	U88036	brain-specific organic anion transporter	-7,9	15	L12380	ADP-ribosylation factor 1 (ARF1)	4,4	20
	M95591	squalene synthetase	-4,0	15	M35052	mitochondrial ATP synthase B	4,0	16
	D63834	monocarboxylate transporter	-3,8	15	D10854	aldehyde reductase	3,1	16
	X06984	aldolase C	-3,7	13	U75581	fatty acid- binding protein (adipocyte)	-5,4	20
	U25651	muscle 6-phosphofructokinase	-2,8	12	M12919	aldolase A	-3,5	20
	U02096	fatty acid-binding protein (brain)	-2,4	12				
motility	U60096	integrin beta 4 precursor	-2,9	14	U25684	thymosin beta 15	6,7	18
					U06755	acidic calponin	2,7	15
ion channel	J04629	Na/K-transp. ATPase (ATP1B2)	-4,4	16				
	M14512	Na/K ATPase, alpha(+) isoform	-2,9	12				
signal transduction	M36317	thyrotropin-releasing hormone (TRH)	24,8	16	D38222	tyrosine phosphatase-like protein	6,5	20
	M20373	neuropeptide Y (NPY) precursor	8,4	16	L13151	ras-GTPase-activating protein	6,3	18
	J03624	neuropeptide Y (NPY)	3,5	13	M95738	Na/Cl- dependent GABA transporter 3	4,6	19
	U55192	inositol phosphatase (SHIP)	6,4	14	M96601	taurine transporter	4,4	19
	M33962	neuronal pentraxin receptor	3,0	12	M85299	sodium/hydrogen exchange prot.1	4,1	17
	L06482	retinoid X receptor alpha	2,6	14	M17525	GTP-binding protein (G-alpha-8)	3,8	16
	AF005099	protein tyrosine phosphatase (PTPase)	2,6	11	D17445	PKC inhibitor protein-1	3,5	16
	M91590	beta-arrestin 2 (ARRB2)	2,5	11	M20637	phospholipase C delta 1 (PLC-III)	2,8	15
	M95735	syntaxin B	-7,7	13	D38224	annexin IV (ANX4)	-3,4	16
	X57764	endothelin receptor (ET-B)	-5,9	14	L20822	syntaxin 5 (STX5)	-2,9	20
	M88595	glycine transporter	-4,5	16				
	M17527	adenylate cyclase-inhibit. G prot.	-3,3	13				
	L35921	GTP-bind.protein G(i)/G(s)/G(o)	-2,7	13				
	U02983	secretogranin 3 (Sg3)	-2,5	11				
	L14851	neurexin III-alpha	-2,5	11				
inflammation / chemotaxis	X69903	IL-4 receptor	4,0	16	X16956	beta-2-microglobulin	7,5	14
	M10072	leukocyte common antigen precursor	3,8	16	U73142	MAP kinase p38	3,4	14
	U22414	MIP-1alpha	3,6	13	M92340	IL-6 receptor beta chain	3,3	16
					M64986	high mobilitiy group protein 1	2,8	14
proteolysis	X63434	urokinase plasminogen activator (uPA)	23,5	16	M65253	matrix metalloproteinase (MMP10)	6,9	14
	D90404	cathepsin C	4,3	16	L31883	tissue inhibitor of metalloproteinase -1	4,2	14
	M24067	plasminogen activator inhibitor 1 (PAI-1)	3,7	14	M23697	tissue-type plasminoge n activator (tPA)	3,8	17
	AF010306	cathepsin K	2,9	15	X52783	proteasome subunit C5	3,3	16
	D50694	26S protease regulatory subunit 7	2,1	11	M61142	thimet oligopeptidase (THOP1)	3,0	16
	M32247	alpha-1 antitrypsin	-4,0	11	U62897	carboxypeptidase D	2,8	16
stress response	X62404	glutathione peroxidase (epididymal)	2,9	11	L15079	multidrug resistance protein 2 (MDR2)	5,3	19
	Z27118	heat shock protein 70 k-Da (HSP70)	2,3	11	L22191	GLU-CYS ligase regulatory subunit	4,9	17
	U14007	aquaporin 4 (AQP4)	-15,2	16	S68987	SET alpha + beta isoform	4,0	15
	AF007775	aquaporin 8	-5,4	11	L38615	glutathione synthetase	3,5	16
	S83436	glutahione S-transferase subunit 13	-2,9	12	X02904	glutathione S-transferase P subunit	2,9	15
	X62660	glutathione transferase subunit 8	-2,6	12				
cell cycle/apoptosis	X64589	G2/M-specific cyclin B1	3,9	16	D14014	G1/S-specific cyclin D1	8,6	20
	U05341	p55cdc	2,7	15	D16309	G1/S-specific cyclin D3	8,0	20
	M64723	clusterin (CLU)	-3,6	14				
transcription					L29259	elongation factor SIII P15 subunit	5,0	19
					L20681	c-ets-1 proto-oncogene protein	3,2	16
translation	X14210	ribosomal protein S4	2,4	12	X53504	ribosomal protein L12	4,8	14
					K03250	40S ribosomal protein S11	4,3	16
					X51707	40S ribosomal protein S19	3,3	16
					J02646	translation initiation factor 2 alpha	2,9	15
myelination	U62803	lecithin cholesterol acyltransferase (LCAT)	-7,1	12	D17512	cysteine-rich protein 2	2,4	15
					U07683	cerebroside synthase	-7,5	16
					M69139	peri pheral myelin protein 22 (PMP22)	-6,5	20
					K03242	myelin P0 protein precursor (P0)	-4,1	16

Table 2. Genes with tissue-specific expression and regulation (>twofold, in >2/3 of the cross-comparison datasets) at the lesion site 4 days after nerve crush were listed for the optic and sciatic nerve, respectively. Within functional categories the genes were ranked according to their average up- or downregulation and the respective number of significant cross-comparison datasets.

buffered saline for 10 min at 4°C, followed by acetone 50/100/50 %, 2 minutes each at 4°C. After 30 min incubation with 0.3 % H_2O_2 nonspecific binding sites were blocked with 3 % normal horse or goat serum in PBS for 30 min at room temperature according to the primary antibody used. Serial sections were incubated overnight at 4 °C with a mouse mAb ED 1 against

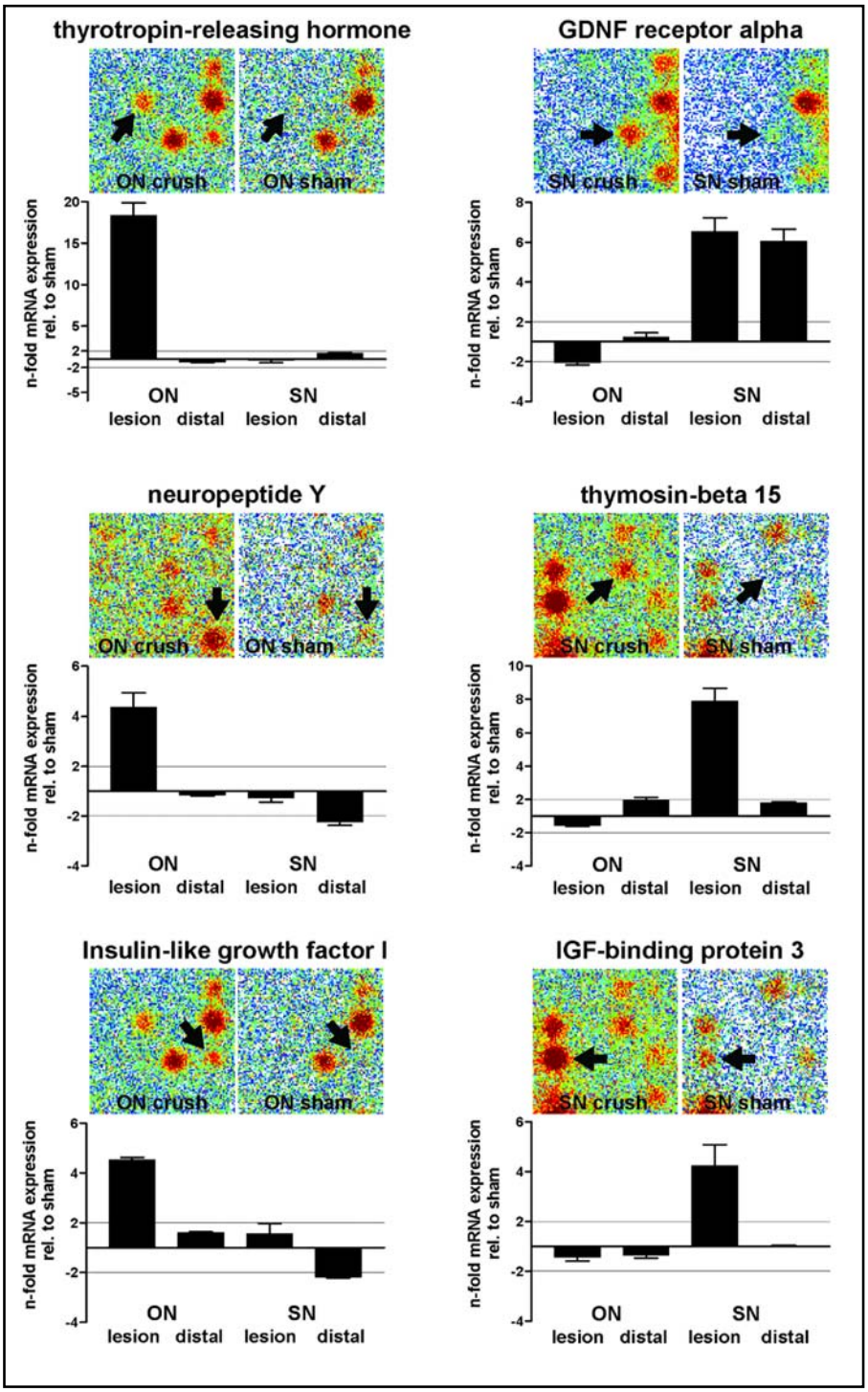

Fig. 2. Results from the array analysis 4 days after nerve crush were confirmed and further quantified by real-time PCR on the same mRNA samples. Representative genes with specific regulation in the optic nerve (left column) and sciatic nerve (right column) are shown with the results of the array hybridization and Q-PCR at the lesion site and additionally in the distal nerve stump. Significant up- or downregulation was defined by a more than 2-fold expression change (dashed line) relative to sham levels. Each Q-PCR was repeated twice and the mean and standard error of the mean were calculated. GDNF, glial cell line-derived neurotrophic factor; IGF, insulin-like growth factor.

phagocytic macrophages (1:2000; Serotec, Oxford, UK), a mouse monoclonal glial fibrillary acidic protein (GFAP) antibody (1:500; Chemicon, CA, USA) against astrocytes, and a polyclonal antibody against the water channel aquaporin 4 (1:500; Sigma, MO, USA) followed by corresponding secondary antibody incubation and detection by the Vectastain ABC Elite kit reagents (Vector Laboratories, CA, USA) with diaminobenzidine as substrate according to the manufacturer's protocol.

Double labeling immunofluorescence was performed for GFAP and AQP4 using fluorochrome-conjugated secondary antibodies instead of the ABC kit reagents (Alexa 488-conjugated goat anti-mouse IgG for GFAP; Alexa 594-conjugated goat anti-rabbit IgG for AQP4; both from Molecular Probes, Eugene, OR, USA).

Results

Array analysis: Overall quantitative aspects

In the array analysis, we found a roughly similar number of significantly regulated genes in ON (67 out of

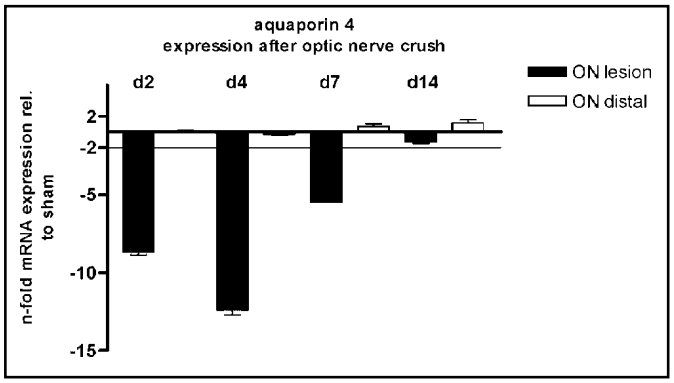

Fig. 3. Aquaporin 4 gene expression changes in the optic nerve (ON) lesion and distal nerve stump relative to sham-operated nerves. Each Q-PCR was repeated twice at days 2, 4, 7 and 14 post nerve crush and the mean and standard error of the mean were calculated.

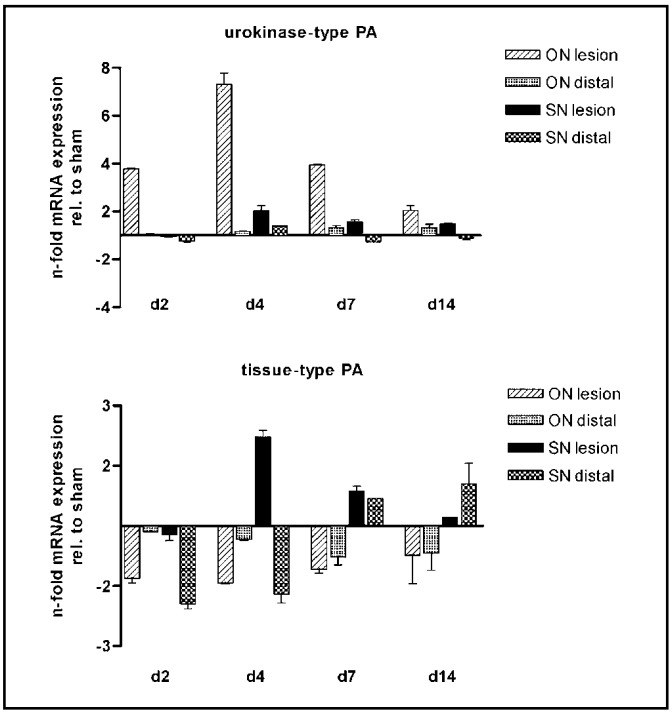

Fig. 4. Gene regulation for the urokinase- and tissue-type plasminogen activators (PA) were quantified at the lesion site and the distal nerve stump at days 2, 4, 7 and 14 post sciatic nerve (SN) and optic nerve (ON) crush by Q-PCR. The mean and standard error of the mean of duplicate Q-PCR results are shown in n-fold relation to sham-operated nerves, respectively.

1185 genes, corresponding to 5.7 % of all genes represented on the array filter) and SN lesions (69/1185 genes, 5.8 %), respectively.

A total of 19 genes, corresponding to 28 % of all gene expression changes, showed an identical regulation pattern in ON and SN injury (Fig. 1, Table 1). Upregulation was found in more than 75% of these uniformly regulated genes (15/19) and comprised genes related to inflammation (Interleukin-18, cytosolic phospholipase), chemotaxis (CXCR4, C5a receptor), and proteolysis (cathepsins B, D, and L). Only four genes were uniformly downregulated in both paradigms. In line with previous findings, downregulated genes mainly represented myelin genes [13] as well as the juxtaparanodal voltage gated potassium channel [14] thereby validating our experimental approach.

In contrast to the uniformly regulated genes, system-specific expression changes were identified for 48 genes in ON and 50 genes in SN crush lesions (Fig. 1, Table 2). Among these differentially regulated genes, upregulation predominated in SN lesions (43 up, 7 down) whereas many more downregulated genes were identified in the ON (23 up, 25 down). System-specific gene regulation was most frequently observed in the categories of signal transduction, trafficking and metabolism, but also prominent for some genes related to growth and differentiation, proteolysis, and stress response (Table 2).

System-specific gene regulation studied by Q-PCR

Representative genes showing differential regulation in ON and SN tissue were further studied by means of Q-PCR. Among the ON-specific regulated genes particular strong upregulation was found for TRH and somewhat weaker induction for NPY and IGF-I (Fig. 2). Of note, Q-PCR confirmed reciprocal induction of IGF-I and IGF-binding protein 3 (IGFBP-3) in ON and SN crush injury, respectively, with 4-5 fold induction of IGF-I in the optic nerve lesion whereas IGFBP-3 was selectively induced in the SN model. In addition to the crush site, we also analyzed mRNA expression in the distal nerve stump. We found that all gene changes in the ON were restricted to the crush site whereas gene regulation in the SN to some extent involved the distal nerve stump as well.

The glial cell line-derived neurotrophic factor (GDNF) receptor alpha was studied as a growth factor-related gene showing exclusive induction in the SN crush model. Q-PCR analysis (Fig. 2) confirmed a 6-fold upregulation at the crush site. GDNF receptor alpha upregulation occurred to a similar extent in the degenerating distal nerve stump. In contrast, the cytoskeleton-related gene thymosin-beta 15 and IGFBP-3 as further examples of SN-specific gene regulation were exclusively induced at the crush site but remained absent from the distal nerve stump.

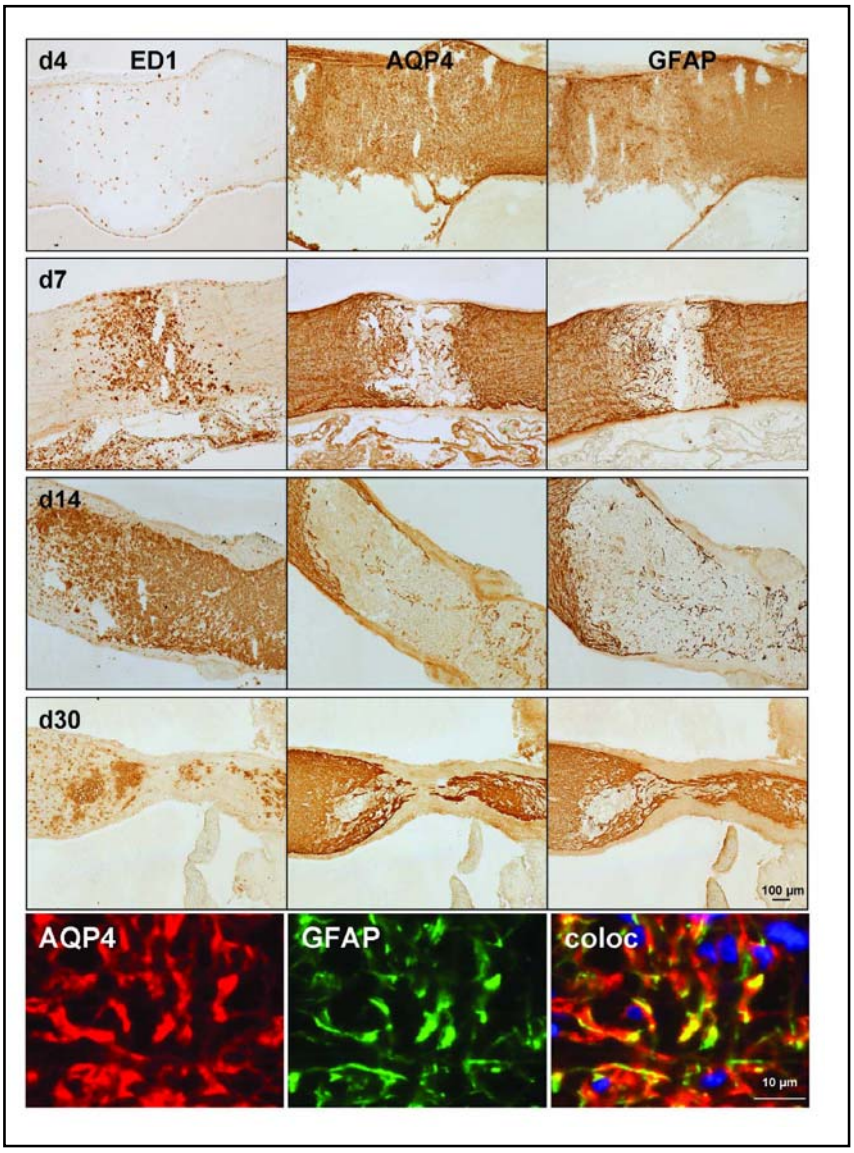

Fig. 5. Immunohistochemistry of the crushed optic nerve showed oedematous swelling at the lesion site at day 4 after injury with strong infiltration by ED1 positive activated macrophage/microglia at days 7 to 14, subsiding at day 30. At the inflamed lesion site aquaporin 4 (AQP4) reactivity was strongly decreased and paralleled by the loss of glial fibrillary acidic protein (GFAP) positive astrocytes. At day 30 AQP4 and GFAP positive astrocytes reappeared. Serial adjacent cryostat sections (10 µm). Bottom row: Immunfluorescence double labeling showing coexpression of AQP4 and GFAP in astrocytes at a perilesional site of an optic nerve (day 4 after injury).

Constitutive expression of the water channel aquaporin 4 (AQP4) was essentially restricted to the ON with a more than 1000-fold higher expression in unlesioned ON compared to only marginal expression in the naïve SN. After crush injury no significant change occurred in the SN. Contrastingly, in the ON time course analysis by Q-PCR (Fig. 3) showed strong downregulation of AQP4 in the ON lesion at day 4 with gradual normalization within the subsequent 10 days.

The array analysis furthermore suggested contrasting patterns of tPA and uPA expression with tPA induction being specific for SN and uPA induction exclusively found in ON lesions (Table 2). In accordance with the array results, Q-PCR revealed strong upregulation of uPA mRNA in ON crush, but not in SN lesions (Fig. 4). Conversely, tPA mRNA was increased in the SN, whereas it was actually downregulated in the ON lesion. Further time course analysis showed maximum induction of tPA in the SN and uPA in the ON lesion site at day 4 with a gradual return to normal levels 14 days after injury. As for the other ON-specific gene regulations, uPA induction in the ON was restricted to the crush site at all time points. Contrastingly, in the SN model, tPA also showed delayed upregulation in the distal nerve stump at 7 and 14 days after injury. The plasminogen activator inhibitor PAI-1 was constitutively expressed in the SN without significant regulation after injury, whereas it was upregulated at the lesion site in the ON (Table 2).

Immunohistochemistry for aquaporin-4

To corroborate our mRNA data at the protein level, we performed immunohistochemistry for AQP4 using a specific polyclonal antibody against rat AQP4 on longitudinal cryostat sections of crushed ON (Fig. 5). In unlesioned control ON, AQP4 immunoreactivity was mainly, if not exclusively, associated with astrocytes

identified by anti-GFAP antibody labeling on serial sections. AQP4 expression by astrocytes was confirmed by double labeling immunofluorescence showing extensive coexpression of AQP4 and the GFAP marker. In crush-injured nerves, AQP4 staining disappeared in the lesion site at days 4 and 7, paralleled by the disappearance of astrocytes and strong infiltration of macrophages seen on serial sections stained for the astrocyte marker GFAP or the macrophage antigen ED1. At days 14 and 30, AQP4 immunoreactivity was reexpressed on some astrocytes within the lesion site.

Discussion

Our comparison by cDNA array, Q-PCR, and immunohistochemistry represents the first systematic approach to the molecular response at the lesion site following crush injury to central vs. peripheral nerves in vivo. Previous gene expression studies have addressed gene expression in models of CNS injury without comparison to the corresponding PNS lesion condition [15]. Other genomic studies have mostly focused on molecular responses of neuronal cell bodies [1, 16-18] or distal nerve segments undergoing Wallerian degeneration [19-21], but have largely neglected the primary site of crush injury. This contrasts to experimental and clinical observations indicating that one major barrier to regenerating axons in CNS injury presents already at the primary injury site and that experimental strategies promoting axon growth across the injury site result in unimpeded axon extension through the distal fiber tract [22]. The comparison of molecular responses at the lesion site between the central and peripheral nervous system might therefore reveal new targets of therapeutic intervention enabling axon regeneration in CNS injury.

Regulation of inflammatory genes

Following localized injury to nerve fiber tracts, anterograde Wallerian degeneration of the nerve segment distal to the lesion occurs rapidly in the PNS but slowly and incompletely in the CNS. This is accompanied by early infiltration of the distal nerve stump by macrophages in the PNS whereas macrophages are only scarcely recruited during Wallerian degeneration in the CNS [23, 24]. In contrast, macrophage reactions at the primary lesion site occur vigorously and to a similar magnitude in both the CNS and PNS [25]. Accordingly, our present study revealed a similar extent of inflammatory gene induction at the lesion site in the crushed ON and SN with common upregulation of the CD53 antigen, C5a receptor and platelet-activating factor receptor involved in the chemoattraction and activation of leuko- and phagocytes. In line with previous results [26] we also found the proinflammatory cytokine IL-18 commonly upregulated, which is presumably caused by the infiltration of IL-18-positive macrophages. As an important exception from the otherwise similar induction of inflammatory gene products, we have previously shown that the cytokine-like protein osteopontin is exclusively expressed by macrophages in CNS, but not PNS crush lesions [27]. It is thus conceivable that at least at the time point studied in our present study there are more subtle differences in the molecular programming of lesion-associated macrophages in the CNS vs. PNS which might nevertheless have implications for the inhibition of axon outgrowth in CNS injury.

Aquaporin 4 downregulation in the optic nerve lesion

The water channel AQP4 was strongly expressed by astrocytes in the naïve ON in contrast to an only marginal expression in the SN. Crush injury induced a profound downregulation of AQP4 restricted to the ON lesion with lowest levels reached at day 4 and gradual normalization thereafter. This was paralleled by the disappearance of GFAP-positive astrocytes (Fig. 3) from the ON lesion. Thus, the loss of AQP4 occurred as a consequence of severe local CNS injury leading to a loss of astrocytes from the lesion site. These findings are in line with previous studies showing transient disappearance of astrocytes and downregulation of AQP4 in various CNS lesion models [28, 29]. In spinal cord injury, AQP4 downregulation was suggested to worsen vasogenic oedema formation [30]. On the other hand, AQP4 knock-out mice had a functional benefit after spinal cord injury [31] suggesting a neuroprotective effect of AQP4 downregulation. In human glioma AQP4 expression was associated with vasogenic oedema formation but seemed not neuroprotective [32]. Our immunohistochemistry at day 30 showed that the scarring ON lesion was partly repopulated by AQP4- and GFAP-positive astrocytes. Interestingly, recent studies point to a role of AQP4 in astrocyte migration and glial scar formation by facilitating cytoskeleton cell changes [33, 34]. AQP4 expression is also lost in severe necrotic and cavitary multiple sclerosis and infarct lesions [35]. Recently AQP4 autoantibodies were discovered as a diagnostic marker for Neuromyelitis optica [36] and passive transfer studies suggested a critical relevance of the autoantibodies for disease

pathogenesis [37, 38].

Growth factor related gene regulation

Apart from a similar upregulation of transforming growth factor (TGF)-beta in both systems, we found a selective upregulation of the glial cell line-derived neurotrophic factor receptor (GDNFR) alpha in the SN but not ON model. Previous studies have shown that GDNFR-alpha becomes trophically active only by TGF-beta mediated transport to the cell membrane [39]. In other systems TGF-beta 1 increases on a transcriptional level the expression of neurotrophic insulin-like growth factor (IGF-I) and its binding proteins (IGFBP) [40]. Interestingly, we found that these genes were differentially regulated with selective upregulation of IGFBP-3 in the SN and of IGF-I in the ON crush lesion. Beyond the modulation of IGF activities IGFBPs exert pleiotropic IGF-independent signaling effects via integrin receptors and are involved in transcriptional regulation [41]. Of note, IGFBP-3 downregulates the mRNA expression of uPA independently of the IGF-I receptor [42]. In contrast, IGF-I upregulates uPA [43]. The differential regulation patterns suggest crossregulatory effects of growth factors on proteases involved in tissue remodeling after nerve injury which might at least partially explain the differential regulation of tPA and uPA observed in our present study (Fig. 4).

Differential plasminogen activator regulation

Proteolysis by activation of plasminogen [8] and matrix metalloproteinases [44] is an essential prerequisite for growth cone extension and axonal outgrowth through regeneration inhibiting components of the injury site, adhesive inflammatory cells, debris and structurally altered extracellular matrix. Our results reveal differential regulation of the two main plasminogen activators with a selective upregulation of uPA in the ON and tPA in the SN lesion. In the SN tPA is expressed in regenerating axons and Schwann cells and axonal regeneration is delayed in knock out mice for tPA, uPA and plasminogen [45, 46]. In line with our results it has been shown that beneficial fibrinolysis after SN crush is mainly tPA mediated [47]. Of note, in these studies the expression of PAI-I remained unchanged which further underscores our findings of a constitutive PAI-I expression in the SN without significant regulation. Our results furthermore showed a delayed upregulation in the distal nerve stump at days 7 and 14 after injury which probably reflects the proceeding of the regrowing axon. Contrastingly, in the ON we found a mild downregulation for tPA while PAI-I and uPA were upregulated with restriction to the lesion site. This differential regulation pattern might be mediated by the p75 neurotrophin receptor which is expressed in normal and crush injured ON [48, 49] and was recently shown to downregulate tPA independent of neurotrophin signaling while it upregulates PAI-I expression and leaves uPA activity unaffected [50]. Evidence from facial nerve axotomy likewise points to the induction of uPA rather than tPA in CNS injury [51] but the specific role of uPA in CNS nerve regeneration has not been comprehensively studied. Numerous findings in heart [52], vessels [53], skin [54], and other peripheral organs [55] indeed suggest differential involvement of uPA and tPA in wound healing with fibrogenic responses mainly exerted via uPA. Thus the differential expression of uPA and tPA might be relevant for tissue remodeling following CNS and PNS injury and may represent a new therapeutic target for recently developed specific uPA inhibitors [56].

Local upregulation of neuronal mRNA

Unexpectedly, our array analysis revealed lesion-associated mRNA upregulation for genes of likely neuronal origin such as NPY, TRH and thymosin-beta 15. As the neuronal cell bodies are remotely located these mRNAs must be expressed in either glial cells or axons. The latter would be in line with several demonstrations of mRNA transportation from the neuronal soma to the axon and extrasomatic mRNA translation in the axon [57]. The presence of local mRNA bypasses the somatic production of too complex or toxic proteins and their rather slow axonal transport. Furthermore, injury-induced local mRNA translation is involved in retrograde signaling from the lesion to the neuron [58] and growth cone formation as a prerequisite for axon regeneration requires active local protein synthesis and proteolysis [59, 60].

Our array analysis revealed a more extensive induction of mRNAs associated with transcription and translation including ribosomal proteins in the SN in line with immunolocalization studies showing almost undetectable ribosomal protein levels in axotomized adult ONs in contrast to abundant levels in cut peripheral sensory axons [60]. We found that thymosin-beta 15 (Tb15) was specifically increased in the SN lesion site. The three thymosin isoforms beta 4, 10 and 15 are small actin-binding proteins with multiple cell type-specific functions. Tb15 is the isoform most specific to the nervous system [61]. In adult rats, Tb15 is found in postmitotic migratory neurons and is induced after cerebral ischemia presumably reflecting axonal sprouting [62]. Interestingly, adult Zebrafish retinal ganglion cells that in contrast to

mammals restore functional axon connections after ON lesions, showed thymosin-beta mRNA at the lesion site with a maximal expression at day 4 and a distal shift along the axon which correlates temporally with the progression of growth cones and did not colocalize with macrophages [63]. Furthermore, studies in the mollusc Aplysia indicate that thymosin-beta is locally synthesized in regenerating axons following axotomy [64]. Thymosin-beta promotes axonal branch formation in developing rat brain [65]. In development, suppression of thymosin-beta by siRNA reduces branching [65] and results in malformation of axonal tracts in Zebrafish [66] while it enhances elongation of outgrowing neurites in young snail neurons [67]. The contradictory effects observed in developing and regenerating neurons might relate to species- or model-dependent differences but could also indicate that an adapted local regulation of thymosin-beta is required for optimal axon regeneration. Recently extracellular ligands such as neurotrophins and myelin components were shown to locally up- or downregulate axonal mRNAs in adult axons [68]. Our results of a specific Tb15 upregulation in the SN lesion underscores its relevance in adult rat axonal regeneration and is probably of local axonal origin.

Our finding of a strong local upregulation of neuropeptides such as NPY and TRH in the ON lesion further underscores an active participation of axons in the local gene expression at site of nerve fiber injury. TRH and analogues were shown to have neuroprotective properties in traumatic brain and spinal cord injury and to modulate posttraumatic mRNA expression. Of note, TRH has been reported to downregulate aquaporin 4 mRNA [69]. The cellular source of TRH in the lesioned CNS is unknown. Retinal cells and ON axons of amphibians are TRH immunoreactive [70], but little is known about TRH expression in mammalian ONs. Although TRH mRNA expression has been observed in astroglial cells of adult rat spinal cord [71] our immunohistochemical results indicate that astrocytes are probably not the source of TRH expression because they progressively disappeared from the crush lesion. Thus, the TRH mRNA in the ON lesion is likely to be of axonal origin.

Conclusion

Our comparative approach to lesion-associated gene expression in optic and sciatic nerve crush injury highlights a potential role of differential growth factor, water channel, and plasminogen activator gene expression for an either hostile or supportive microenvironment for axonal regeneration. An unexpectedly strong participation of putative axonally localized mRNAs was found and represent potential therapeutic targets for local RNA interference recently shown to be effective in mammalian peripheral axons [72]. The functional and potential therapeutic implications of local gene regulations at sites of nerve injury are still incompletely understood and warrant further study.

Abbreviations

AQP4 (aquaporin 4); CNS/PNS (central/peripheral nervous system); GDNFR (glial cell line-derived neurotrophic factor receptor); GFAP (glial fibrillary acidic protein); IGF (insulin-like growth factor); IGFBP (IGF-binding protein); IL (interleukin); ON (optic nerve); PAI (plasminogen activator inhibitor); Q-PCR (quantitative real-time polymerase chain reaction); SN (sciatic nerve); tPA/uPA (tissue/urokinase-type plasminogen activator); TGF (transforming growth factor); Tb15 (thymosin-beta 15); TRH (thyrotropin-releasing hormone); NPY (neuropeptide Y).

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