Proteomics of the injured rat sciatic nerve reveals protein expression dynamics during regeneration

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Running title: Proteomics of the regenerating peripheral nerve

number of text pages including tables: 20
number of words in abstract (178), introduction (472) and discussion (1500)
number of figures: 4
number of tables: 2

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ABSTRACT

Using proteomics, we investigated the temporal expression profiles of proteins in rat sciatic nerve after experimental crush. Extracts of sciatic nerves collected at 5, 10 and 35 days after injury were analyzed by two-dimensional gel electrophoresis and quantitative image analysis. Of the ~1500 protein spots resolved on each gel, 121 showed significant regulation during at least one time point. Using cluster analysis these proteins were grouped into two expression profiles of down-regulation and four of up regulation. These profiles mainly reflected differences in cellular origins in addition to different functional roles. Mass spectrometric analysis identified 82 proteins pertaining to several functional classes, i.e., acute phase proteins, antioxidant proteins and proteins involved in protein synthesis/ maturation/ degradation, cytoskeletal (re)organization and in lipid metabolism. Several proteins not previously implicated in nerve regeneration were identified, e.g., translationally-controlled tumor protein, annexin A9/31, vitamin D-binding protein, α-crystallin B, α-synuclein, dimethylargininases and reticulocalbin. Real-time PCR analysis of selected genes showed which were expressed in the nerve vs the dorsal root ganglion neurons. In conclusion, this study highlights the complexity and temporal aspect of the molecular process underlying nerve regeneration and points to the importance of glial and inflammatory determinants.

KEYWORDS:

Regeneration / Two-dimensional gel electrophoresis / quantitative PCR / MALDI-TOF/TOF / Mass spectrometry
INTRODUCTION

Injury to a peripheral nerve induces cellular and molecular changes in the injured neurons and in the microenvironment of their axon stumps which together support axonal regeneration (for a review see (1-3). During the first week after injury, Wallerian (anterograde) degeneration occurs whereby distal axons and their myelin sheaths are phagocytosed by invading macrophages (4). Schwann cells, deprived of axonal contact play a key role in regulating macrophage recruitment (5,6). Moreover, following injury, Schwann cells proliferate and migrate to provide a growth-permissive substrate onto which axons regenerate through the expression of cell adhesion molecules and growth factors (7-10). Thus, the molecular and cellular changes that underly peripheral nerve regeneration are complex and identification of proteins involved will contribute significantly to our understanding of neuroregeneration.

To study the molecular mechanisms that govern regeneration of the sciatic nerve, global gene expression profiling of dorsal root ganglia (DRG) and sciatic nerve has been employed recently. These studies revealed that the regeneration-associated genes can be categorized into several groups, including cytoskeletal proteins, neurotransmitter metabolizing enzymes, neuropeptides, growth factors, and signal transduction molecules in the DRG (11-14), as well as genes encoding proteins involved in inflammation, proliferation and myelination in the nerve (15-17).

Proteomic analysis of the protein expression pattern of the regenerating nerve is in many ways additive to gene expression profiling. It analyzes proteins and protein isoforms directly, and detects proteins derived from the nerve, neuronal cell bodies, and also from external sources such as the circulation. A quantitative and qualitative assessment of the proteome can be achieved by combining two-dimensional gel electrophoresis (2DGE) generating a high resolution, quantitative protein expression map, and mass spectrometry (MS) to identify regulated proteins (18,19). Previous metabolic labeling experiments employing 2DGE (20-23) indicated several proteins regulated by nerve injury of which only one up-regulated protein, apolipoprotein E (APOE), was identified (23).

In the present study, using 2DGE, we quantitatively analyzed the temporal protein expression profiles in sciatic nerve segments distal to a crush injury versus contralateral control segments. Peptide sequencing by tandem MS led to the identification of proteins involved in a wide range of functions related to nerve injury and recovery. Our results underscore the role of Schwann cells and macrophages as well as circulatory proteins in the immediate response to injury as well as throughout the course of the regeneration process.
MATERIALS AND METHODS

Animals and Surgery. All surgical and animal care procedures were carried out according to the local guidelines of the experimental animal committee. Adult male Wistar rats (±220g) (Harlan, The Netherlands) were housed in group cages, maintained on a 12h light/12h dark cycle and food and water were available ad libitum. Rats were anaesthetized using 1.8% isoflurane (in 0.3 l/min O₂; 0.6 l/min N₂O). The sciatic nerve was exposed at mid-thigh level and crushed for 30 sec by closing a hemostatic forceps with grooved jaws. At the distal rim of the lesion a suture (Ethicon 10.0; Ethicon, Norderstedt, Germany) was placed through the superficial epineurium to identify the precise position of the crush site. Animals were sacrificed at 5, 10 and 35 days (n=6 per time point) after surgery by decapitation. All animals were sacrificed at the same hour of day to prevent the possibility that changes in protein levels or gene expression can be attributed to circadian rhythm. After removal of the sciatic nerve, nerve segments of 1 cm (~10 mg w/w) proximal and distal of the crush site, and a comparable piece of the contralateral control nerve were dissected and stored at -80°C. Dorsal root ganglia at levels L4, L5 and L6 were dissected, frozen and stored at -80°C.

Sample preparation for 2DGE. All chemicals were electrophoresis grade from Sigma, unless stated otherwise. Nerve segments (1 cm) were chopped on an ice-cold glass plate into 1 mm pieces and transferred into an eppendorf tube on ice and homogenized in 250 µl buffer (40 mM Tris-base, 0.5% Triton X-100, 10% glycerol, 5 mM EDTA, 65 mM DTT and 2 mM pefabloc) using a plastic electronic potter device. The homogenate was left on ice for 15 min followed by centrifugation at 4°C. The supernatant was transferred to a separate tube to which urea (8M), CHAPS (4%) and IPG buffer (3-10 NL, 0.5%, Amersham Biosciences) were added yielding a volume of ~400 µl. A Bradford procedure was used to determine the protein concentration in the samples.

Two-dimensional gel electrophoresis and protein staining. Immobilized pH gradient (IPG) gels (Immobiline DryStrip 18 cm, 3–10 NL, Amersham Biosciences) were rehydrated with 370 µl of solubilized sample (~350 µg) and focused overnight (O/N) (65 kVh, 20°C). Immediately after focusing, IPG strips were wrapped in plastic foil and stored at -80°C. Prior to SDS-PAGE, IPG strips were equilibrated in 6 M urea/ 2% SDS/ 1% DTT/ 50 mM Tris, pH 8.4/ 30% glycerol for 15 min, followed by equilibration in 6 M urea/ 2% SDS/ 2.5% iodoacetamide/ 50 mM Tris, pH 8.4/ 30% glycerol for 15 min. The second dimension separation was done O/N using the Isodalt System (Amersham Biosciences) in 1.5 mm 11% gels (Duracryl, Genomic Solutions, CA, USA) run at 25 mA per gel at 15°C. Immediately after electrophoresis, gels were fixed in 5% acetic acid/ 50% methanol for 1 hr and silver stained (26). Preparative gels containing ~700 µg of protein were fixed overnight in 40% ethanol/2% acetic acid. Subsequently, gels were washed three times for 1 hr in 2% acetic acid and stained with sypro-ruby (Biorad) O/N. After staining, gels were washed for 30 min in 10% methanol/7% acetic acid followed by washing in water. The gels were scanned in a laser scanner (Fuji) at a wavelength of 473 nm. Subsequently, the sypro-ruby stained gels were stained O/N with colloidal Coomassie brilliant blue (G250 in 50% ethanol, 3% phosphoric acid).
Quantitative analysis of 2D protein maps and cluster analysis of regulated proteins. The silver stained 2D gels were scanned using a densitometer (Biorad) and comparative spot pattern analysis across multiple gels was accomplished using PDQuest image analysis software (version 7.0, Biorad). Statistical comparisons (analysis sets using a student t-test, p<0.05) were made between replicate groups, comprising four to six gels of sufficient quality per time point after nerve crush (contralateral control, N=4; 5 days after crush, N=4; 10 days after crush, N=6; 35 days after crush, N=4). Analysis of protein expression profiles in the proximal nerve was carried out at one time point (at 5 days after injury, N=3). After transformation of the normalized protein spot intensities by taking the log value of the ratio of 5d, 10d and 35d post lesion and control, protein expression profiles were clustered by means of a Self Organising Map (SOM) algorithm (24,25). To this end, Genecluster 2.0 (http://www-genome.wi.mit.edu/cancer/software/genecluster2/gc2.html), developed by the Whitehead Insitute/MIT Center for Genome research, was used.

Protein identification by mass spectrometry. The protein spots of interest were manually excised from the sypro-ruby/coomassie double stained 2D gels, washed, and in-gel digested with trypsin (porcine modified, Promega, at 5 ng/µl) O/N at 37°C, as described (26) with some minor modifications. Tryptic peptides were extracted from the gel pieces in 1 volume 0.1% TFA while vortexing for 5 min followed by sonication for 5 min. Crude digest mixtures were concentrated and desalted using µC18 ziptips (Millipore). Subsequently the bound peptides were eluted in 5 µl 5% formic acid in 60% methanol for electrospray MS or in 1.5 µl matrix (5 mg α-cyano 4-hydroxycinnamic acid / ml in 50% acetonitrile/ 0.1% TFA) for MALDI-MS. For MS, the samples were loaded in a nanoelectrospray capillary (pulled from borosilicate capillary GC 100F-10 with a micro-capillary puller) or spotted onto a matrix-assisted laser desorption ionization (MALDI) target.

Tandem mass spectrometry was performed on the 4700 Proteomics Analyzer (Applied Biosystems). This MALDI TOF/TOF® instrument consists of a MALDI source with a 200 Hz neodinium YAG laser operating at 355 nm. MS and MS/MS were performed as described previously (27). Both MS and MS/MS spectra were searched against the NCBI database, using mascot software from matrix science (www.matrixscience.com) to identify the proteins. The protonated trypsin auto-digest products at m/z 842.510 and 2211.104 were used for internal calibration of the MALDI-MS spectra. The MALDI-MS resolution for the peptides was ~10000, and the mass accuracy 0.01-0.02 Da. The MS/MS resolution was 3000-6000. No internal standard was used for calibration of the MS/MS spectra. Therefore, for database searching of MS and MS/MS data, the mass tolerance was set at 0.03 Da and 0.3 Da, respectively.

RNA isolation and cDNA synthesis. Total RNA was isolated from sciatic nerve distal to the crush lesion, from sciatic nerve of the contralateral side, and from DRG L4-6 from the lesioned and contralateral side of each animal (n=3 per time-point). RNA isolation was performed (28), however omitting sarcosyl in the GTC solution. Sarcosyl was added after homogenization to a final volume of 0.05%, followed by an additional chloroform extraction. RNA was checked by gel electrophoresis and by photospectrometry. For each time-point equimolar amounts of RNA of 3 animals were pooled, then split in two batches (A an B), which were processed for independent DNase-I treatment.
(20 U, Boehringer Mannheim) and subsequent cDNA synthesis. After DNase treatment, RNA was checked for integrity by gel electrophoresis. Of each DNase treated RNA sample 5 µg total RNA was primed with 100 pmol random hexamers (Eurogentec) and reverse transcribed with MMLV superscript (300 U, Gibco BRL) according to the protocol of the manufacturer. Equal amounts of A and B cDNA were mixed and stored at -20 °C.

**Quantitative PCR.** Real-time quantitative PCR (qPCR) was performed on a Perkin-Elmer ABI PRISM 7700 sequence detection system. PCR conditions and SYBR green reagents (SYBR core reagent kit; Applied Biosystems) were used in a reaction volume of 10 µl using transcript-specific primers (300 nM) on cDNA (corresponding to ~20 ng total RNA). Three reference genes were selected for the two tissue types analysed by performing a variance analysis of the Cycle Threshold (Ct) value of 10 genes known to vary little between tissue types and treatments. The average Ct value of HPRT, Sr317 and NSE was used to normalize the sciatic nerve data. For DRG, L37a, β-Actin and GAPDH were used.

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RESULTS

Quantitative analysis of 2D protein maps and cluster analysis of regulated proteins

To study regeneration of peripheral nerve after injury, proteome analysis was performed at 5, 10 and 35 days following sciatic nerve crush. In this time-frame various processes occur (reviewed by (2,29)) that are essential for successful regeneration. The injury-induced processes include Wallerian degeneration/demyelination in the first 2 weeks (30), Schwann cell proliferation peaking at day 4 after crush (17), macrophage infiltration starting at 3-4 days and peaking at 7 days after crush (17,30), axonal regeneration (the first neurites cross the distal boundary of the crush site between 2-5 days after crush leading to functional recovery at 3-4 weeks) and remyelination starting 1-2 weeks after nerve crush (17).

The sciatic nerve proteome contained about 1500 detectable protein spots per silver-stained 2D gel. Figure 1 shows an example of a 2D gel of a control nerve (1A) and nerve distal to the crush site (1B). Computer-assisted comparative analysis of the stained protein maps of distal nerve segments at 5, 10 and 35 days after nerve crush and contralateral control segments, revealed a total of 74 spots that were significantly increased in intensity and 47 spots that were significantly decreased (N=4 for the control, 5 and 35 days after crush and N=6, 10 days after crush; p<0.05) at least one of the timepoints. No significant differences in spot intensities were observed between the proximal nerve segment (at 5 days after injury, N=3) and the contralateral control segment. The differentially expressed proteins were clustered using self-organizing maps into six categories of distinct temporal regulation profiles (Table I). Expression profiles 1 (12 spots) and 2 (35 spots) contained proteins of decreased intensities and differed in the timing and extent of down-regulation and recovery to pre-injury levels. Expression profiles 3 (23 spots), 4 (19 spots), 5 (22 spots) and 6 (9 spots) contained up-regulated proteins that differed both in the extent and timing of up-regulation. Figure 2 shows for each expression profile, a selected spot in zoom sections of the 2D gels, as examples of the different temporal regulation clusters.

Identification of differentially regulated proteins after nerve injury.

To identify proteins with altered expression in injured sciatic nerves, the spots were excised from the silver-stained gels and from gels that were double stained with sypro-ruby and colloidal coomassie blue. The double-stained gels gave a higher recovery of tryptic peptides than the silver stained gels, which facilitated the identification of lower abundant proteins. The extracted tryptic peptides were analyzed using MS and database searching of the peptide mass fingerprints. Most protein identities were further confirmed by sequence analysis using tandem MS (Table I and Table II for details of the protein identification). A substantial number (82 out of 121) of the regulated proteins could be identified and they are categorized according to their function in Table I. With respect to the relative contribution of different functional groups of proteins, the proportion of metabolic proteins is relatively high in the group of down-regulated proteins whereas the proportion of acute-phase proteins and proteins involved in protein maturation and degradation and lipid metabolism is relatively high in the group of up-regulated proteins (Fig. 3).
Down-regulated proteins associated with Schwann cell dedifferentiation and Wallerian degeneration. In cluster 1, protein levels were strongly down-regulated peaking at 10 days after crush and low protein levels still persisted at 35 days after injury (Table I). Cluster 1 contained two proteins normally expressed in myelinating Schwann cells, namely CRAB, a small heat shock protein (HSP) abundant in myelin and PERAX, that were down-regulated by ~66-fold and ~9-fold, respectively, after nerve crush.

Expression profile 2 included proteins that were moderately down-regulated at 5 and 10 days after crush and exhibited recovered expression at 35 days after nerve injury. Several neuronal proteins such as CRMP2, DDH2, phosphatidylethanolamine binding protein (PEPB), α-synuclein (SYNA) and enolase γ (ENOG) clustered in expression profile 2 (Table I), which is consistent with Wallerian degeneration and subsequent axon regeneration.

Up-regulated proteins associated with inflammation, Schwann cell activation, migration and macrophage infiltration. Circulatory/acute phase proteins consistent with breakdown of the blood-nerve barrier and inflammation were well represented in expression profiles 3 and 5, of early/transient, and more sustained up-regulation, respectively (Table I). These proteins included haptoglobin (HP), hemopexin (HPX), alpha1-macroglobulin (APLHA1M), vitamin D binding protein (VTDB), transferrin (TTHY) and the fibrinonogen α and β chains (FGA and FGB). TTHY was detected at a molecular weight of ~39 kD which is larger than the calculated molecular weight of 15.7 kD.

Expression profile 4 of strong up-regulation that peaks on day 5 after crush contained the galactoside-binding lectin, galectin-3 (GAL3, 15-fold induction at day 5), that is expressed in Schwann cells and macrophages upon injury (31). Another strongly up-regulated protein is the lysosomal thioprotease, CATB, that is expressed in macrophages and microglia (32-34). The most abundant 22 kD form (16-fold induction at 10 days post-crush) clustered in expression profile 4 whereas the 26.5 kD form (77-fold induction peaking at 5 days post-crush) clustered in expression profile 6. Consistent with Schwann cell migration, proteins involved in cytoskeletal reorganization, i.e., tropomyosin γ (TPMsk3) and tropomyosin α (TPM4), also clustered in expression profile 4 (together with GAL3).

Furthermore, expression profile 4, as well as 3 and 5, contained several proteins involved in protein synthesis and maturation, such as 40S ribosomal protein SA (RPSA), elongation factor 1β (EF1β), two protein disulphide isomerases (PDI/ERP59 and PDI/ER60), endoplasmic reticulum (ER) protein 29 (ERP29), and the multiple EF hand, low-affinity calcium-binding proteins reticulocalbin (RCN2), and calumenin (CALU). The up-regulation of these proteins may reflect Schwann cell activation and proliferation.

Differential up-regulation of lipid binding proteins. We found increased levels of two isoforms of APOA1, APOA4, APOD and four isoforms of APOE after injury. Interestingly, the temporal protein regulation profiles were APO-type specific, with APOA1 variants clustering in expression profile 5 of mild and sustained up-regulation, APOA4 in profile 3 of mild and transient up-regulation, and APOE isoforms and APOD clustering in profile 6 of strong and sustained up-regulation. APOE was detected as a row of 4 spots. Like in humans (35), the train of 3 adjacent APOE spots in our 2D gels may correspond to allelic variants as well.
Shifts in abundancy of thioredoxin peroxidase isoforms. We detected altered abundance of variant forms of the antioxidant enzymes proteins thioredoxin peroxidase (TDPX) 1 and TDPX2 (36); (37). The acidic forms of both TDPX1 and TTDPX2 were decreased by two-fold, whereas the basic form of only TDPX2 was increased by four-fold. This shift in abundancy of TDPX2 variants possibly indicates a change in oxidation state (36).

Analysis of gene expression of altered proteins identified using proteomics,
Gene expression analysis of some selected proteins (indicated in Table I) was performed to distinguish between the origin of expression in relation to the neuronal (cell bodies in the dorsal root ganglion) versus peripheral sites (Schwann cells and other cells present or invading the nerve). Quantitative PCR showed that most of the selected genes exhibited regulation in the nerve and (almost) no regulation in the DRG (Fig. 4). For example, qPCR analyses confirmed the up-regulation of galectin-3 (GAL3), the apolipoproteins APOD and APOE, cathepsin B (CATB), calmodulin (CALM), the tropomyosins TPMsk3 and TPM4, voltage-dependant anion channel 1 (VDAC1) and translationally-controlled tumor protein (TCTP) as well as the marked down-regulation of α-crystallin B (CRAB) and peraxin (PERAX) (Fig. 4). Thus, the changes in gene expression of these proteins match the observed changes in protein expression, and their regulation is probably caused by peripheral cells, most likely Schwann cells and invading macrophages. The mRNA levels of collapsin response mediator protein-2 (CRMP2), dimethylargininase 2 (DDH2) and heat shock protein 27 (HSP27) did not show any changes in the nerve although they showed profound down-regulation at the protein level. This is in agreement with the fact that these proteins are of neuronal origin and synthesized in the DRG. We did not detect any mRNA for APLHA1M, VTDB, TTHY and α1-antitrypsin (ATA1) in the sciatic nerve, which is in agreement with the fact these proteins are derived from the circulation.
DISCUSSION

Using proteomics, we investigated the effect of peripheral nerve injury on the global protein expression patterns in sciatic nerve at 5, 10 and 35 days after injury. We performed cluster analysis of the temporal expression profiles. In general, the clusters of strong up- or down-regulation (clusters 1, 4, and 6) mainly reflected changes in protein synthesis locally in the nerve (presumably in Schwann cells and invading macrophages) and the clusters of mild regulation (2, 3 and 5) revealed mainly alterations in levels of proteins originating from distant sites (e.g., the nerve or circulation). The identified regulated proteins function in diverse pathways such as inflammation, secretory protein synthesis, lipid metabolism and cytoskeletal reorganization. These proteins might well be involved in the orchestration of Wallerian degeneration, axon regeneration and remyelination.

Initially, disruption of axonal contact upon nerve injury causes Schwann cells to dedifferentiate and proliferate. In line with this, two proteins of cluster 1, known to be expressed in myelinating Schwann cells, e.g., PERAX and CRAB, were strongly down-regulated after crush and relatively low levels of CRAB and PERAX persisted at 35 days after injury. This marked down-regulation is in line with our qPCR data and underscores the dedifferentiation of Schwann cell that occurs upon injury-induced disruption of axonal contact and myelin degradation by infiltrating blood-derived macrophages (38,39). PERAX is a component of the membrane ensheathing the axon and regulates myelin thickness (40). Mutations in the PERAX gene cause demyelination in Charcot-Marie-Tooth disease. Importantly, CRAB induction was found associated with neuroinflammation and cell death in CNS injury (41,42). Therefore, marked and prolonged CRAB down-regulation observed upon PNS injury might play a role in restricting inflammation and/or promoting regeneration.

After peripheral nerve injury, axon degeneration and myelin degradation proceed rapidly by infiltration of blood-derived macrophages that clear axonal and myelin debris (38,39). Therefore, as expected, the levels of axonal proteins that all clustered in profile 2 of down regulation, were decreased at 5 and 10 days after injury and their levels increased as regeneration occurred. Unexpectedly, the mRNA levels of SYNA and DDH1 (but not DDH2), previously described to be neuronally expressed (43,44), were also strongly down-regulated in sciatic nerve after crush. We propose that these proteins may be synthesized locally by e.g. Schwann cells, or alternatively de novo within the regenerating axons by axonal mRNAs. DDH1 is found in cluster 1 of down-regulation together with Schwann cell dedifferentiation markers, which supports the first hypothesis. DDH1 is a regulator of nitric oxide synthase and SYNA is known as a synaptic protein with homology to the chaperone 14-3-3 (45). Notably, the SYNA gene has been implicated in neurodegenerative diseases and the protein accumulates in amyloid plaques and Lewy bodies, the pathological hallmarks of Alzheimer's and Parkinson's disease, respectively (46). The functional significance of down-regulation of SYNA in the sciatic nerve upon crush remains to be established.

Concomitant with axon degeneration, several proteins are strongly up-regulated distal to the crush site. One of the strongly induced proteins found in cluster 4 is GAL3, which may have two different functions in nerve regeneration. Firstly, GAL3 on activated Schwann cells and macrophages mediates lectin-mediated phagocytosis of myelin (31) and, secondly, extracellular GAL3 supports adhesion and neurite outgrowth (47). Another strongly up-regulated protein, clustering in the expression profile of GAL3, is the lysosomal thiol protease CATB. CATB is
expressed in macrophages and microglia, and participates in intracellular degradation of proteins (for a review see (32-34). The peak level of up-regulation of the CATB gene and protein at 5-10 days after crush is consistent with macrophage infiltration into the distal sciatic nerve, and suggests that it might contribute to regeneration through degradation of axonal debris.

Furthermore, we detected the up-regulation of TCTP and ANXA9/31. TCTP is a regulator of cell growth and is up-regulated by growth stimuli (48,49). The induced TCTP protein and mRNA peaking at 5 days after nerve crush, may be part of the mitotic response of Schwann cells after nerve injury. ANXA9/31 belongs to a novel subfamily of annexins (50) that lacks type II calcium-binding sites and is highly expressed in differentiating tissues (51). ANX1 in PNS macrophages has been implicated as an endogenous regulator of inflammation in nerve repair (52,53). Whether ANXA9/31 exerts a similar function remains to be investigated.

Several recently discovered small ER proteins ie., ERP29 and the multiple EF hand, low-affinity calcium-binding proteins CALU and RCN2, were included in the functional group of protein synthesis and maturation that was up-regulated 5-10 days after crush. They are all implicated in secretory protein synthesis (reviewed by (54) and may be important for protein folding and maturation of eg., cytokines and growth factors in activated Schwann cells. In addition, CALU and RCN2 have been implicated in calcium-dependent cell adhesion (55) and CALU in immunological defense (56). Whether these alternative functions of RCN2 and CALU are of importance for peripheral nerve regeneration, remains to be established.

Schwann cell proliferation and migration into the injury area of the distal nerve involves an extensive transformation of cell shape and motility. The up-regulated protein and mRNA expression of TPMsk3 and TPM4 peaking at 5 days after crush, may be important for this. TPMs are actin-binding proteins that differentially stabilize actin filaments by protecting them from the activity of severing proteins. Altered patterns of expression of TPM isoforms have been observed in transformed fibroblasts, human cancers (Pawlak and Helfman, 2001) and in neuronal development (Gunning et al., 1997).

During nerve regeneration, large amounts of lipids are required for axonal regeneration and remyelination. We found differentially increased levels of APOA1, APOA4, APOD and APOE after injury, including allelic variants of APOA1 and APOE. Expression profile 6 contained APOE and APOD that are synthesized in macrophages and endoneurial fibroblasts, respectively, which is in agreement with our qPCR data that indicate a marked upregulation in the nerve. In contrast, APOA1 and APOA4 have been postulated to enter the injured nerve from the circulation (57), which is consistent with our qPCR data that failed to detect their mRNAs in the nerve as well as with the clustering in expression profiles that contain numerous circulatory proteins. Various studies have established a role for APOE in re-utilization of cholesterol from degenerating nerves for expansion of axonal membranes and myelin production (58-62). The strong and prolonged induction of APOD and APOE found here underscores their role in membrane synthesis and remyelination during the late regeneration phase.

Injury leads to a breakdown of the blood-nerve barrier, resulting in leakage of blood-derived proteins into the nerve. We detected accumulation of the acute phase proteins HP and HPX as well as the plasma proteins VTDB, ALPHA1M and TTHY. HP- and HPX-haemoglobin complexes are cleared via a specific macrophage receptor (63) which may protect the cells of the regenerating nerve against the oxygen radical toxicity of free haemoglobin. HPX
may derive from the circulation but can also be induced locally in Schwann cells and invading macrophages in injured peripheral nerves (64). VTDB is a multifunctional protein that may augment chemotaxis of macrophages (65) and is a precursor for macrophage-activating factor (66) and might in the injured sciatic nerve attract and activate hematogenic macrophages. Alpha-macroglobulins are broad-range protease inhibitors that trap proteinases, and may bind to a variety of substrates. These complexes are endocytosed and degraded in the lysosomal system (67). Therefore, the increased levels of a C-terminal fragment of ALPHA1M after nerve crush may reflect increased lysosomal breakdown. Interestingly, ALPHA1M has been shown to enhance NGF-mediated neurite outgrowth and differentiation of PC12 cells (68) and might have a role in nerve regeneration. Furthermore, our proteomics data showed accumulation of fibrinogens FGA and FGB after nerve crush. Consistent with our findings, fibrin(ogen) deposition in the matrix has been found during sciatic nerve degeneration and a role as an inhibitor of Schwann cell differentiation and peripheral nerve myelination has been demonstrated (69).

Besides circulatory proteins with antioxidant functions, we detected altered abundance of the antioxidant enzymes TDPX1 and TDPX2 (36); (37) and glutathione peroxidase (GPX). TDPX2 was originally identified as a 23 kD stress-inducible protein in macrophages (70), and is widely expressed in the CNS (71). The increased levels of antioxidant enzymes after nerve crush may indicate a neuroprotective role.

In this study, a considerably greater number of proteins was resolved than in previous studies employing 2DGE of sciatic nerve (22,72), probably due to improvements in separation methodology and sample preparation. However, a limitation of proteomics remains its sensitivity. For instance, we did not detect altered abundance of low-abundant proteins such as receptors, cytokines and growth factors, which are known to be induced in Schwann cells upon nerve injury. However, we did identify many novel proteins regulated by sciatic nerve crush in vivo. Their identification is the first essential step in elucidating their functional role in the nerve repair process. An evaluation of the cellular localization, as well as loss and gain-of-function interventions are the next steps to causally relate our findings to the process of nerve regeneration.
REFERENCES

FIGURE LEGENDS

Figure 1. A differential 2D gel protein display of control and injured sciatic nerve. Proteins of rat sciatic nerve were separated by 2DGE (first dimension, 18 cm 3-10 NL IPG strips, second dimension 11% duracryl in the isodal system) and visualized by silver staining. Representative examples of 2D gels from a contralateral control nerve (Fig. 1A) and nerve section distal to the crush injury, 5 days post-crush (Fig. 1B). Labeled spots indicate spots that show crush-induced changes in protein levels. Down-regulated spots are marked in 1A and up-regulated spots in 1B. SA, serum albumin; A, β-actin.

Figure 2. Typical examples of temporal changes in protein levels as caused by nerve crush. Selected regions of the 2D gels display protein spots that exhibit alterations in levels that are typical for each of the six temporal regulation profiles as shown in Table I. The bar graphs show the average alterations in protein levels of each indicated spot of the control (N=4), at 5 days (N=4), 10 days (N=6) and 35 days (N=4) after nerve crush. Error bars indicate coefficient of variation. The regulated spots indicated by the arrow were identified as CRAB (spot 8001), HSP27 (spot 4105), TCTP (spot 0108), CATB (spot 2101), ALPHA1M (spot 3208) and APOD (spot 0111). For details of the protein identification and abbreviations see Table II.

Figure 3. Functional groups of sciatic nerve proteins regulated by crush injury. Pie charts showing down-regulated (A) and up-regulated (B) proteins as a fraction of total proteins down- or up-regulated after crush.

Figure 4. Gene regulation in sciatic nerve and DRG after crush as assessed by quantitative PCR on a selection of genes encoding nerve-injury regulated proteins and proteomics. A-B: mRNA levels in sciatic nerve distal to the lesion (A) and DRG (B) as compared to the contralateral side at 5, 10 and 35 days after injury. The $^2\log$ value of the ratio of 5d, 10d and 35d post lesion and control is plotted. The average Ct value of HPRT, Sr317 and NSE was used to normalize the sciatic nerve data. For DRG, L37a, β-Actin and GAPDH were used. C: Protein levels in the sciatic nerve. The normalized protein spot intensities were transformed by taking the $^2\log$ value of the ratio of 5d, 10d and 35d post lesion and control. For most gene products, mRNA regulation was found mainly in the sciatic nerve, not in the DRG. In some cases, peaks in regulation at the RNA level occur at a timepoint earlier than peaks in protein regulation. For abbreviations of protein names see Table II.
Fig. 2.
Figure 3
Fig. 4.