Abstract

Chronic denervation is one of the key factors that affect nerve regeneration. Chronic axotomy deteriorates the distal nerve stump, causes protein changes, and renders the microenvironment less permissive for regeneration. Some of these factors/proteins have been individually studied. To better delineate the comprehensive protein expression profiles and identify proteins that contribute to or are associated with this detrimental effect, we carried out a proteomic analysis of the distal nerve using an established delayed rat sciatic nerve repair model. Four rats that received immediate repair after sciatic nerve transection served as control, whereas four rats in the experimental group (chronic denervation) had their sciatic nerve repaired after a 12-week delay. All the rats were sacrificed after 16 weeks to harvest the distal nerves for extracting proteins. Twenty-five micrograms of protein from each sample were fractionated in SDS-PAGE gels. NanoLC-MS/MS analysis was applied to the gels. Protein expression levels of nerves on the surgery side were compared to those on the contralateral side. Any protein with a P value of less than 0.05 and a fold change of 4 or higher was deemed differentially expressed. All the differentially expressed proteins were further stratified according to the biological processes. A PubMed search was also conducted to identify the differentially expressed proteins that have been reported to be either beneficial or detrimental to nerve regeneration. Ingenuity Pathway Analysis (IPA) software was used for pathway analysis. The results showed that 709 differentially expressed proteins were identified in the delayed repair group, with a bigger proportion of immune and inflammatory process-related proteins and a smaller proportion of proteins related to axon regeneration and lipid metabolism in comparison to the control group where 478 differentially expressed proteins were identified. The experimental group also had more beneficial proteins that were downregulated and more detrimental proteins that were upregulated. IPA revealed that protective pathways such as LXR/RXR, acute phase response, RAC, ERK/MAPK, CNTF, IL-6, and FGF signaling were inhibited in the delayed repair group, whereas three detrimental pathways, including the complement system, PTEN, and apoptosis signaling, were activated. An available database of the adult rodent sciatic nerve was used to assign protein changes to specific cell types. The poor regeneration seen in the delayed repair group could be associated with the down-regulation of beneficial proteins and up-regulation of detrimental proteins. The proteins and pathways identified in this study may offer clues for future studies to identify therapeutic targets.

Key Words: chronic axotomy; chronic denervation; delayed repair; distal nerve; functional recovery; nerve regeneration; peripheral nerve; prolonged denervation; proteome; sciatic nerve; sciatic nerve transection

Introduction

Many factors, including the patient’s age, type of trauma, and level of injury, can affect regeneration and functional recovery following nerve repair. Another critical prognostic factor is the timing of nerve repair. Prompt intervention is more effective than delayed intervention in restoring function (Mackinnon, 1989). However, immediate repair is oftentimes not possible due to concomitant injuries, infections, and late presentation. Functional recovery is compromised in patients whose nerve repair is delayed (Fu and Gordon, 1995; Samii et al., 2003; Han et al., 2015). The relatively poor clinical outcomes of delayed repair are generally attributed to atrophy of the denervated muscles (Anzil and Wernig, 1989). Additionally, chronic axotomy and chronic denervation significantly reduce the success of axonal regeneration (Fu and Gordon, 1995; Sulaiman et al., 2002). The decline in regenerative capacity corresponds to the decline in brain-derived neurotrophic factor...
Identifying the proteome profiles of the chronically denervated nerves can reveal more therapeutic targets to overcome the negative effects of axotomy and chronic denervation. Studies focused on proteomic analyses of rat sciatic nerve transection injury, the most common model in nerve regeneration research, have been reported. Aiki et al. (2018) found that protein expression changes were site (proximal or distal) and stage (post-nerve transection time) specific after rat sciatic nerve transection: the number of identified proteins successively increased in both the proximal and distal stumps at 5, 10, and 35 days after injury. In the study by Bryan et al. (2012), the expression of 15 proteins known to be involved in various aspects of the regenerative process including growth factors, extracellular matrix (ECM) proteins, and adhesion and motility proteins was profiled in a 1 cm rat sciatic nerve conduit repair model over a 28-day regeneration period. They also showed that protein expression changes were site (proximal, within the conduit, or distal) and stage (earlier or later) specific (Bryan et al., 2012). Vergara et al. (2018) performed proteomic analysis on cross-sections of rat sciatic nerve at 20 days after nerve transaction and immediate repair and identified 201 differentially expressed proteins that belonged to four significantly enriched canonical pathways: EIF2 signaling, LXR/RXR activation, acute phase response signaling and actin cytoskeleton signaling. To the best of our knowledge, there has not been a report about differential proteome profiling of promptly repaired and delayed repaired nerves. In this study, we used the established delayed rat sciatic nerve repair model to compare the proteomes maintained between chronically and nonchronically axotomized nerves via label-free proteomics analysis to identify proteins that are potentially detrimental or beneficial to nerve regeneration.

Materials and Methods

Animal group assignment, surgery, and postoperative testing

The study was approved by the Institutional Animal Care and Use Committee (IRB) of Mayo Clinic (IRB number A8814-14). All animal care and study procedures were carried out in accordance with the guidelines of NIH (National Institute of Health), USDA (United States Department of Agriculture), and AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International). Eight adult male Lewis rats (Harlan Laboratories, Indianapolis, IN) with a body weight of around 200 g (range from 192 to 225 g) were included in this study. They were kept on a 12-hour light/12-hour dark cycle in an animal room, where they had free access to food and water. The rats were randomly allocated to the immediate repair group (n = 4) and the delayed repair group (n = 4) using the random number generator. The delayed repair group was used to establish the chronic regeneration model, while the immediate repair group was regarded as the control group.

Rats underwent all surgical and postoperative evaluation/tissue harvest procedures under anesthesia with an intraperitoneal injection of 80 mg/kg ketamine (Ketaset III; Fort Dodge Animal Health, Fort Dodge, IA, USA) and 5 mg/kg xylazine (AnaSed; Lloyd Laboratories, Shenandoah, IA, USA) and 5 mg/kg xylazine (AnaSed; Lloyd Laboratories, Shenandoah, IA, USA) and 5 mg/kg xylazine (AnaSed; Lloyd Laboratories, Shenandoah, IA, USA) and 5 mg/kg xylazine (AnaSed; Lloyd Laboratories, Shenandoah, IA, USA). The surgical procedures were done under sterile conditions. Rats in the immediate repair group underwent no surgery. Rats in the delayed repair group underwent two survival surgeries as previously described (Wu et al., 2013). In the first surgery, the proximal nerve stump was turned around and embedded into the neighboring muscles after sciatic nerve transaction. The distal nerve stump was tagged 10 mm from the lower edge of the obturator tendon to prevent retraction. The incision was closed layer by layer. The second surgery was done 12 weeks later when the sciatic nerve was exposed again to mobilize the proximal and distal nerve ends which were trimmed and sutured together directly without tension. Postoperative pain management included a single dosage of subcutaneous injection of 0.05 mg/kg long-lasting buprenorphine (Buprenorphine Hydrochloride Injection III, Hospira, Lake Forest, IL, USA), supplemented by 30 mL Tylenol (Children’s Mapap, Livonia, MI, USA) in every 473 mL of drinking water that started 48 hours preoperatively and lasted 7 days postoperatively.

Sixteen weeks after the nerve repair, a nerve conduction study was carried out in all the rats to record compound muscle action potential (CMAP) from muscles innervated by the two sciatic nerve branches, the tibial nerve and peroneal nerve using Nicolet Viking IV (Viasys Healthcare, Madison, WI, USA) (Wu et al., 2013). Each recording was repeated twice to ensure reproducibility. The average of the amplitude and latency of CMAP recorded from tibial nerve innervated muscle (Tamp and Tlat, respectively) and the average of the amplitude and latency of CMAP recorded from peroneal nerve innervated muscle (Pamp and Plat, respectively) from the two repeats were logged. After the nerve conduction study before the animal was euthanized, the tibialis anterior muscle and the triceps surae muscle from both hind limbs were harvested and weighed using a digital balance (Mettler Toledo, Columbus, OH, USA). Wet muscle weight recovery was calculated by dividing the muscle weight on the operated side by the muscle weight on the contralateral side and expressed as a percentage. The mean and standard deviation of both CMAP data and muscle wet weight recovery data were calculated for each group.

Nerve sample processing for proteomics

After muscle harvest, the rats were euthanized and transcardially perfused with chilled 0.9% saline to expel any hematogenous components from the nerve samples. Sciatic nerves from both the surgical side and the contralateral normal side were harvested from the repair site to its muscle entry site and snap-frozen in liquid nitrogen. All nerve samples were stored at −80°C for later use.

Preparation of SDS-PAGE Gels

Each nerve segment, while still frozen, was transferred to a 0.5 mL 1.4 mm ceramic bead tube (Bertin Technologies, Green Bay, WI, USA) containing 100 µL lysis buffer (0.5% SDS, 0.5 mM MgCl₂, 20 mM Tris (pH 8.2), benzonase, Halt protease inhibitor). Tissue was homogenized twice at 5000 rpm for 30 seconds each using a MiniBeadbeater BB4 (BioSpec Products, Bartlesville, OK, USA) using bovine serum albumin (BSA) as a standard. Samples were frozen at −80°C until they needed to be thawed for electrophoresis.

SDS-PAGE and protein staining

Nerve lysate samples containing equal amounts of protein (5 µg) were dried on a vacuum centrifuge (Savant SpeedVac model SPD111V, Thermo Fisher Scientific, Waltham, MA, USA) and resolubilized in 30 µL of Laemmli buffer/5% beta-mercaptoethanol. After being heated for 10 minutes at 85°C, each sample was loaded on a 10.5–14% Criterion gel (Bio-Rad Laboratories, Hercules, CA, USA). Two pairs of lysates from nerves of the surgical side and contralateral side from each group (immediate repair and delayed repair), for a total of 8 samples, were run per gel. Electrophoresis was performed for 12 minutes at 140 V and 50 minutes at 200 V. Gels were fixed in 10% acetic acid and stained in 2.5% Coomassie Blue for 1 hour and washed in acetic acid/water that started 48 hours preoperatively and lasted 7 days postoperatively.

Protein identification by nano-scale liquid chromatography-tandem mass spectrometry

Gel cuts from each lane were divided into approximately 1 mm cubes and digested with trypsin largely as previously described (Hogan et al., 2014). Gel sections were destained, reduced with tris(2-carboxyethyl)phosphine (TCEP), and alkylated with iodoacetamide. Then, 150 ng of sequencing-grade trypsin (Promega, Madison, WI, USA) was added to each sample, and the mass spectrometry analysis was performed using an Ultima LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) in negative ion mode. The instrument was operated in a data-dependent fashion to acquire full scan spectra and CID spectra for the 10 most abundant ions. The resulting MS/MS spectra were submitted to the in-house Mascot search engine (www.matrixscience.com). The search parameters were set as follows: Sequence database, Uniprot mouse proteomes; enzyme, trypsin; oxidation (M) and carbamidomethyl (C) were set as fixed modifications; and one methionine oxidation was set as variable modification. The peptide and MS/MS ion tolerances were set at 20 ppm and 0.5 Da, respectively. The peptide and protein false discovery rates were set at 1%.
samples were incubated overnight at 37°C. After digestion, peptides were first acidified with 10 μL of 4% trifluoroacetic acid (TFA) and then incubated for 30 minutes, after which the supernatant was transferred to new PCR tubes. A second extraction was performed with 60 μL acetonitrile (ACN) for 30 minutes, and the product was added to the first extract. The extracts were vacuum concentrated to dryness and stored at −80°C for LC-MS/MS and analysis. LC-MS/MS measurements were performed by gel section using a data-dependent method on a Q-Exactive (Thermo Fisher Scientific Inc., Waltham, MA, USA) interfaced to a Dionex Ultimate® 3000 RSLCnano liquid chromatography as previously described (Ayers-Ringler et al., 2016). Dried tryptic peptides from each gel section were reconstituted in 100 μL of a reconstitution solution (2% FA, 0.1% TFA, and 0.002% Zwittergent 3–16) and the autosampler was used to load sample aliquots (10 μL for gel sections A, B, and C; 5 μL for gel section D; 15 μL for gel sections E and F) that were preconcentrated on a 0.25 μL reversed-phase OptiPak trap (Optimize Technologies, Oregon City, OR, USA) custom-packed with 5 μm, 200 Å Magic C8 stationary phase (Bruker-Michrom, Auburn, CA, USA). Peptides were washed for 4 minutes at 10 μL/minute with a mixture containing 0.2% FA and 0.05% TFA, and the trap was then placed in line with the analytical column via a 10-port valve. Peptides were separated on a 40 cm, 100 μm internal diameter self-packed PicoFrit® (NewObjective, Woburn, MA, USA) column packed with Poroshell 120S EC-C18, 2.7 μm stationary phase (Agilent, Santa Clara, CA, USA). A mobile phase gradient of 2–30% over 70 minutes (phase A) followed by a 30–50% gradient over 30 minutes (phase B), was used at a flow rate of 400 nL/min, where mobile phase A was 2% ACN in high-performance liquid chromatography grade water (Honeywell Burdick & Jackson, Muskegon, MI, USA) with 0.2% FA and mobile phase B was ACN/isopropanol/water (80/10/10 by volume) with overall 0.2% FA. Eluting peptides were electrosprayed from the column tip into the Q-Exactive massrometer, where MS1 scans over the m/z range of 350–2000 were recorded at 70,000 resolving power (measured at m/z 200) using an AGC (automatic gain control) value of 3E6. Tandem mass spectra (MS2) were recorded for the 15 most abundant multiply charged ions (≤25e, NCE (normalized collision energy) = 26, fixed m/z of 140, maximum fill time of 50 ms, isolation window of 3 m/z, isolation offset of 0.5 m/z, 60-second dynamic exclusion).

Bioinformatic analysis of label-free differential expression using LC-MS/MS data

We utilized a label-free peptide MS1 intensity-based method for finding differentially expressed proteins between the operated side and the contralateral side. All MS/MS were matched against a composite mouse protein sequence database containing the UniProt reference proteome (downloaded June 2015, https://www.uniprot.org/proteomes/) and sequences of common contaminants (e.g., trypsin, keratin, cotton, wool). Reversed protein sequences were applied to MS/MS to estimate false discovery rates (FDRs) of protein identification. First, we utilized our MyriMatch-IDPicker-SwiftQQA in-house pipeline to assess the quality of the raw LC-MS/MS data (Ma et al., 2009; Tabb et al., 2007). For this purpose, MyriMatch was configured to derive semitryptic peptides from the protein database while matching the MS/MS present in a run. The MyriMatch software was configured to use 10 ppm m/z tolerance when matching both precursor and fragment ions. MyriMatch also considered the following variable modifications for the search: carbamidomethylation of cysteine (+57.023 Da), oxidation of methionine (+15.994 Da), and N-terminal pyroglutamic acid (-17.023 Da). IDPicker (Ma et al., 2009) filtered the resulting peptide-spectrum matches at a 2% FDR and assembled them into protein identifications. SwiftQQA processed the raw spectra and identified peptides to extract quality metrics (such as mass error of identified peptides, total ion current of acquired MS and MS/MS scans, mass error drift with retention time, and quality of the acquired and identified MS/MS) associated with each LC-MS/MS run. Runs whose quality control metrics were within expected ranges (determined based on historical yeast lysate LC-MS/MS runs) were considered for peptide intensity quantification. All LC-MS/MS analyses present in this study met the quality assurance criteria. A previously published MaxQuant-based (version 1.5.1, https://www.maxquant.org) protocol was utilized to detect the peptides and proteins present in each sample and record their intensities (Cox and Mann, 2008; Cox et al., 2014). The software was configured to use 20 ppm m/z tolerance for precursors and fragments while performing peptide-spectrum matching. The software derived semitryptic peptides from the aforementioned protein sequence database while searching for the above-described variable modifications, augmented to include protein n-terminal acetylation (+42.01 Da). MaxQuant filtered the peptide and protein identifications at a 1% FDR, grouped protein identifications into groups, and reported protein group intensities.

Data analysis

Statistical analysis

No statistical methods were used to predetermine sample sizes; however, our sample sizes are similar to those reported in previous publications (Aiki et al., 2018; Vergara et al., 2018). An in-house script written in the statistical programming language R (version 3.1.2) was utilized to perform differential expression analysis of the detected protein group intensities between the samples for any two experimental groups of interest. For this purpose, the protein group intensities of each sample were log2 transformed and normalized using the quantile method (Bolstad et al., 2003). For each protein group, the normalized intensities observed in the comparative groups of samples were modeled using a Gaussian-linked generalized linear model (Nelder and Wedderburn, 1972). A one-way analysis of variance was used to test the differentially expressed proteins between a pair of experimental groups. Differential expression p-values were FDR corrected using the Benjamini-Hochberg-Yekutieli procedure (Benjamini and Hochberg, 1995). Protein groups with a corrected p-value of ≤ 0.05 and an absolute log2 fold change of ≥ 4 (where 0.0 signifies no change) were considered significantly differentially expressed. Independent-sample t-tests were performed using SPSS software (version 19.0, SPSS Inc., Chicago, IL, USA) to evaluate nerve conduction and wet muscle weight data. P-values of less than 0.05 were considered significant.

Biological process & pathway analysis

Ingenuity Pathway Analysis (IPA, build version 389077M, Licensed by Qiagen, a multi-national company headquartered in Hilden, Germany) was used to analyze the proteins identified as significantly different by the bioinformatic analysis described above. The ratios, p-values, and FDRs from these resulting proteins were uploaded to IPA with their corresponding UniProt identifiers. The Disease and Functions algorithm was used to determine the significantly enriched terms between the immediate repair group and the delayed repair group. Additionally, the pathway and network functions were used to identify the top pathways most likely to have been impacted.
Protein expression profile in the distal sciatic nerve
Each distal nerve proteome contained 5754 detectable proteins on the surgical side and contralateral side from both groups (immediate repair and delayed repair). To identify proteins that were differentially expressed, the relative abundance measured by mass spectrometry was compared between nerve samples (n = 8) of the surgery side from both immediate and delayed repair groups and nerve samples (n = 8) of the contralateral side from both groups. All the expression levels of such identified proteins were compared among the above groups to screen out the proteins with significant change using a P-value less than 0.05. Such proteins with a statistically significant change were further analyzed from the biological perspective to make the analysis biologically meaningful. Therefore, a fold change threshold was determined by analyzing the characteristics of protein expression profile between the operated side and contralateral side in all 8 rats, since nerve transection and repair surgery could be regarded as a notable biological factor and should lead to significant protein profile changes compared to the contralateral intact nerve. The comparative analysis between the surgery side and the contralateral side showed an apparent natural break point at a 4-fold change (Figure 1). This natural break point was probably caused by the biological factor, namely, nerve transection and repair surgery. The threshold for differential expression was therefore set as a 4-fold change and a P-value less than 0.05.

Further comparison of the protein profiles of the surgery side nerve samples to the contralateral side samples in the immediate repair group revealed that 270 proteins were significantly downregulated and 208 proteins were upregulated, while the comparison of protein profiles of nerve samples of the surgery side to those of the contralateral side in the delayed repair group revealed 392 significantly downregulated and 317 upregulated proteins. Comparative analysis of the protein profiles of the surgery side nerve samples in the immediate repair group and the surgery side samples in the delayed repair group revealed 104 proteins that were significantly downregulated and 77 proteins that were significantly upregulated (Figure 2).

Biological processes and key proteins
The differentially expressed proteins in either the immediate repair group or the delayed repair group were assigned to subcategories according to the biological function they are associated with. Six biological processes associated with nerve regeneration were identified: inflammatory response, cell proliferation, cell migration, cell apoptosis, axon regeneration, and lipid metabolism. Some of the proteins were involved in multiple processes and assigned to more than one biological process. More proteins of differentially expressed proteins that were associated with such afore-mentioned biological processes in the immediate repair group were inflammatory response 5.89%, cell proliferation 33.89%, cell migration 15.16%, cell apoptosis 22.95%, axon regeneration 6.32% and lipid metabolism 15.78%, respectively. While, similar percentages in the delayed repair group were 12.93%, 32.93%, 16.73%, 22.59%, 2.31% and 12.52%, respectively (Figure 3). When comparing the percentages of biological processes between the immediate repair and delayed repair groups, we found that the inflammatory response process was increased in the delayed repair group, while axon regeneration processes were decreased. In the delayed repair group, 69/95 differentially expressed proteins related to the inflammatory response process were upregulated and 26/95 were down-regulated, while 6/17 differentially expressed proteins related to axon regeneration were upregulated and 11/17 were down-regulated. In the immediate repair group, 12/28 differentially expressed proteins related to the inflammatory response process were upregulated and 16/28 were down-regulated, while 14/30 differentially expressed proteins related to axon regeneration were upregulated and 16/30 were down-regulated.

Table 2 | The wet muscle weight recovery of chronic denervation (delayed repair) and non-chronic denervation (immediate repair) groups

| Group                | TS recovery (%) | TA recovery (%) |
|----------------------|-----------------|-----------------|
| Immediate repair     | 70.81±8.90      | 79.60±11.65     |
| Delayed repair       | 45.43±8.20*     | 47.89±15.82*    |

*P < 0.05, vs. immediate repair group (independent-sample t-tests). Data were expressed as mean ± SD of each group (n = 4). SD: Standard deviation; TS: triceps surae muscle; TA: tibialis anterior muscle.

Figure 2 | Overview of the differentially expressed proteins between samples for any two sets of interest.

The bar graph on the left shows the number of identified proteins that were differentially expressed when comparing samples (n = 4) of the surgical side to samples (n = 4) of the contralateral side in the immediate repair group, as defined by a fold change of more than 4 and a P-value of less than 0.05. The bar graph in the middle shows the number of identified proteins that were differentially expressed when comparing samples (n = 4) of the surgical side to samples (n = 4) of the contralateral side in the delayed repair group. The bar graph on the right represents the number of identified proteins that were differentially expressed when comparing samples (n = 4) of the surgical side in the immediate repair group to samples (n = 4) of the surgical side in the delayed repair group. IS-IC, DS-DC, IS-DS mean IS vs. IC, DS vs. DC, IS vs. DS, respectively. DC: Delayed repair contralateral side; DS: delayed repair surgery side; IC: immediate repair contralateral side; IS: immediate repair surgery side.

Figure 3 | Biological process analysis of differentially expressed proteins.

Pie charts showing functional groups as a fraction of differentially expressed (either up-regulated or down-regulated when comparing surgery side to contralateral side) proteins in the immediate nerve repair group and delayed nerve repair group. In the delayed repair group there were more proteins belonging to the biological process of immune/inflammatory response and fewer proteins pertaining to axon outgrowth and lipid metabolism processes.

Furthermore, we analyzed the differentially expressed proteins by searching the literature to identify the proteins that are related to the key processes in nerve injury and regeneration. Multiple key proteins were identified to be involved in the inflammatory response, cell proliferation, cell apoptosis, cell migration, axon regeneration, and lipid metabolism processes (Table 3). The differential expressions of these key proteins, either up-regulation or down-regulation, in the aforementioned processes were listed in the Additional Table 1 and shown in the heatmap (Figure 4). By comparing fold change expression levels between surgery side samples in the immediate repair group to surgery side samples in the delayed repair group, we identified the top three differentially expressed proteins in each of the biological processes. The top three differentially expressed proteins in the inflammatory response process were S100A8, PLA2G4A, and S100A9, which were all up-regulated in the nerves that have been subjected to chronic denervation. The top three differentially expressed proteins in the
Cell proliferation

MAPK11 (Fragoso et al., 2003; Hossain et al., 2012), CASP6 (Monnier et al., 2011), S100A8 (Chernov et al., 2015), IGFBP5 (Simon et al., 2015), S100A9 (Chernov et al., 2015), MSLN (Roet et al., 2013), SPAST (Wood et al., 2006; Butler et al., 2010), SHH (Martinez et al., 2015), LIMK1 (Endo et al., 2003; Koch et al., 2014), BCL2 (Kohno et al., 2011; Seon et al., 2018), MADD (Hao et al., 2010), MPZ (Lemke et al., 1985; Giese et al., 1992), IGFR1 (Joshi et al., 2015; Leon et al., 2017), PTGDS (Trimmer et al., 2014), ARHGA24 (Nguyen et al., 2012), C6 (Ramaglia et al., 2009), SFRP1 (Kele et al., 2012), EGR2 (Decker et al., 2006), POSTN (Shimamura et al., 2012; Shih et al., 2014; Matsunaga et al., 2015), and C3 (Matsunaga et al., 2015). Among these key proteins, some were differentially expressed in the cell migration process. The top three differentially expressed proteins were MAPK11, down-regulated; and the other two up-regulated in the nerves that have been subjected to chronic denervation. Among the top three differentially expressed proteins were IGF1R, which were all significantly down-regulated in the nerves that have been subjected to chronic denervation. The top three differentially expressed proteins in the axon regeneration process were CAMKK1, MPZ, and IGFR1, which were all significantly down-regulated in the nerves that have been subjected to chronic denervation. The top three differentially expressed proteins in the lipid metabolism process were S100A8, MSLN, and S100A9, which were all up-regulated in the nerves that have been subjected to chronic denervation. The top three differentially expressed proteins in the inflammatory response were S100A8, S100A9, and MAPK11, down-regulated; and the other two up-regulated in the nerves that have been subjected to chronic denervation. The top three differentially expressed proteins in the cell apoptosis process were S100A8, S100A9, and MAPK11, down-regulated; and the other two up-regulated in the nerves that have been subjected to chronic denervation.
The impact of the key proteins on nerve regeneration. Further organizing these enriched proteins, 5 pathways were predicted to be activated, and 17 pathways were predicted to be inhibited. The roles of the 22 pathways in nerve regeneration were further refined by IPA analysis. The resource is an openly accessible online resource which was established through the transcriptional profiling of mouse sciatic nerve at the single-cell level (Gerber et al., 2021). This dataset contained the analysis of cells at early postnatal development (P1) and cells at the adult stage (P60) using single-cell RNA sequencing of sciatic nerve cells. Using the search function of this database we looked at the gene cluster and differential expression of the previously identified genes of interest across epineurial cells, perineurial cells, endothelial cells, immune cells, and vascular smooth muscle cells in age P60 sciatic nerve plots. The appearance of a violin plot in the expression level plot indicated that the P-value of the gene of interest in the specific cell cluster was less than 0.05 and this gene was considered to be differentially expressed in the delayed repair and immediate repair groups.

Table 4 | Key proteins that were assigned to sciatic nerve cells

| Key protein | Cells |
|-------------|-------|
| MSLN       | PnC   |
| MPZ        | SC    |
| EGR2       | SC    |
| DHH        | SC    |
| MADD       | Per/EC|
| SYNOP       | Per/EC|
| LGALS8     | Per/EC|
| SFRP1      | EpC, PnC|
| HSPB8      | EpC, EC1|
| LYN        | IC, Per/EC|
| NTTN1      | EpC, PnC|
| POSTN      | EpC, PnC, Per/EC|
| IGF1R      | EC1, Per/EC, Per/VSMC|
| IGFBP5     | EpC, EC1, EC2, Per/EC, Per/VSMC|
| IGFBP6     | EpC, EC1, EC2, Per/EC, Per/VSMC|
| LYN        | IC, Per/EC|
| LGALS8, POSTN, CD63, MADD, SYNOP, LGALS8, LYN, POSTN, IGF1R, IGFBP5, TXNIP, NFIB, CD63, APD, ARRP19|
| APDQ       | SC, EC, PnC, IC, EC1, EC2, Per/EC, Per/VSMC|
| ARRP19      | SC, EC, PnC, IC, EC1, EC2, Per/EC, Per/VSMC|

Table 5 | The cells in the sciatic nerve and the key proteins assigned to each cell type

| Cells                                  | Key protein                                  |
|----------------------------------------|----------------------------------------------|
| SC                                     | MPZ, EGR2, DHH, TXNIP, NFIB, CD63, APD, ARRP19|
| EpC                                    | SFRP1, HSPB8, NTTN1, POSTN, IGFBP5, TXNIP, NFIB, CD63, APD, ARRP19|
| EnC                                    | IGFBP5, TXNIP, NFIB, CD63, APD, ARRP19|
| PnC                                    | MSLN, SFRP1, NTTN1, POSTN, IGFBP5, TXNIP, NFIB, CD63, APD, ARRP19|
| IC                                     | LYN, TXNIP, CD63, APD, ARRP19|
| EnK                                    | IGFBP5, TXNIP, NFIB, CD63, APD, ARRP19|
| Per/EC                                 | MADD, SYNOP, LGALS8, LYN, POSTN, IGF1R, IGFBP5, TXNIP, NFIB, CD63, APD, ARRP19|
| Per/VSMC                               | LGALS8, POSTN, IC, EC1, EC2, Per/EC, Per/VSMC|

Canonical pathways analysis

All the differentially expressed proteins were uploaded to the IPA dataset for canonical pathways analysis. Sixty-five canonical pathways were significantly regulated in the immediate repair group (Additional Figure 1). By IPA analysis, 5 pathways were predicted to be activated, and 17 pathways were predicted to be inhibited. The roles of the 22 pathways in nerve regeneration were further confirmed by searching Pubmed. We found that the signaling pathways that were related to nerve regeneration were calcium signaling, LXR/RXR activation, protein kinase A signaling, and IL-6 signaling. Concerning the activation status of the above pathways in the immediate repair group, LXR/RXR activation was predicted to be
activated whereas the other three pathways were inhibited. Fifty-two canonical pathways were significantly regulated in the delayed repair group (Additional Figure 2). Using IPA analysis, 8 signaling pathways were predicted to be activated, and 21 signaling pathways were predicted to be inhibited. The signaling pathways that were related to nerve regeneration were LXR/RXR activation, acute phase response signaling, complement system, CNTF signaling, ERK/MPK signaling, apoptosis signaling, IL-6 signaling, and FGF signaling. Concerning the activation status of the above pathways in the delayed repair group, the complement system pathway, PTEN signaling pathway, and apoptosis signaling pathway were predicted to be activated whereas the rest seven pathways were inhibited.

Discussion

In this study, we examined the proteomes of repaired nerves that have or have not been subject to chronic denervation. Poorer regeneration and functional recovery were seen in the chronic denervation group, reflected by nerve conduction study and wet muscle weight recovery study that showed prolonged CMAP latency, reduced CMAP amplitude, and decreased muscle mass restoration. This difference in outcomes was underlined by differences in the protein expression profiles. The observations of different levels of protein expression reflect the state of the proteomic environment at the time of the observation. These establish associations between a poor or detrimental, delayed repair and a successful or beneficial immediate repair. These cannot be interpreted as having a cause-and-effect relationship. Upregulation of certain proteins, e.g. myelin proteins may be markers or the result of successful regeneration. In addition, the heterogeneity of the cellular environment and proportion of various cells within the injured nerve increases the difficulty of assigning specific causal roles to individual proteins.

To approach this problem, we categorized the differentially expressed proteins according to the biological processes relevant to nerve regeneration. This initial immune/inflammatory response is essential in clearing the wound and creating a permissive environment for the extension of re-growth cones to promote nerve regeneration (Sonigra et al., 1999; Bradke et al., 2012; Vergara et al., 2018). PKA pathway plays an important role in axon outgrowth. Pharmacological elevation of cAMP, as a mediator, accelerated neurite outgrowth in cultured motoneurons by activating the cAMP-PKA axis (Gordon et al., 2010). The inhibition of these two pathways in the immediate repair group 16 weeks post-repair indicated a phase that passed early regeneration. The only activated key pathway in the immediate repair group was the LXR/RXR pathway. This pathway was involved in the regulation of lipid metabolism and transport. A prior study indicated the importance of lipid metabolism in nerve regeneration (Sonigra et al., 1999; Bradke et al., 2012; Vergara et al., 2018).

Furthermore, the canonical pathway analysis also predicted differences in the key signaling pathways between the two groups. One signaling pathway, LXR/RXR activation, was activated and 3 pathways including calcium signaling, protein kinase A (PKA) signaling, and IL-6 signaling were inhibited in the immediate repair group. In the delayed repair group, seven pathways including CNTF signaling, IL-6 signaling, FGF signaling, LXR/RXR activation, acute phase response signaling, Rac signaling, and ERK/MPK signaling were inhibited while three pathways including apoptosis signaling, PTEN signaling, and complement signaling were activated. Calcium signaling can affect events including cytoskeletal dynamics and local protein translation, transport and trafficking in the growth cone to promote nerve regeneration (Sonigra et al., 1999; Bradke et al., 2012). A previous study showed that the inhibition of this pathway by C6 deletion accelerated axonal regeneration and functional recovery after sciatic nerve injury (Ramaglia et al., 2009). Prolonged activation of the complement system might be detrimental to nerve regeneration. Shi et al. reported that Rac-MAPK pathway worked as an important pathway for the response of Schwann cells to peripheral nerve injury promoting nerve regeneration (Shin et al., 2013). Newbern et al. demonstrated that activated ERK/MPK signaling in Schwann cells could stimulate Schwann cell dedifferentiation in vivo to promote nerve regeneration. Inhibition of these two pathways in delayed repair could be detrimental. Moreover, the apoptosis signaling pathway was activated in the delayed repair group, which was also inhibited. A previous study showed that acute phase response signaling was initiated immediately after peripheral nerve injury to promote nerve regeneration (Yi et al., 2015). The complement system, the fourth most enriched canonical signaling pathway, was activated in the delayed repair group. A study showed that the inhibition of this pathway by C6 deletion accelerated axonal regeneration and functional recovery after sciatic nerve injury (Ramaglia et al., 2009). Prolonged activation of the complement system might be detrimental to nerve regeneration. Shi et al. reported that Rac-MAPK pathway worked as an important pathway for the response of Schwann cells to peripheral nerve injury promoting nerve regeneration (Shin et al., 2013). Newbern et al. demonstrated that activated ERK/MPK signaling in Schwann cells could stimulate Schwann cell dedifferentiation in vivo to promote nerve regeneration. Inhibition of these two pathways in delayed repair could be detrimental. Moreover, the apoptosis signaling pathway was activated in the delayed repair group, which was consistent with the increase in proteins associated with the biological process of apoptosis. Inhibition of PTEN signaling could accelerate sciatic nerve regeneration by enhancing axon outgrowth (Christie et al., 2010). In the delayed repair group, this pathway was activated.
in primary Schwann cells induced expression of the gene encoding glial fibrillary acidic protein which is required for proper regeneration of the injured peripheral nerve (Lee et al., 2009a, b). Hausott et al. indicated that FGF could promote peripheral and central nerve regeneration. The inhibition of CNF, IL-6 and FGF signaling pathways in the delayed repair group could potentially contribute to poorer nerve regeneration.

There are several limitations in this proteomics study. First, only one time point 16 weeks after repair surgery was examined. The temporal changes in the protein profiles were not explored. The proteome only reflects the state the nerve was in when it was harvested. One could argue that the proteome of the nerve in the immediate repair group is expected to contain more beneficial proteins or fewer detrimental proteins since nerve regeneration is more complete and the regeneration process is more advanced. Vice versa for the proteome of the nerve in the delayed repair group. However, these trends do not predict causal relationships. Up to now, proteomic studies have mainly focused on nerve injury without repair (Aiki et al., 2018; Wei et al., 2020), or observed over 20 days or 28 days after immediate repair (Bryan et al., 2012; Vergara et al., 2018). Both key proteins and pathways that were identified in these studies differed from our proteomics findings. Our study is unique in that the nerve in our model has been subjected to chronic denervation before repair, which is a common yet challenging clinical scenario for nerve regeneration and functional restoration. A second limitation is the relatively small sample size. Third, to facilitate the analysis of such a large data set, we categorized all the differentially expressed proteins by biological processes. However, many key proteins functioned in more than one biological processes. The interactions of such proteins were not explored in this study, thus leading to a simplified analysis. Similarly, all the indicated pathways were analyzed separately, without considering signaling interactions. Four, identification of key proteins that are beneficial or detrimental to nerve regeneration was achieved by literature search using relevant key words. Given the fact that one protein can have multiple terminology by the genes that encode them, it was inevitable that some key proteins were missed during the search.

The identification of trends in gene categories that we used did result in the recognition of a relatively small number of proteins that were differentially regulated in the poor or successful regeneration condition. Numbers were further restricted by using available databases to present these proteins to specific cell types that are present in peripheral nerves. Future studies to address mechanistic relationships between proteins and success of regeneration may use secretome studies of individual cell types in vitro, that have been genetically modified to up or down-regulate specific proteins or factors.

Conclusions

This proteomics study demonstrated distinctly different protein profiles in the nerves that are repaired immediately and that are repaired after a delayed nerve repair, which is commonly seen in the clinical practice with less ideal functional outcomes. Future studies employing secretome proteomics that account for cellular composition in protein profiling can reveal potential therapeutic targets to improve the functional recovery.

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**Additional Table 1** Differentially expressed key proteins relevant to the inflammatory response process in the chronic denervation (delayed repair) and the non-chronic denervation (immediate repair) groups

| Key proteins | IS-IC (FC) | DS-DC (FC) | IS-DS (FC) |
|--------------|------------|------------|------------|
| CD63         | 20.09      | 1.33       | 14.89      |
| IKBKG        | 16.45      | 2.83       | 4.51       |
| CBL          | -2.54      | -4.64      | 1.95       |
| MPZ          | -1.39      | -14.10     | 12.57      |
| S100A8       | -6.08      | 26.13      | -30.77     |
| S100A9       | -8.09      | 18.16      | -20.49     |
| LIMK1        | -1.37      | -4.64      | 3.85       |
| RAB27A       | 7.13       | 12.92      | -0.91      |
| SFRP1        | 6.19       | 13.60      | -1.56      |
| POSTN        | 12.52      | 12.77      | -6.13      |
| PTGDS        | -11.20     | -17.76     | 5.83       |
| PLA2G4A      | -12.50     | 20.76      | -26.92     |

DC: delayed repair contralateral side; DS: delayed repair surgery side; FC: fold change; IC: immediate repair contralateral side; IS: immediate repair surgery side.
### Additional Table 2: Differentially expressed key proteins relevant to the cell proliferation process in the chronic denervation (delayed repair) and the non-chronic denervation (immediate repair) groups

| Key proteins | IS-IC (FC) | DS-DC (FC) | IS-DS (FC) |
|--------------|------------|------------|------------|
| SPAST        | -15.54     | -10.36     | 6.82       |
| ARHGAP24     | 17.23      | 1.28       | 12.30      |
| EGR2         | 17.33      | 12.93      | 1.05       |
| CD63         | 20.09      | 1.33       | 14.89      |
| MADD         | -3.03      | -21.44     | 18.60      |
| IGF1R        | -8.59      | -19.89     | 10.69      |
| MPZ          | -1.39      | -14.09     | 12.57      |
| DHH          | -2.41      | -4.87      | 2.14       |
| HSPB8        | -2.57      | -4.70      | 1.87       |
| CBL          | -2.54      | -4.64      | 1.95       |
| GJB1         | -1.85      | -4.51      | 2.66       |
| CASP6        | -5.32      | 12.26      | -8.86      |
| CASP3        | 10.25      | 14.70      | 0.62       |
| S100A8       | -6.08      | 26.13      | -30.77     |
| S100A9       | -8.09      | 18.16      | -20.49     |
| IGFBP1       | 0.12       | 20.10      | -18.54     |
| LIMK1        | -1.37      | -4.64      | 3.85       |
| RAB27A       | 7.13       | 12.92      | -0.91      |
| SFRP1        | 6.19       | 13.60      | -1.56      |
| POSTN        | 12.52      | 12.77      | -6.13      |
| SHH          | -4.81      | -10.58     | 5.10       |
| MAPK11       | -14.80     | -35.17     | 19.48      |
| PTGDS        | -11.20     | -17.76     | 5.83       |
| APOD         | 4.76       | 4.80       | -0.44      |
| MSLN         | -11.33     | 13.88      | -23.77     |

DC: delayed repair contralateral side; DS: delayed repair surgery side; FC: fold change; IC: immediate repair contralateral side; IS: immediate repair surgery side.
**Additional Table 3** Differentially expressed key proteins relevant to the cell apoptosis process in the chronic denervation (delayed repair) and the non-chronic denervation (immediate repair) groups

| Key proteins | IS-IC (FC) | DS-DC (FC) | IS-DS (FC) |
|--------------|-----------|------------|-----------|
| SMURF1       | -17.98    | -7.75      | -9.18     |
| NFIB         | -11.33    | 14.58      | -24.48    |
| IKBKG        | 16.45     | 2.83       | 4.51      |
| EGR2         | 17.33     | 12.93      | 1.05      |
| MADD         | -3.03     | -21.44     | 18.60     |
| IGF1R        | -8.59     | -19.89     | 10.69     |
| MPZ          | -1.39     | -14.10     | 12.57     |
| LGALS8       | 0.77      | -13.86     | 8.48      |
| PAK3         | -3.04     | 2.62       | 2.19      |
| HSPB8        | -2.57     | -4.70      | 1.87      |
| CNTF         | -0.10     | -4.18      | 2.20      |
| CBL          | -2.54     | -4.64      | 1.95      |
| GJB1         | -1.85     | -4.51      | 2.66      |
| CASP6        | -5.32     | 12.26      | -8.86     |
| CASP3        | 10.25     | 14.70      | 0.62      |
| RAB27A       | 7.13      | 12.92      | -0.91     |
| SFRP1        | 6.19      | 13.60      | -1.56     |
| S100A9       | -8.09     | 18.16      | -20.49    |
| IGF1BP5      | 0.12      | 20.10      | -18.54    |
| C6           | 13.77     | 21.37      | -0.74     |
| S100A8       | -6.08     | 26.13      | -30.77    |
| POSTN        | 12.52     | 12.77      | -6.13     |
| CAMKK1       | -4.28     | -18.65     | 14.79     |
| MAPK11       | -14.80    | -35.17     | 19.48     |
| PTGDS        | -11.20    | -17.76     | 5.83      |
| SHH          | -4.81     | -10.58     | 5.10      |
| APOD         | 4.76      | 4.80       | -0.44     |

DC: delayed repair contralateral side; DS: delayed repair surgery side; FC: fold change; IC: immediate repair contralateral side; IS: immediate repair surgery side.
### Additional Table 4
Differentially expressed key proteins relevant to the cell migration process of the chronic denervation (delayed repair) and the non-chronic denervation (immediate repair) groups

| Key proteins | IS-IC (FC) | DS-DC (FC) | IS-DS (FC) |
|--------------|------------|------------|------------|
| CD63         | 20.09      | 1.33       | 14.89      |
| IKBKG        | 16.45      | 2.83       | 4.51       |
| EGR2         | 17.33      | 12.93      | 1.05       |
| MPZ          | -1.39      | -14.10     | 12.57      |
| LIMK1        | -1.37      | -4.64      | 3.85       |
| RAB27A       | 7.13       | 12.92      | -0.91      |
| SFRP1        | 6.19       | 13.60      | -1.56      |
| S100A8       | -6.08      | 26.13      | -30.77     |
| S100A9       | -8.09      | 18.16      | -20.49     |
| C6           | 13.77      | 21.37      | -0.734     |
| PTGDS        | -11.20     | -17.76     | 5.83       |
| EPHB2        | 14.91      | 24.44      | -2.94      |
| ITGB2        | 22.91      | 17.93      | 0.40       |
| TXNIP        | 20.81      | 17.54      | -1.18      |
| LYN          | 21.26      | 19.28      | -1.59      |
| ADGRG6       | -10.54     | -17.48     | 7.17       |

DC: delayed repair contralateral side; DS: delayed repair surgery side; FC: fold change; IC: immediate repair contralateral side; IS: immediate repair surgery side.
Additional Table 5 Differentially expressed key proteins relevant to the axon regeneration process of the chronic denervation (delayed repair) and the non-chronic denervation (immediate repair) groups

| Key proteins | IS-IC (FC) | DS-DC (FC) | IS-DS (FC) |
|--------------|------------|------------|------------|
| EGR2         | 17.33      | 12.93      | 1.05       |
| SPAST        | -15.54     | -10.36     | 6.83       |
| IGF1R        | -8.59      | -19.89     | 10.69      |
| MPZ          | -1.39      | -14.10     | 12.57      |
| SHH          | -4.81      | -10.58     | 5.10       |
| PAK3         | -3.04      | -5.26      | 2.19       |
| HSPB8        | -2.57      | -4.70      | 1.87       |
| LIMK1        | -1.37      | -4.64      | 3.85       |
| GJB1         | -1.85      | -4.50      | 2.66       |
| CNTF         | -0.10      | -4.18      | 2.20       |
| CASP6        | -5.31      | 12.26      | -8.86      |
| CASP3        | 10.25      | 14.70      | 0.62       |
| CAMKK1       | -4.28      | -18.65     | 14.79      |
| SYNPO        | 15.54      | 17.40      | -6.99      |
| NTN1         | 16.96      | 13.87      | -1.20      |
| SMURF1       | -17.98     | -7.75      | -9.18      |
| ARPP19       | -12.67     | -26.14     | 8.56       |

DC: delayed repair contralateral side; DS: delayed repair surgery side; FC: fold change; IC: immediate repair contralateral side; IS: immediate repair surgery side.
**Additional Table 6** Differentially expressed key proteins relevant to the lipid metabolism process of the chronic denervation (delayed repair) and the non-chronic denervation (immediate repair) groups

| Key proteins | IS-IC (FC) | DS-DC(FC) | IS-DS (FC) |
|--------------|------------|------------|------------|
| IGF1R        | -8.59      | -19.89     | 10.69      |
| PTGDS        | -11.20     | -17.76     | 5.83       |
| TRPV1        | -8.14      | -14.97     | 7.09       |
| LGALS8       | 0.77       | -13.86     | 8.48       |
| DHH          | -2.41      | -4.87      | 2.14       |
| LIMK1        | -1.37      | -4.64      | 3.85       |
| CBL          | -2.54      | -4.64      | 1.95       |
| CNTF         | -0.10      | -4.18      | 2.20       |
| RAB27A       | 7.13       | 12.92      | -0.91      |
| CASP3        | 10.25      | 14.70      | 0.62       |
| S100A9       | -8.09      | 18.16      | -20.49     |
| S100A8       | -6.08      | 26.13      | -30.77     |
| APOD         | 4.76       | 4.80       | -0.44      |

DC: delayed repair contralateral side; DS: delayed repair surgery side; FC: fold change; IC: immediate repair contralateral side; IS: immediate repair surgery side.


Additional Figure 1 Significantly regulated pathways in the immediate repair group.

Sixty-five canonical pathways were identified by IPA pathway analysis. Five pathways (denoted by a circle that precedes the pathway name) were predicted to be activated, and 17 pathways (denoted by a square that precedes the pathway name) were predicted to be inhibited. $-\log (P \text{ value})$ was calculated by Fisher's exact test right-tailed. Orange colored bars indicate predicted pathway activation with a positive z-score (the circle preceding the pathway way name indicates that particular pathway is activated). Blue colored bars indicate predicted pathway inhibition with a negative z-score (the square preceding the pathway way name indicates that particular pathway is inhibited). The orange points connected by a thin line represent the Ratio. The ratio was calculated as follows: the number of proteins in a given pathway that met the cutoff criteria, divided by the total number of proteins that make up that pathway and that are in the reference protein set.
Additional Figure 2 Significantly regulated pathways in the delayed repair group.

Fifty-two canonical pathways were identified by IPA pathway analysis. Eight signaling pathways (denoted by a circle that precedes the pathway name) were predicted to be activated, and 21 signaling pathways (denoted by a square that precedes the pathway name) were predicted to be inhibited. –log (P value) was calculated by Fisher's exact test right-tailed. Orange colored bars indicate predicted pathway activation with a positive z-score (the circle preceding the pathway way name indicates that particular pathway is activated). Blue colored bars indicate predicted pathway inhibition with a negative z-score (the square preceding the pathway way name indicates that particular pathway is inhibited). The orange points connected by a thin line represent the Ratio. The ratio was calculated as follows: the number of proteins in a given pathway that met the cutoff criteria, divided by the total number of proteins that make up that pathway and that are in the reference protein set.