Transection injury differentially alters the proteome of the human sural nerve

Short title: Injury alters the human sural nerve proteome

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Abstract

Regeneration after severe peripheral nerve injury is often poor. Knowledge of human nerve regeneration and the growth microenvironment is greatly lacking. We aimed to identify the regenerative proteins in human peripheral nerve by comparing the proteome before and after a transection injury. In a unique study design, we collected from the same participants, samples from naïve and degenerating sural nerve. Naïve and degenerating (two weeks after injury) samples were analyzed using mass spectrometry and immunoassays. Using a correlation matrix, we found significantly altered levels following the nerve injury. Mass spectrometry revealed that post-injury samples had 672 proteins significantly upregulated and 661 significantly downregulated compared to naïve samples (q < 0.05, |FC| > 2). We used Gene Ontology pathways to highlight groups of proteins that were significantly

upregulated or downregulated with injury-induced degeneration and regeneration. Significant protein changes in key pathways were identified including growth factor levels, Schwann cell de-differentiation, myelination downregulation, epithelial-mesenchymal transition, and axonal regeneration pathways. Having proteome signatures of human peripheral nerves of both the uninjured and the degenerating/regenerating state may serve as biomarkers to aid in the future development of repair strategies and in monitoring neural tissue regeneration.

Introduction

After severe peripheral nerve injury, there is poor regeneration and functional recovery. Understanding the transcriptome[1], proteome, and the mechanisms that underlie peripheral nerve regeneration is essential to further develop peripheral nervous system (PNS) regenerative therapies and to improve clinical outcomes. Even though there is a great deal that is known about peripheral nerve regeneration and the growth-permissive microenvironment in animal models, results on human nerves is sparse and inconclusive[2,3]. Multiple components contribute to peripheral nerve repair after injury notably Schwann cells[4-7], fibroblasts, endothelial cells, and immune cells such as macrophages[8-10]. An injured peripheral nerve undergoes three main processes for recovery and reestablishment of a functional connection with its distal end[11]. First, within 24-48 hours after transection injury of the nerve, Wallerian degeneration starts in which the distal end of the transection undergoes axonal degeneration. Afterward, the myelin is degraded as macrophages infiltrate this area. Secondly, axonal regeneration begins, leading to the third step of end-organ re-innervation. Macrophages and Schwann cells play major roles in mediating degeneration and regeneration.

Animal models of peripheral nerve regeneration demonstrate that Schwann cells have remarkable plasticity. They rapidly adapt to injury through extensive cellular reprogramming that

transforms their myelinating phenotype into a reparative phenotype[12,13]. Studies have delineated two phases of the Schwann cell injury response: 1) clearing of myelin[4] and de-differentiation of the cell's myelinating phenotype and 2) maturation into the repair Schwann cell phenotype in which the cells promote survival by releasing neuroprotective factors and increasing anti-apoptosis factors. Schwann cells upregulate and release a whole host of neurotrophic and cell survival factors including glial cell-derived neurotrophic factor [14,15], neurotrophin-3 (NT-3), nerve growth factor (NGF)[16,17], brain derived neurotrophic factor (BDNF)[18], vascular endothelial growth factor (VEGF), erythropoietin [19], pleiotrophin, and N-cadherin[6,7,20-22].

Human peripheral nerve injury studies are typically limited to collecting nerve tissues *after* an injury (*e.g.* after a trauma) while obtaining healthy, uninjured nerve beforehand has not been as feasible. As such, many aspects of human peripheral nerve regeneration are unknown including the profile of protein upregulation and downregulation. Understanding these changes would help optimize discovery and clinical management strategies for improving PNS repair outcomes.

To address these gaps in knowledge, we performed a comprehensive proteomic analysis to include more than 5000 of the most abundant proteins found in peripheral (sural) nerve *before and after* a transection injury. We hypothesized that transecting a peripheral nerve and leaving it in place for two weeks would increase synthesis in the pathways associated with nerve regeneration. Thus, this proteome profile would provide a database to identify new targets for improving regeneration outcomes.

Materials and Methods

Research Subjects

The University of Kentucky's Institutional Review Board approved the study and the participants provided written informed consent. Sural nerve tissue was collected from 26 participants before and after sural nerve transection *in situ*. Participants were diagnosed with idiopathic PD. Nine were female, 17 were male. Their ages ranged from 50-70 years old (mean 61 years old). Years diagnosed with PD ranged from 2-17 years (mean 10 years). Tables 1 and 2 list participant demographics from both types of proteomic analyses (mass spectrometry and targeted immunoassays). Note that there are three participants whose tissues were used in both mass spectrometry and targeted immunoassay.

Table 1. Mass Spectrometry Participant Demographics

Participant	Age (years)	Years diagnosed	Assigned Birth Sex
1	70	7	Male
2	70	12	Female
3	63	11	Male
4	69	9	Male
5	66	2	Female
6	66	16	Male
7	52	6	Female
8	60	6	Male
9	57	10	Male
10	60	7	Female
11	50	8	Male
12	61	9	Male
13	61	8	Male
14	65	6	Male

Table 1. Mass spectrometry study participant demographics listed include age, years diagnosed with PD, and assigned birth sex.

86

Table 2. Targeted Immunoassays Participant Demographics

Participant	Age (years)	Years diagnosed	Assigned Birth Sex
15	51	17	Male
7	52	6	Female
17	54	16	Male
18	56	10	Male
19	58	12	Female
20	60	13	Female
21	61	15	Female
13	61	8	Male
23	62	4	Male
24	62	7	Male
25	64	16	Male
26	65	8	Male
14	65	6	Male
28	66	9	Female
29	69	8	Female

87 Table 2. Immunoassay study participant demographics listed include age, years diagnosed with PD,
88 and assigned birth sex.

89

90 **Peripheral Nerve Transection and Tissue Collection**

91 Our approach in transecting the peripheral nerve and collecting tissue has been previously
92 described[1,23,24]. Fig 1 illustrates the timing of the transection and tissue collections. Briefly, sural
93 nerve samples were collected at two different time points: a naïve non-injured sample collected during
94 the first stage of surgery followed by a post-transection (injured) sample from the second stage of
95 surgery two weeks later (. To collect the naïve tissue, the neurosurgeon identified the neurovascular
96 bundle containing the sural nerve in the ankle. Two sutures were tied around the nerve, 1 cm apart. The
97 section of nerve just proximal to the sutures was transected, and a 1-cm segment was excised for
98 analysis. Individual nerve fascicles were separated, snap-frozen, and stored for assays as in Welleford
99 et al.[1].

To collect post-transection tissue for both implantation in the clinical trial and proteomic analysis, the ankle incision was reopened after two weeks, suture markers were located, and a new 1-2 cm segment was collected from the distal nerve stump for analyses and implantation as part of the trial design. Individual nerve fascicles were separated, snap-frozen, and stored for assays[1].

Fig 1. Study Overview Schematic to illustrate the timing and location of the sural nerve injury and tissue collection

Proteomics Analyses

Mass spectrometry proteomics

TMT analysis was used which is a global, unbiased method. As many proteins/peptides as possible were detected and searched against a species-specific database. Total protein was isolated from peripheral nerve tissue samples. Protein samples were prepared for mass spectrometric analysis, and MS/MS sequence and tandem mass tag reporter ion data was collected using a state-of-the-art Orbitrap Eclipse instrument. Quantitative analysis was performed to obtain a comprehensive proteomic profile.

Proteins were identified and quantified using MaxQuant and visualized with Scaffold using 1% false discovery thresholds at both the protein and peptide level. The data was checked for quality and normalized using an in-house ProteiNorm Shiny app, a tool for a systematic evaluation of normalization methods, imputation of missing values and comparisons of different differential abundance methods.

CME bHPLC TMT Methods – Orbitrap Eclipse:

Proteins were reduced, alkylated, and purified by chloroform/methanol extraction prior to digestion with sequencing grade modified porcine trypsin (Promega). Tryptic peptides were labeled using tandem mass tag isobaric labeling reagents (Thermo) following the manufacturer's instructions and combined into three 11-plex sample groups with a pooled reference sample in each group. Each labeled peptide multiplex was separated into 46 fractions on a 100 x 1.0 mm Acquity BEH C18 column using an UltiMate 3000 UHPLC system (Thermo) with a 50 min gradient from 99:1 to 60:40 buffer A:B ratio under basic pH conditions, and then consolidated into 18 super-fractions. Each super-fraction was then further separated by reverse phase XSelect CSH C18 2.5 μ m resin on an in-line 150 x 0.075 mm column using an UltiMate 3000 RSLCnano system (Thermo). Peptides were eluted using a 60 min gradient from 98:2 to 60:40 buffer A:B ratio. Eluted peptides were ionized by electrospray (2.2 kV) followed by mass spectrometric analysis on an Orbitrap Eclipse Tribrid mass spectrometer (Thermo) using multi-notch MS3 parameters with real-time search enabled. MS data were acquired using the FTMS analyzer in top-speed profile mode at a resolution of 120,000 over a range of 375 to 1500 m/z. Following CID activation with normalized collision energy of 35.0, MS/MS data were acquired using the ion trap analyzer in centroid mode and normal mass range. Using synchronous precursor selection, up to 10 MS/MS precursors were selected for HCD activation with normalized collision energy of 65.0, followed by acquisition of MS3 reporter ion data using the FTMS analyzer in profile mode at a resolution of 50,000 over a range of 100-500 m/z.

Buffer A = 0.1% formic acid, 0.5% acetonitrile

Buffer B = 0.1% formic acid, 99.9% acetonitrile

Both buffers adjusted to pH 10 with ammonium hydroxide for offline separation

143 Mass Spectroscopy Statistical Analysis:

144 Each protein in the data set had a log fold change and false discovery rate (FDR) adjusted p-
 145 value. For the proposed experiments, the units used for statistical comparison were normalized MS3
 146 TMT reporter ion intensities. We used the limma package for statistical analysis, which is a moderated
 147 t-test that accounts for the data variance. We ran a paired sample model using limma. The final results
 148 contained the log2 fold change, unadjusted p-values, and FDR adjusted p-values. Significance threshold
 149 is a Fold change > 2 and a FDR adjusted p-value < 0.05. Each data set had its own characteristics so we
 150 performed QC checks of data prior to running statistical analyses to ensure the most appropriate
 151 normalization method and statistical test was run.

152

153 Correlation matrix:

154 Mass spectrometry quantification (log 2 scale, Cyclic Loess normalized) data included 5,573
 155 rows for different proteins mapped back to their gene symbol representations for 28 paired (naïve and
 156 injured) samples from 14 subjects.

157

158 Pre-statistical procedures:

159 **‘0’ values:** Seventeen percent of the data space (# rows * # samples = 156,044) was occupied
 160 by ‘0’ values. These ‘0’ values could represent low levels (below the detection threshold), no levels, or
 161 technical error[25]. In proteomic data, ‘0’s are considered missing-not-at-random’ (MNAR), and
 162 various strategies have been proposed to address this property of the data[26]. Here, we used a two-step
 163 procedure to address the ‘0’s. First, the number of ‘0’s on each row were quantified and rows with < 20
 164 / 28 ‘0’s (i.e., at least seven reliable signal intensity measures on the row) were retained for further

analysis (5,508 rows of data were retained). Second, within this filtered data set, the remaining 0's were treated as missing values.

Annotation: Fourteen protein symbols with date-like names (from the MAR, MARC, SEP, and SEPT families) were updated to reflect newer non-date-like protein symbols (e.g., SEP15 was changed to SELENOF) to avoid their accidental conversion to date codes in spreadsheet programs. Additionally, 434 instances of 'repeated' annotations (that is, more than one row reporting the same gene symbol) were identified. Among these, the repeated protein symbol with the highest average level was retained for analysis, resulting in a final data set of (28 samples * 5074) uniquely protein symbol mapped proteins with reliable signal intensities.

174

Volcano plot:

For the total of 5074 proteins included in the analysis, log 2 fold changes (x axis) were plotted against the log 10 p-values (y axis) for each protein. Highly stringent p-value ($\leq 1E-10$) and fold change (FC) ($\geq |16|$; or as expressed on the log 2 scale, $\geq |4|$) cutoffs were used to highlight proteins with the largest and most significant changes.

180

GO pathway analyses and heatmap generation:

Gene ontology pathways that were specifically related to peripheral nerve injury and regenerative processes were analyzed. The visualized mapped proteins represent the significantly differentially expressed proteins from each pathway. The proteins that corresponded to the GO pathways that were significantly upregulated or downregulated based off the p value and Log 2 FC were graphed in clustered hierarchical heatmaps using Ward's hierarchical clustering.

Heatmaps were created in JMP software (SAS Institute, Cary NC). The protein and its fold change were mapped using hierarchical clustering. The range of fold changes were illustrated with a red/blue gradient and 0 was set at the center with gray. Note that participants' samples that lacked either naïve or injured tissue results (e.g. because of insufficient amounts of tissue) were removed from analysis so as to not misrepresent fold changes. These are marked with a black and white grid pattern in the heatmaps.

Immunoassays for proteins of interest: ELISA and Luminex

Certain proteins of interest were not detected because they were not in high enough abundance to be detected by mass spectrometry. For these proteins of interest, such as growth factors, we used enzyme-linked immunosorbent assay (ELISA) and multiplex Luminex® immunoassays (Cincinnati Children's Hospital Flow Cytometry Core) to detect their change after injury. We compared naïve and injured tissues, from 15 participants, three of these participants' tissues were also used in mass spectrometry analysis (see Tables 1 and 2).

Analyte concentrations in the sample supernatants were determined by ELISA according to manufacturer's protocol. The ELISA and antibody sources and dilutions were: Cerebral dopamine neurotrophic factor (CDNF) (Abcam, Cambridge, MA), samples were diluted 1:100. Nuclear factor erythroid 2-related factor 2 (NRF2) (ThermoFisher Scientific, Carlsbad, CA), samples diluted 1:2. Nerve Growth Factor Receptor (NGFR) (ThermoFisher Scientific, Carlsbad, CA), samples were diluted 1:2. B cell lymphoma 6 (BCL-6) (MyBiosource, San Diego, CA), samples were neat. EPO concentrations in the sample supernatants were determined by using Milliplex™ Multiplex kits (MilliporeSigma, Darmstadt, Germany) and BDNF, Beta-NGF, Platelet-Derived Growth Factor

(PDGF)-AA, VEGF, GDNF, NT-3, PDGF-BB, PDGF-AB were determined by Human Magnetic Luminex Assays (R&D Systems, Minneapolis, MN) according to manufacturer's protocol.

211

212 Analysis:

213 For each protein analyzed with the immunoassay, the mean difference between the naïve and
214 injured samples and the 95% confidence interval (Excel, Microsoft) is reported.

215

216 Availability of Data and Materials

217 Datasets are available at (<https://uknowledge.uky.edu/>).

218

219 Results

220 A clear proteomic distinction between naïve and injured nerve

221 Protein levels were significantly altered after nerve injury (Fig 2A) based on
222 Pearson's *r* correlation. The Pearson's correlation *r*-value for each sample, correlated with every other
223 sample is displayed in a correlation matrix.

224 We calculated Lin's Concordance Correlation Coefficient (CCC), which quantifies degree of
225 similarity among different samples. Results indicated strong positive, and highly similar profiles from
226 each sample compared to the average of all samples within either the pre-injury or post-injury groups
227 (Fig 2B). CCCs in Pre-injury (CCC= 0.936 +/- 0.0065) and Post-injury (CCC = 0.93 +/- 0.012)
228 conditions were greater than the 0.8 typically interpreted as showing strong similarity, and thus indicates
229 generally strong homogeneity among samples within the pre- and post-injury groups.

Among the largest and most significant protein synthesis changes of the *whole* mass spectrometry dataset, the volcano plot shows 17 proteins that were downregulated (blue) and 41 upregulated proteins (red) after injury (Fig 2C). The volcano plot cutoffs were more stringent than in each individual Gene Ontology (GO) heatmap and highlight the most upregulated and downregulated proteins of the whole data set.

Fig 2. Clear Proteomic Changes Between Naïve and Injured Nerve **A.** Protein levels were significantly altered after nerve injury based on Pearson r correlation. The Pearson's correlation R-value for each sample, correlated with every other sample is displayed in a correlation matrix of naïve and injured samples (matrix scale orange R = 1: more similar, green R = -1: less similar). **B.** Lin's CCC demonstrated strong homogeneity among samples within the pre- and post-injury groups **C.** The volcano plot shows proteins that were downregulated (blue) and upregulated (red) after injury

Significant changes in protein changes shown in Gene Ontology (GO) pathways and heatmaps

Mass spectrometry analysis of naïve and injured sural nerve returned a total of 5573 of the most abundant proteins in the tissues. When comparing naïve to injured nerve, 672 proteins were significantly upregulated and 661 were significantly downregulated. We used heatmaps to highlight significantly changed GO pathways related to tissue injury degeneration and regeneration.

Growth Factors

Mass Spectrometry: Fig 3 shows all of the proteins that were significantly upregulated or downregulated ($q < 0.05$, $|\text{FC}| > 2$) from the GO pathway, Growth Factor Activity (GO:0008083). Six proteins were significantly downregulated after transection, and five proteins were significantly upregulated after injury (Fig 3). Out of 163 unique genes listed in this GO pathway, 11 corresponding proteins (7%) were significantly differentially expressed when comparing naïve and injured tissue.

Immunoassay: Immunoassays were used to quantify the change in protein content of growth factors of interest that were not abundant enough to be detected via mass spectrometry. These factors of interest included EPO (mean difference: -17.37 pg/ml, $n = 13$), PDGF-AA (+1.58 pg/ml, $n=13$) and PDGF-BB (+1.81 pg/ml, $n=15$), VEGF (+2.96 pg/ml, $n=15$), BDNF(+5.07 pg/ml, $n=15$), NGFR (+0.18 ng/ml, $n=14$), beta-NGF (+38.63 pg/ml $n=12$), CDNF (62.2 pg/ml $n=15$), and GDNF (+51.97 pg/ml, $n=7$), with the 95% confidence interval (CI) of the mean difference between injured and naïve tissues of PDGF-BB (-0.02, 3.65), PDGF-AA (-0.26, 3.42), EPO (-97.6, 62.9), VEGF (0.27, 5.66), BDNF (0.45, 9.68), CDNF (-5.6, 130.1), beta-NGF (6.19, 71.09), NGFR (0.03, 0.32), and GDNF (18.48, 85.45)(Fig 3B).

Fig 3. Changes in growth factors after injury based on the GO pathway “Growth Factor Activity
A. Mass Spectrometry: Analysis of all the protein products that were significantly upregulated or downregulated ($\text{Log}_2(\text{FC})$ range: -6 to 6). Proteins are organized by Ward hierarchical clustering. **B. Immunoassay:** Mean (triangle) differences, between naïve and injured tissue samples, and 95% CI for EPO, PDGF-AA and -BB, VEG , BDNF, NGFR, beta-NGF, and GDNF. Red markers represent mean difference CIs that were above zero and gray ones represent mean difference CIs that include zero.

Mass Spectrometry: Fig 4 shows all of the proteins that were significantly upregulated or downregulated from the GO pathway “Growth Factor Binding” (GO: 00019838). Seven proteins were significantly downregulated after transection, and 13 proteins were significantly upregulated after transection. Out of 137 unique genes listed in this GO pathway, 20 gene protein products (15%) were significantly differentially expressed when comparing naïve and injured tissue.

Fig 4. Changes in growth factor binding after injury based on the GO pathway, “Growth Factor Binding.” : Analysis of all the protein products that were significantly upregulated or downregulated from the GO pathway (Log2(FC) range: -6 to 8)

Myelination

Mass Spectrometry: Fig 5 shows all of the protein products that were significantly upregulated or downregulated from the GO pathway “myelination” (GO: 0042552). Twenty five proteins were significantly downregulated after transection (19% of 132), and 6 proteins (5% of 132) were significantly upregulated after transection. Out of 132 unique genes listed in this GO pathway, 31 gene protein products (24%) were significantly differentially expressed when comparing naïve to injured nerve.

Fig 5. Changes in myelination after injury based on the GO pathway “Myelination.” Analysis of all the protein products that were significantly upregulated or downregulated from the GO pathway (Log2(FC) range: -12.5 to 5)

295 Schwann cell phenotypic changes

296 Schwann Cell Differentiation

297 Mass Spectrometry: Fig 6A shows all of the protein products that were significantly upregulated
298 or downregulated from the GO pathway “Schwann cell differentiation” (GO: 0014037). Ten proteins
299 were significantly downregulated after injury, and no proteins were significantly upregulated after
300 injury. Out of 39 unique genes listed in this GO pathway, 10 gene protein products (26%) were
301 significantly differentially expressed when comparing naïve to injured nerve.

302 Immunoassay: We quantified the content of total c-JUN (mean difference: +0.03 OD=450,
303 n=15, CI: 0.02, 0.06) and its activated form phospho c-JUN (+0.01 OD=450, n=15, CI: -0.0007, 0.0138)
304 in the naïve and injured tissue(Fig 6A).

305

306 Epithelial to Mesenchymal Transition (EMT)

307 Mass Spectrometry: Fig 6B shows all of the protein products that were significantly upregulated
308 or downregulated from the GO pathway “EMT” (0001837). Four proteins were significantly
309 downregulated after injury, and 6 proteins were significantly upregulated after injury. Out of 146 unique
310 genes listed in this GO pathway, 10 gene protein products (7%) were significantly differentially
311 expressed when comparing naïve to injured nerve.

312

313 **Fig 6. Changes in Schwann cell phenotype after injury based on the GO pathway “Schwann cell**
314 **differentiation” A. Mass spectrometry:** Aall the proteins that were significantly upregulated or
315 downregulated (Log2(FC) range: -4 to 4) **B. Immunoassay:** We quantified the protein expression of c-
316 JUN and its activated form phospho c-JUN, in the naïve and injured tissue and show the mean difference

and 95% CI bands. Red markers represent mean difference CIs that were above zero and gray ones represent mean difference CIs that include zero. **C.** Graph of mass spectrometry analysis of all the protein products that were significantly upregulated or downregulated from the GO pathway, “EMT.”

320

321 **Anti-apoptosis**

Mass Spectrometry: Fig 7 shows all of the protein products that were significantly upregulated or downregulated from the pathway “Negative Regulation of Apoptotic Processes” (GO: 0043066). Thirty nine proteins were significantly downregulated after injury, and 42 proteins were significantly upregulated after injury. Out of 890 unique genes listed in this GO pathway, 81 gene protein products (9%) were significantly differentially expressed when comparing naïve to injured nerve. Because we used samples from participants with PD, we particularly examined levels of α -synuclein (SNCA) as aggregates of α -synuclein are associated with PD and deposits of phosphorylated α -synuclein[27] are found in the sural nerve of people with PD. We observed a significant reduction (FC: -1.97 , $p = 9.2 \times 10^{-7}$) in levels of α -synuclein following injury.

Immunoassay: Additionally we examined for NRF2 (mean difference: $+13.96$ pg/ml, $n=14$, CI: -3.39 , 31.30) and BCL-6 ($+0.62$ ng/ml, $n=15$, CI: 0.24 , 1.01) where the 95% CI for BCL-6 was above zero in the injured tissue compared to naïve tissue (Fig 7)

334

Fig 7. Anti-apoptosis factor changes after injury based on the GO pathway, “Negative Regulation of Apoptotic Processes.” **A.** Mass spectrometry analysis of all the protein products that were significantly upregulated or downregulated (Log2(FC) range: -10 to 10) **B.** Immunoassay: Quantified

338 NRF2 and BCL-6 changes after injury. Red markers represent mean difference CIs that were above zero
339 and gray ones represent mean difference CIs that include zero.

340

341 **Response to Axonal Injury**

342 Mass Spectrometry: Fig 8A shows all of the protein products that were significantly upregulated
343 or downregulated from the GO pathway, “response to axonal injury” (0048678). This is defined as “any
344 process that results in a change in state or activity of a cell or an organism (in terms of movement,
345 secretion, enzyme production, gene expression, etc.) as a result of an axon injury stimulus”. Twelve
346 proteins were significantly downregulated after injury, and 4 proteins were significantly upregulated
347 after injury. Out of 75 unique genes listed in this GO pathway, 16 gene protein products (21%) were
348 significantly differentially expressed when comparing naïve to injured nerve.

349

350 **Axonal Regeneration**

351 Mass Spectrometry: Fig 8B shows all of the protein products that were significantly upregulated
352 or downregulated from the GO pathway, “Axonal regeneration” (0031103). This is defined as “The
353 regrowth of axons following their loss or damage.” Nine proteins were significantly downregulated after
354 injury, and one protein was significantly upregulated after injury. Out of 44 unique genes listed in this
355 GO pathway, 10 gene protein products (23%) were significantly differentially expressed when
356 comparing naïve to injured nerve tissue.

357

358 **Fig 8. Axonal response after injury** A. Graph of mass spectrometry analysis of all the protein products
359 that were significantly upregulated or downregulated from the GO pathway, “Response to Axonal

360 Injury” (Log2(FC) range: -10 to 4) **B.** Graph of mass spectrometry analysis of all the protein products
361 that were significantly upregulated or downregulated from the GO pathway, “Axonal Regeneration”
362 (Log2(FC) range: -10 to 2)
363

364 Discussion

365 The goal of this study was to determine how protein levels change after human peripheral nerve
366 injury. Our study used closely matched internal controls, namely participants’ own naïve and injured
367 peripheral nerve samples to show how the nerve responds with degenerative and regenerative processes.
368 The nerve transection was a necessary event for our underlying trial, an approach which harnesses the
369 regenerative capacity of the peripheral nerve for treating neurodegenerative diseases. Our group was
370 able to transect a peripheral nerve, activate degeneration and regeneration in human sural nerve all *in*
371 *situ* at the ankle, and collect tissue samples from both conditions, which would not have been ethically
372 possible before this trial. Sural nerve biopsies are common neurosurgical procedures that can result in
373 localized pain and paresthesias[28]. Participants in our studies reported these adverse events acutely but
374 found them tolerable and not bothersome over time. The findings here provide an overview of the
375 proteomic changes in peripheral nerve degeneration/regeneration and could serve as a basis for targeted
376 therapies to promote nerve regeneration and improve clinical outcomes.

377

378 Myelination and schwann cell phenotype after injury

379 Schwann cells play a major role in supporting peripheral nerves through myelination,
380 maintaining axons, and guiding axons in regeneration after injury. Schwann cells from peripheral nerve

381 tissue have remarkable plasticity in that they rapidly adapt to injury through cellular transformation into
382 a reparative phenotype in two stages. First, upon injury or transection, the transcription factor c-JUN is
383 upregulated, and Schwann cells de-differentiate from a myelinating phenotype into an immature
384 phenotype, undergoing EMT[29,30]. Second, Schwann cells transform into a reparative phenotype and
385 release trophic factors and cytokines to support neuronal survival, and axon regeneration[7,30-32].

386 Our proteomic results support many of the processes reported in the literature in animal models.
387 Myelin-related proteins such as MPZ, MBP, and MAG are downregulated two weeks after injury (Fig
388 5)[30]. After injury, the first step of degeneration is clearing the myelin[4]. Schwann cells then de-
389 differentiate. As such, we observed the downregulation of S100B which is expressed by myelinating
390 Schwann cell[33].

391 c-JUN, is the major transcription factor that activates the Schwann cell repair program by first
392 downregulating the myelinating Schwann cell phenotype[6,7,31,32,34]. Both c-JUN and its activated
393 form, phosphorylated c-JUN, were present more abundantly two weeks after injury than in the naïve
394 tissue (Fig 6A). Interestingly, in our previous work with this transection paradigm[1], *JUN* mRNA levels
395 were not detectable at two weeks suggesting that the peak for mRNA had diminished while the
396 synthesized protein still remained.

397 c-JUN initiates Schwann cell de-differentiation into a progenitor-like stage, and upregulates
398 stem cell factors such as Sox2, Notch1, Oct6 as a transition between two states. The Schwann cells
399 undergo aEMT-like process in which cells become more like multipotent stem cells, and release
400 neurotrophic factors to support cell survival[2]. *JUN* is rarely expressed in normal, non-injured human
401 nerve tissue[3]; however after injury, it was elevated in human nerve samples denervated for 4–50 days
402 and up to 200 days[29]. In the early response to injury, in many tissues, activation of EMT and stemness

is associated with increased cell motility, proliferation, phenotypic flexibility, tissue remodeling and differentiation flexibility[35,36], all which are important in repair and neuroprotection.

Schwann cells transform from the myelinating phenotype to a reparative phenotype via EMT mechanisms[29,30]. Our results show that proteins expressed by Schwann cells such as PRX and LAMA2[37,38] were downregulated after injury. We showed the upregulation of proteins in the EMT GO pathway, including SFRP2 (Fig 6). SFRP2 and SFRP4 are typically associated with concomitant EMT gene expression in a pan-cancer study[39]. The adaptor molecule, disabled-2 (DAB2) promotes EMT[40], and two weeks after injury, the protein was also upregulated in our results[7,30-32] (Fig 6B).

Growth Factors After Injury

The response to injury creates a neuroprotective microenvironment with the production of neurotrophic factors. Our results here support our hypothesis and previous findings that growth factor levels, specifically neurotrophic factors, are higher after nerve injury[7,30-32]. Two weeks after injury, we observed higher mean levels in the injury samples vs. the naïve samples of growth factors: BDNF, CDNF, VEGF, GDNF, NGF, PDGF-AA and PDGF-BB (Fig 3B). The protein-level profile of individual neuroprotective factors can vary greatly with time. After injury in mice, GDNF peaks around 7 days, and BDNF levels peak around 2–3 weeks[31]. Trophic factors involved in neurite outgrowth, cytoprotection, and neuronal survival such as mesencephalic astrocyte-derived neurotrophic factor (MANF) have also been reported to increase after peripheral nerve injury[41,42]. Interestingly, some growth factors such as CNTF and FGF1 that have been shown in the literature[43-45] to promote nerve regeneration were significantly decreased after the two week injury paradigm, aligning with our

findings. This could indicate that the two week timepoint is past the peak expression time for these factors in humans.

Other notable protein level increases from our findings include Glia Maturation Factor Gamma (GMFG) and tissue inhibitor of metalloproteinase-1 (TIMP1). TIMPs play a major role in extracellular matrix (ECM) and tissue remodeling through its action as a potent inhibitor of ECM proteases such as matrix metalloproteinase 9 (MMP9). In a sciatic nerve axotomy model, *Timp1* was found to be the top 6th gene that was induced[46]. Further supporting its role, mice with the *Mmp9* gene knocked out showed greater numbers of de-differentiated/immature myelinating Schwann cells in the injured nerve. This model also suggests that the *Mmp9/Timp1* axis guides myelinating Schwann cell differentiation and the molecular assembly of myelin domains during nerve regeneration[46].

Data Interpretation

Important limitations apply to the interpretation of these results. We used naïve and injured nerve tissue from participants with PD which may differ from non-PD tissue. Synucleinopathies are characteristic in people with PD in both their central and peripheral nervous systems. α -synuclein aggregates have been found in the sciatic nerves and pharyngeal nerves of post-mortem patients with PD[47]. Of note, our results from mass spectrometry show that the α -synuclein protein was significantly downregulated after transection injury. The transection did not cause further upregulation of α -synuclein. Furthermore, as people with PD have a higher incidence of neuropathy[48,49], we recognize that using tissue from participants with PD introduces the concern of neuropathies. The presence of a neuropathy was not an exclusionary criterion for the underlying clinical trial and most participants in the trial did not report a history of neuropathy.

446 As in Welleford et al.[1], the design of the underlying clinical trial necessitated a delay in flash
 447 freezing the samples, more so for the injury samples, that could affect the protein profile. However, the
 448 direction of changes in protein levels, after injury, in the GO pathways of myelination, EMT, and axonal
 449 regeneration suggest that elements of the proteome in the injured state are in concordance to reported
 450 changes in animal studies [2,15,16,31]. The underlying clinical trial design from where these samples
 451 were obtained limited our ability to adequately control for the existence of neuropathy, comorbidities
 452 that might influence regeneration, age, disease duration, genetics of participants or even the sample
 453 freezing time differences among individuals; thus, for interpreting the analyses, we quantified the degree
 454 of similarity among different samples by calculating Lin's CCC (Fig 2B). We found a strong
 455 homogeneity among samples within the naïve and injured groups.

456 Therefore, even with these limitations, we think this study provides insight on the proteomic
 457 profile of degenerating/regenerative nerves two weeks after an injury and contributes a database of
 458 useful information for establishing biomarkers of peripheral nerve injury in humans.

459

460 **Summary**

- 461 ◦ We present a proteomic picture of the degenerative and regenerative processes in humans after
 462 peripheral nerve injury *in situ*
- 463 ◦ We provide a database for peripheral nerve repair proteins
- 464 ◦ Our results support many processes reported in animal models—down-regulation of
 465 myelination, Schwann cell de-differentiation, and upregulation of growth factors

466

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B

C

Growth factors**A** **B**

Growth factor binding

Myelination

EMT + Schwann cell differentiation

A Schwann cell differentiation

B EMT

Anti-apoptosis

AResponse to axonal injury **B**Axonal regeneration