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**Native paraneurial tissue and paraneurial adhesions alter nerve strain distribution
in rat sciatic nerves**

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additionally contributed to the research design and manuscript writing. All authors edited
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1 **Abstract:**

2 Paraneurial adhesions have been implicated in the pathological progression of
3 entrapment neuropathies. Surgical decompression of adhesions is often performed, with
4 the intent of restoring nerve kinematics. The normal counterpart of adhesions, native
5 paraneurium, is also thought to influence nerve deformation and mobility. However,
6 influences of native or abnormal paraneurial structures on nerve kinematics have not
7 been investigated. We measured regional strains in rat sciatic nerves before and
8 immediately after decompression of native paraneurial tissue, and before and after
9 decompression of abnormal paraneurial adhesions, which formed within 6 weeks of the
10 initial decompression. Strain was significantly higher in the distal-femoral than the mid-
11 femoral region of the nerve before either decompression. Decompression of native and
12 abnormal paraneurial tissue removed this regional strain difference. Paraneurial tissues
13 appear to play a major role in distributing peripheral nerve strain. Normal nerve strain
14 distributions may be reconstituted following decompression, even in the presence of
15 paraneurial adhesions.

16

17 **Introduction:**

18 Peripheral nerves are dynamic structures that must withstand significant mechanical
19 forces while reliably conducting electrical impulses. Because nerves traverse
20 articulating joints and frequently follow paths outside of the normal plane of joint motion,
21 they stretch and glide during movement, while maintaining structural and functional
22 integrity.(Topp and Boyd, 2006) Animal models have demonstrated that when nerves
23 are subjected to prolonged or excessive strain above physiological thresholds, their
24 electrical function is compromised.(Clark et al., 1992; Tanoue et al., 1996; Wall et al.,
25 1992) In humans, excessive traction related to mechanical impingement is believed to
26 contribute to the progression of entrapment neuropathies, such as carpal and cubital
27 tunnel syndromes.(Mackinnon, 2002; Mahan et al., 2015; Ogata and Naito, 1986;
28 Rempel et al., 1999; Tanoue et al., 1996)

29

30 Peripheral nerves are believed to have several adaptations to protect against excessive
31 strain. One such protective mechanism, observed in both animal and human cadaveric
32 models, is the increased compliance of nerves at joint regions compared with non-joint
33 regions (Mason and Phillips, 2011; Phillips et al., 2004), to accommodate increased
34 strains in the vicinity of articulating joints.(Boyd et al., 2005; Topp and Boyd, 2006;
35 Wright et al., 2001; Wright et al., 1996) This heterogeneity in nerve compliance is
36 observed in nerves both *in vivo* and *ex vivo*, and allows them to accommodate the
37 increased physiologic strain associated with synovial joint motion without resulting in
38 mechanical failure. The structural basis for this heterogeneous strain profile has not
39 been fully elucidated. Mason and Phillips revealed that rat median nerves possess
40 subtle differences in collagen fibril geometry between joint and non-joint regions,
41 possibly explaining these biomechanical differences.(Mason and Phillips, 2011)
42 However, the same study also indicated that such structural variance is not conserved
43 across all nerves,(Mason and Phillips, 2011) suggesting the possibility of additional
44 adaptations that regulate local compliance.

45

The paraneurium, also referred to as mesoneurium, is another possible modulator of local nerve compliance. It is composed of connective tissue that attaches the peripheral nerve to its surrounding nerve bed, and also carries the nerve's extrinsic segmental blood supply.(Lundborg, 1979) It has been hypothesized that the paraneurium serves a mechanical function, guiding the trajectory of peripheral nerves within their beds and facilitating low-friction nerve "gliding".(Butler, 2000; Mazal and Millesi, 2005; Millesi et al., 1995; Smith, 1966) Moreover, the paraneurium has a pathologic counterpart frequently encountered in a surgical or other clinical setting: adhesions,(Millesi et al., 1993) which often describe abnormal connections between two tissue structures, such as two loops of bowel.(Smith, 1966) In peripheral entrapment neuropathies such as cubital tunnel syndrome, paraneurial adhesions are often implicated as causal agents of neural impingement and associated changes in traction, abnormally tethering the nerve to its surrounding structures.(Abe et al., 2005; Foran et al., 2016; Ochi et al., 2014; Topp and Boyd, 2006) Additionally, there is concern that adhesions eventually contribute to intraneural fibrosis, resulting in intraneural structural changes with deleterious mechanical and functional consequences(Ikeda et al., 2003; Yamamoto et al., 2010), as well as increased risk for recurrent entrapment (Botte et al., 1996; McCall et al., 2001; Steyers, 2002). The mainstay of surgical management of these syndromes often involves some form of neurolysis or decompression,(Foran et al., 2016; Millesi et al., 1993) with the intent of relieving mechanical effects of traction and entrapment on the nerve.

Despite empirical evidence supporting a mechanical role for the paraneurium, as well as a well-documented role for adhesions in the progression of entrapment neuropathy, influences of native or abnormal paraneurial tissue on peripheral nerve strain have not been examined. In this study we used a rat sciatic nerve model to investigate the effect of both normal paraneurial tissue and paraneurial adhesions, and the effect of surgical neurolysis of these tissues, on peripheral nerve strain.

Methods:

Rat surgeries and image analysis:

All procedures were completed under protocols approved by our institutional IACUC. Twelve 10-week-old male Lewis rats (Harlan (now Envigo), Placentia, CA) were studied in this experiment. Rats were kept in standard group housing, 3 rats to a cage, with a normal diet and water. The rats were induced and maintained under anaesthesia at 10 weeks of age with inhalational isoflurane delivered via nose cone. A 15 mm incision was made on the posterolateral thigh overlying the sciatic nerve with aseptic technique. The right or left sciatic nerve was chosen randomly for study by a random number generator (RANDOM.ORG). Using a gluteus splitting technique, the sciatic nerve was then carefully exposed using a combination of sharp and blunt dissection under a dissecting microscope from the level of the quadratus femoris muscle to the trifurcation of the tibial, common peroneal, and sural nerves. Care was taken not to disturb the paraneurial attachments of the nerve to the underlying nerve bed. Using a tissue marking pen, 5 epineurial markers were placed at about 4mm increments with the most proximal marker at the level of the quadratus femoris muscle and most distal marker at the level of the trifurcation (Figure 1). The region between the first and third marker was designated as “mid-femoral,” while the region between the third and fifth marker was defined as “distal-femoral.” A sterile ruler was placed into the wound to set scale, and pictures were then taken with the knee flexed to 90° and ankle maximally plantarflexed (relaxed configuration) and the knee extended to 0° and the ankle maximally dorsiflexed (stretched configuration). Next, the paraneurial connections from the level of the quadratus femoris to the trifurcation were sharply dissected with tenotomy scissors (decompression), with the knee in full flexion to minimize strain on the nerve during decompression. The knee and ankle were then placed into the two configurations above and pictures were again taken. The wound was then closed in layers with absorbable, braided 4-0 Vicryl suture (Ethicon, Somerville, NJ, USA).

Six weeks after the index surgery, rats were sacrificed with carbon dioxide and cervical dislocation, and the sciatic nerve was again exposed in the manner above under a

dissecting microscope. In all animals, adhesions were discovered connecting the sciatic nerve to the surrounding muscle, muscle fascia, fat, and nerve bed (Figure 1) in a distribution greater than the original native paraneurial tissues seen at week zero. Epineurial markers were placed as above using a tissue marking pen, at 4mm increments from the level of the quadratus femoris to the level of the trifurcation. Adhesive tissue was perforated, but not released, to access the underlying nerve for labeling. The knee and ankle were ranged through the same two joint configurations as above and pictures were taken. Next, the adhesions from the level of the quadratus femoris muscle to the trifurcation were sharply and fully dissected with scissors (decompression), freeing the nerve from all soft tissue connections in this region. The knee and ankle again were then placed into the two configurations above and pictures were taken. With all images obtained, nerves were harvested from just above the level of the quadratus femoris and just below the level of the trifurcation and placed into a freezer at -80° for future sectioning and analysis. Six sciatic nerves were also harvested from 10-week old Lewis rats to serve as controls. Contralateral controls were not used, due to the possibility of compensatory changes resulting from ipsilateral nerve damage. Images were analyzed using ImageJ (NIH) and strain was calculated based on marker-to-marker distances in the stretched position relative to the relaxed position. (Foran et al., 2016; Mahan et al., 2015) Edges of each ink dot in each joint configuration were used as fiducial markers, to improve accuracy of measurement. Average strain across the entire exposed region, maximum strain between any two adjacent markers, and regional nerve strain in the proximal and distal regions were calculated.

Nerve sectioning, trichrome, and immunohistochemistry

All harvested nerves were thawed at -20°, embedded in Optimal Cutting Temperature compound, and flash frozen in liquid nitrogen cooled isopentane. Several 10 µm cross-sections were obtained using a cryostat (Leica CM 3050 S) proximally at the level of the first tissue marker (at the level of the quadratus femoris) and distally at the level of the fifth tissue marker (the level of the trifurcation). Sections were maintained in a freezer at -20° between sectioning and staining.

133

134 A proximal and distal section of each nerve underwent trichrome staining to allow for
135 analysis of gross structural morphology and connective tissue content. Slides were
136 thawed at room temperature for 15 minutes and then placed into neutral buffered
137 formalin. They were then stained with Harris Modified Hemotoxylin (HHS128, Sigma-
138 Aldrich, St. Louis, MO) for 4 minutes, followed by Gömöri's Trichrome Stain (#24205,
139 Polysciences, Inc., Warrington, PA) for 5 minutes. Slides were then successively
140 washed in acetic acid, alcohol, and citrisolv, and finally mounted with Permount
141 medium.

142 A proximal and distal section of each nerve also underwent immunohistochemical
143 staining for phosphorylated neurofilaments (SMI31; axonal marker) and laminin (basal
144 lamina marker). Sections were fixed with 10% formalin, permeated with 0.2% Triton X-
145 100, and blocked using a solution of 10% normal goat serum and 3% BSA in PBS. For
146 laminin and neurofilament staining, sections were incubated with primary antibodies
147 against laminin (1:500, Sigma Aldrich) and phosphorylated neurofilament (1:1000,
148 SMI31, Sigma-Aldrich) for 1 hour at room temperature. After rinsing with PBS, sections
149 were then incubated with Alexa Fluor-594 goat anti-mouse and Alexa-Fluor-488 goat
150 anti rabbit (1:200, Life Technologies) for 1 hour at room temperature. Samples were
151 imaged using a widefield fluorescence microscope (Leica DM 6000 B) to analyze
152 neurofilament localization and the internal structure of the nerve.

153 Axon counts were performed using a semi-automated approach, from 63X images of
154 SMI-31 labeled sections. Images were binarized using Image J (NIH), and axon
155 densities calculated by summing axon numbers from three randomly selected regions of
156 interest (ROI) within each section, and dividing by the summed area of these ROIs.
157 Axon counts were then estimated for the whole nerve by multiplying axon density by the
158 intra-epineurial area of each section, measured from 20x images. Given the variability in
159 attached extra-epineurial connective tissue following nerve excision, area-based
160 measurements of connective tissue remodeling were unreliable. Therefore, trichrome
161 images were scored semi-quantitatively to determine the integrity of the epineurium and
162 the infiltration of adhesion tissue into the nerve. Blinded images and scoring criteria

163 were presented to 5 individuals with knowledge of basic nerve architecture, and images
164 were scored based on a scale from 1 to 5. This scale reflects the circumferential extent
165 of fusion between extra-epineurial tissue and the interior of the nerve, indicated by the
166 loss of a clear external epineurial boundary (Table 1 and Figure 2). Sample images for
167 each score were presented to individuals as training, prior to blind testing of the full set
168 of images. Average scores from all 5 individuals for each image were used for statistical
169 analysis.

171 ***Statistical analysis***

172 Average and maximum measured strains were compared using 2x2 two-way repeated
173 measures ANOVA with factors of time (0 or 6 weeks) and surgical intervention (pre- or
174 post-decompression). Proximal and distal strains were compared using 2x2 two-way
175 repeated measures ANOVA with factors of surgical intervention (pre- or post-
176 decompression) and region (proximal or distal), or factors of time point (0 or 6 weeks)
177 and region (proximal or distal). Student's t-test was used to test for significant
178 differences between epineurial integrity scores ($\alpha = 0.05$). Data are given as Mean
179 (SEM).

Results:

Nerve structure

At week 0, prior to decompression, nerves were slack with neutral knee and ankle, but incurred tension upon knee extension and ankle dorsiflexion (Figure 1A-B). At week 6 (42 days), the nerve trajectory in both neutral and stretched configurations was markedly altered, and overlying connective tissue was observed (Figure 1C-D). These changes did not appear to cause any apparent functional deficits, as assessed by toe-spreading. Gross vascular deficits were also not observed, based on minimal pooling of blood and a consistent coloration of nerves and visible regions of their targeted muscles. To investigate whether these extra-epineurial adaptations resulted in changes to the interior of the nerve, we performed immunohistochemistry. Blind scoring of tissue sections with trichrome labeling showed a significant decrease in epineurial integrity 6 weeks after decompression, compared to controls (Figure 2). However, immunofluorescence labeling of axons (phosphorylated neurofilaments, red) and basal lamina (laminin, green) revealed that connective tissue infiltration into the interior of the treatment group nerves was not observed (i.e., internal epineurial borders remained sharp), and nerve fiber architecture appeared normal in both mid-femoral and distal-femoral regions of the nerve (Figure 3). Axon counts (0 weeks: 5324 (434); 6 weeks: 5846 (312); $p > 0.37$, $n = 4$) and axonal density (0 weeks: 9.9×10^{-3} (2.6×10^{-4}); 6 weeks: 9.8×10^{-3} (6.0×10^{-4}); $p > 0.92$, $n = 4$) were not significantly different.

Nerve Strain

Average and maximum sciatic nerve strains were measured before and after decompression, at 0 and 6 weeks. Average strains (Mean $\pm$ SEM) prior to decompression at week 0 and week 6 were 17.64% (4.5%) and 16.17% (2.14%), respectively. Average strains after decompression at week 0 and week 6 were 18.5% (3.35%) and 17.49% (4.5%), respectively. Maximum strains prior to decompression at week 0 and week 6 were 32.14% (6.35%) and 31.28% (3.9%), respectively. Maximum strains after decompression at week 0 and week 6 were 28.80% (4.15%) and 31.73% (5.02%), respectively. No significant differences were observed in either average or

210 maximum strains before and after decompression at either time point. We did, however,
211 observe significant regional differences in sciatic nerve strain (Figure 4). At 0 weeks,
212 strain in the distal-femoral region (i.e., just proximal to the trifurcation) was significantly
213 higher than that in the mid-femoral region (23.91% (5.4%) vs. 12.41% (4.07%),
214 $P < 0.001$) prior to decompression, but this regional strain difference disappeared after
215 decompression (Figure 4A). Similarly, prior to decompression at 6 weeks, nerve strain
216 in the distal-femoral region was significantly higher than that in the mid-femoral region
217 (20.51% (3.86%) vs. 12.26% (2.23%), $P < 0.05$); again, this regional strain difference was
218 not observed after decompression (Figure 4B).

Discussion:

Several studies have suggested a mechanical role for the paraneurium, in guiding nerve trajectory and in enabling controlled nerve gliding within its bed. (Butler, 2000; Mazal and Millesi, 2005; Millesi et al., 1995; Smith, 1966) Additionally, in an early investigation of peripheral nerve blood supply, Smith observed that nerves *in situ* displayed greater elasticity than those removed from their “mesoneurial” attachments. (Smith, 1966) He noted, “The fact that an isolated nerve has far less capacity for stretching than one *in situ* suggests that it is mainly the mesoneurium which allows for adjustments in length of the nerve.” Later, Millesi also described the apparent mechanical importance of the structure he termed the “paraneurium.” He noted that this structure appeared to be designed to allow for nerve motion and caliber changes. He also recognized that the paraneurium could become fibrotic, forming adhesions: “shrinkage of the fibrotic paraneurium... may cause compression of the entire nerve trunk, like a stocking that is too tight.” (Millesi et al., 1995) Although these investigators inductively reasoned that the paraneurium might modulate tensile nerve mechanics based upon gross observation, the nature of such a role remained to be tested.

In this study, we found that both normal paraneurium and its abnormal counterpart, adhesions, significantly impacted the distribution of strain in rat sciatic nerves. When normal paraneurial tissues were intact, regional differences in strain were observed, with increased strain in the distal-femoral region of the nerve compared to mid-femoral regions. Strikingly, an almost-identical strain gradient was recreated within 6 weeks after the initial decompression, in spite of differences in the trajectory of the nerve within its bed. This gradient was again eliminated the adhesion tissue was decompressed. The elimination of regional differences in strain by surgical decompression indicates that paraneurial attachments play an essential role in directing strain along the course of nerves. The underlying rationale for relative compliance of the more distal region of the nerve is not completely clear, but may result from proximity to the articulating knee joint (cf. (Boyd et al., 2005; Wright et al., 2001; Wright et al., 1996)), compliance required for trajectory changes of a trifurcation, or both.

Our data have several important clinical implications. The first is that surgeries that divide paraneurium or adhesions may have unpredictable consequences. Peripheral nerves are frequently dissected free of their paraneurial attachments during surgery, either to mobilize them for purposes of exposure, or during decompression procedures. However, releasing these nerves from their paraneurial tissues for purposes of mobilization may redistribute strain along the nerve, as recently described for the ulnar nerve, following its release during surgical treatment of cubital tunnel syndrome.(Foran et al., 2016) More persistent redistribution of strain from nerve regions accustomed to high strain to less compliant regions of the nerve may alter nerve susceptibility to structural and functional injury. It is not unreasonable to posit that neuropraxia and nerve injuries of greater severity, which are commonplace after these exposures, may be a result of altered mechanical loading subsequent to neurolysis, rather than a direct mechanical insult from retractors.(McConaghie et al., 2014)

Also compelling is our discovery that adhesion tissue appears to reconstitute strain distributions seen in control nerves, in both orientation and magnitude. This finding raises the possibility that the seemingly “random” generation of fibrous tissue exhibited by adhesions may instead participate in or respond to an organized process that attempts to recreate the normal neural biomechanical environment. This raises the question of whether the formation of adhesion tissue is necessarily pathological. Several strategies have been proposed to limit formation of adhesion tissue. However, rarely is consideration given as to whether paraneurial structures – whether native paraneurium or adhesions – may in some cases represent a positive biological response, possibly in response to mechanical loads experienced, and perhaps even favored, by peripheral nerves.(Anava et al., 2009; Pfister et al., 2004; Simpson et al., 2013) It is worth noting that rats were not immobilized in this study. It may be that immobilization, which is frequently recommended to patients following surgery or traumatic injury, leads to an absence of mechanical stimuli, driving the formation of a pathologic tether. Further research comparing nerve kinematics in the context of adhesion formation with and without immobilization will be needed to investigate this possibility.

An interesting methodological note is that paraneurial adhesion formation in our study required little more than incision, dissection of the paraneurial structures about the nerve, closure with absorbable braided sutures, and the passage of time. Gross observations of fibrotic tissue, as well as histological findings indicating a loss of epineurial integrity, confirm the formation of adhesions, despite use of reproducible and conservative surgical technique. Similar phenotypes have also been previously reported by Millesi, as indicators of adhesion formation.(Mazal and Millesi, 2005; Millesi et al., 1995) The ease of forming adhesion tissue is of interest, as a prior study reported the formation of adhesions in the sciatic nerves of rabbits by both cauterization of the nerve bed as well as suturing of the nerve to the surrounding nerve bed, followed by 5-6 weeks passage of time.(Abe et al., 2005) This latter method of forming adhesions is problematic for study purposes involving biomechanics, as suturing of the nerve to the nerve bed in and of itself results in a mechanical tether that affects future strain measurements.(Mahan et al., 2015) Our method of producing adhesions is highly reproducible and does not require nerve suturing, which may be helpful to other investigators in future studies.

Our study has several limitations. We measured nerve surface (epineurial) strain by measuring displacement of epineurial markings, rather than directly measuring strain with a microstrain gauge or other method. Despite the comparatively low resolution of our approach, we avoided damage to the neural elements, and our nerve strain findings were in keeping with prior studies.(Topp and Boyd, 2006) We also did not measure nerve strain distal or proximal to the exposed region of the sciatic nerve. It is possible that nerve strain following paraneurial decompression was transferred somewhere along the course of an unexposed region of the nerve. Further investigations will be needed to quantify this possibility, and would be aided by the development of noninvasive approaches to imaging less accessible nerve regions. Additionally, while we did not observe any pooling of blood or change in tissue coloration indicative of overt ischemia, more investigation is needed to determine the functional effects of paraneurial decompression on neural electrical conduction and blood flow. A final limitation is the nature of adhesions that were created following surgical decompression of otherwise healthy tissue. Such adhesions likely more closely resemble those formed following

310 surgical dissection than those formed following injury or during neuropathic progression.
311 Such conditions may result in damage to nerve fibers, an altered inflammatory
312 environment, and fibrosis, all of which could impact resultant mechanical sequelae.

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384

385 **Table Legend:**

Score	Criterion
1	Complete extracellular tissue infiltration through the epineurium and into the nerve.
2	An amount of extracellular tissue fusion with the epineurium greater than or equal to 50% of the circumference of the nerve.
3	An amount of extracellular tissue fusion with the epineurium greater than or equal to 20% but less than 50% of the circumference of the nerve.
4	An amount of extracellular tissue fusion with the epineurium less than 20% of the circumference of the nerve.
5	Complete absence of extracellular tissue fusion with the epineurium.

386

387 Table 1. Criteria for assigning Epineurial Integrity Scores, based on trichrome labeling.

388 Sample images for each score are shown in Figure 2. A score of 1 was not recorded for
389 any sections.

Figures

Figure 1. Macroscopic view of nerve trajectories just prior to the initial decompression (A-B, Day 0) and 6 weeks after the initial decompression, but before a second decompression (C-D, Day 42). (A) Nerve trajectory in a relaxed position at Day 0 (hip abducted, knee flexed 90°, ankle neutral). Also indicated on this image are sample epineurial markings used to make strain measurements. Exposed nerve was divided into two regions, mid-femoral and distal-femoral, for regional strain analysis. Ruler is demarcated in mm. (B) Nerve trajectory in a joint configuration inducing strain at Day 0 (hip abducted, knee fully extended (180°), ankle maximally dorsi-flexed). (C) Nerve trajectory in a relaxed position at Day 42 (hip abducted, knee flexed 90°, ankle neutral). (D) Nerve trajectory in a joint configuration inducing strain at Day 42 (hip abducted, knee fully extended (180°), ankle maximally dorsi-flexed).

Figure 2. Trichome labeling was used to assess epineurial integrity 42 days after decompression for sham operated and decompressed nerves. (A-D) Sample images of completely intact (Score 5, A) to largely disrupted (Score 2, D) epineurium, with images shown from each experimental group. Scale bars are 200µm. Epineurial integrity scores were assessed based on criteria provided in Table 1. No tissue sections were scored with “Score 1” in either experimental group. (E) Comparison of semi-quantitative scoring of epineurial integrity reveals decreased integrity after decompression.

Figure 3. Phospho-neurofilament/laminin staining of mid-femoral and distal-femoral regions of nerve cross-sections from sham operated (A-B) and decompressed (C-D) nerves show intact axonal and intra-epineurial structure in both experimental groups. Axons are indicated by phosphorylated neurofilaments (red), and basal lamina is indicated by laminin (green). Insets are shown below the full cross-sectional image. Scale bars are 100µm.

418 Figure 4. Regional rat sciatic nerve strains before and after the initial decompression (A,
419 Day 0) and before and after a second decompression 6 weeks after the initial
420 decompression (B, Day 42). Distal strain is significantly higher than proximal strain
421 ($P<0.001$) prior to decompression at both time points. 2-way repeated measures
422 ANOVA revealed an interaction effect of region and decompression on strain at Day 0
423 ($p<0.027$) and an effect of decompression on strain at Day 42 ($p<0.05$). Asterisks
424 indicate significant differences between individual groups based on post-hoc testing.

425

Figure 1

Figure 2

Figure 3

Figure 4