1   **Paclitaxel Alters Sensory Nerve Biomechanical Properties**

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17   Abstract: Paclitaxel is an effective chemotherapeutic that, despite its common use, frequently
18   causes debilitating peripheral sensory neuropathy. Paclitaxel binds to and stabilizes
19   microtubules, and through unknown mechanisms, causes abnormal microtubule aggregation.
20   Given that microtubules contribute to the mechanical properties of cells, we tested the
21   hypothesis that paclitaxel treatment would alter the stiffness of sensory nerves. Rat sural
22   nerves were excised and soaked in Ringer's solution with or without paclitaxel. Nerves were
23   secured between a force transducer and actuator, and linearly strained. Stress-strain curves
24   were generated, from which tangent moduli and linear elastic moduli were calculated. Paclitaxel
25   treated nerves exhibited significantly higher moduli in both linear and transition regions of the
26   curve. A composite-tissue model was then generated to estimate the stiffness increase in the

cellular fraction of the nerve following paclitaxel treatment. This model was supported experimentally by data on mechanical properties of sural nerves stripped of their epineurium, and area fractions of the cellular and connective tissue components of the rat sural nerve, calculated from immunohistochemical images. Model results revealed that the cellular components of the nerve must stiffen 12x to 115x, depending on the initial axonal modulus assumed, in order to achieve the observed tissue level mechanical changes. Consistent with such an increase, electron microscopy showed increased microtubule aggregation and cytoskeletal packing, suggestive of a more cross-linked cytoskeleton. Overall, our data suggests that paclitaxel treatment induces increased microtubule bundling in axons, which leads to alterations in tissue-level mechanical properties.

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Introduction

Paclitaxel is an effective and common chemotherapeutic that prevents cell division by stabilizing microtubules, thus preventing their depolymerization (Amos and Lowe, 1999). Due to its systemic delivery, paclitaxel also accumulates in peripheral nerves, causing a debilitating sensory neuropathy (Argyriou et al., 2014). Because neurons are post-mitotic, mechanisms underlying paclitaxel-induced neuropathy must differ from those affecting dividing cells. Animal models of disease and patients display dying back of axons, reduced epidermal nerve fiber density (Boyette-Davis et al., 2011), demyelination (Argyriou et al., 2008), altered ion channel activity (Hara et al., 2013), and disruptions in axonal transport. The latter pathway is commonly hypothesized to underlie neuropathic progression; abnormal clustering of microtubules (Masurovsky et

al.,1981; Masurovsky et al.,1983; Turner and Margolis,1984) is thought to impair delivery of vital proteins and organelles by microtubule-based motor complexes to distal axonal reaches.

Beyond their role in transport, microtubules also play important structural and biomechanical functions in axons(Garcia et al.,2012; Ouyang et al.,2013; Peter and Mofrad,2012), which may also be perturbed by paclitaxel. For example, a recent study demonstrated, using atomic force microscopy, that paclitaxel increases the compressive modulus of cell bodies in cultured dorsal root ganglia(Au et al.,2014). Paclitaxel also alters the response of central neurons to damage during high strain-rate loading(Tang-Schomer et al.,2010). Theoretical studies suggest that microtubules may influence tensile axonal biomechanics(Garcia, Pena et al.,2012; Peter and Mofrad,2012).

Whether and how structural and mechanical changes caused by paclitaxel influence biomechanics and physiological function at the whole nerve level has not been tested. Tensile loading is particularly relevant in peripheral nerves, which span articulating joints and can incur substantial regional strains(Aoki et al.,2005; Ochi et al.,2013; Topp and Boyd,2006). However, deformation beyond normal levels impairs action potential conduction(Wall et al.,1992). To protect neurons, strain is partially accommodated by axonal undulations, which straighten upon nerve deformation(Haninec,1986; Rydevik et al.,1990). In axons themselves, maintenance of structural integrity during axonal straightening and subsequent lengthening is likely to be modulated by the cytoskeleton, including microtubules. Increasing microtubule amount, length, stiffness, and bundling through paclitaxel treatment could alter the mechanical response and stability of this delicate biomechanical system, leading to

impaired function. This study uses a pure sensory nerve model to test the hypothesis that paclitaxel causes changes in the tensile properties of peripheral nerves. Our data suggest that paclitaxel influences the structure and biomechanics of sensory nerves, supporting a new hypothesis that altered neuronal biomechanics could contribute to paclitaxel-induced sensory dysfunction.

Methods

Mechanical testing

12 to 20 week old Sprague-Dawley rats were sacrificed and sural nerves were exposed and freed of the surrounding connective tissue bilaterally. A 5mm sural nerve segment just distal to the trifurcation point of the sciatic nerve was tied off with 6-0 nylon sutures *in situ* (fig 1a). Both nerve segments were excised and randomly assigned to be soaked in Ringer's solution (Aqueous solution of NaCl: 120mM, KCl: 4mM, MgSO₄: 0.8mM, NaHCO₃: 16mM, glucose: 20mM, and CaCl₂: 2.2mM, brought to pH 7.4) with or without the addition of 20μM paclitaxel for 2 hours. Each nerve segment was attached to an Aurora 402A force transducer and linear actuator and stretched in a chamber containing the appropriate Ringer's solution.

The length at which the nerve first incurred detectable passive load was recorded as L_0 . Nerves were stretched in increments of 0.2-0.5mm at a rate of ~0.05mm/sec and held for 5min before recording the force output, to allow stress relaxation as previously described(Luna et al.,2013; Shah and Lieber,2003). Because sutures securing the nerve to the force transducer and actuator were initially slack, nerve length was measured from suture to suture from images captured at each incremental deformation

(*fig 1b*). All measurements were made blinded as to paclitaxel treatment. We alternately normalized lengths to the length at which nerve stress was equal to 2g-force/mm² ($L_{\sigma=2}$). Cross-sectional area was calculated from nerve diameters measured from images captured at L_0 , assuming the nerve as a cylinder. Engineering stress-strain (σ - ϵ) curves were then generated, normalizing to L_0 or $L_{\sigma=2}$. Segmental strains were also measured using ink dots along the nerve, to determine the eventual region of failure.

To test the mechanical properties of nerves without their epineurium, sural nerves were excised as above. The tip of the inner sheath, which extruded beyond the epineurial boundary(Walbeehm et al.,2004), was held with forceps and the epineurium was manually peeled away. Mechanical testing was performed as above.

The elastic modulus of each curve was calculated as the slope of the linear region of each stress-strain curve at high strains. To compare tangent moduli at specific lower strains preceding the linear region, each stress-strain curve, with strain normalized to $L_{\sigma=2}$, was plotted from $\epsilon=0$ to the start of the linear region, and fit with an exponential function $\sigma = A*\exp(B*\epsilon)$ as for other biological tissues(Xiong et al.,2008; Yamanari et al.,2012). $A=2$, such that $\sigma=2\text{g-force/mm}^2$, by definition, at $\epsilon=0$. All curves had R^2 values $> .95$. Tangent moduli of non-linear regions of each curve were then calculated by the derivative of the exponential curve fit with respect to strain. The strain at which the tangent modulus of the exponential equation equaled the slope in the linear region was denoted as ϵ_L . The region of the stress-strain curve with $\epsilon<\epsilon_L$ are referred to as the transition region.

Because our higher-resolution mechanical testing apparatus was not designed to measure forces larger than 50 grams-force, we performed additional testing on a separate apparatus to measure the ultimate stress of 5 pairs of sural nerves. Contralateral pairs of ~5 mm segments of sural nerves were bathed in Ringer's solution with or without paclitaxel as above, mounted between a force transducer (Omega, DFG55-5) and a linear actuator, and strained in increments of 0.5 mm every 30 seconds. Maximum force at failure was recorded, and ultimate stress was calculated by dividing the force by the resting cross sectional area of the nerve.

Modeling

The linear portion of the nerve was modeled as a composite tissue comprised of epineurium, inner sheath (perineurium and endoneurium) connective tissue, and a cellular fraction, which were stretched in parallel. The total modulus was expressed as:

$$E_w = A_e E_e + A_{in} E_{in} + A_c E_c \quad (1)$$

where A_i and E_i denote area fractions and modulus, respectively, and subscripts of w , e , in , and c refer to those properties of the whole nerve, epineurium, inner sheath connective tissue, and cells, respectively.

Area fractions of the epineurium and inner sheath were measured from immunohistochemical analysis, and the modulus of the epineurium was then calculated from:

$$E_w = A_e E_e + A_{ic} E_{ic} \quad (2)$$

where A_i and E_i denote area fractions and modulus, respectively, and subscripts of w , e , and ic refer to those properties of the whole nerve, epineurium, and inner sheath combined with cells, respectively.

Transmission electron microscopy

C57/Bl6 mice were anesthetized and sural nerves were exposed bilaterally. Smaller mouse nerves enabled us to obtain a more accurate and comprehensive representation of the entire axonal population compared to rat nerves. Nerves were surrounded by a hydrophobic barrier and immersed in Ringer's solution containing 20 μ M paclitaxel for 2hr. Nerves were then fixed and imaged via electron microscopy. A custom MATLAB script was used to determine the nearest neighbor distance between microtubules and the microtubule density in images of 13 myelinated fibers from paclitaxel-treated and control nerves. Significance between parameters was tested using a Student's t-test ($\alpha < 0.05$).

Immunohistochemistry and Gomori's Trichrome.

10 μ M frozen rat sural nerve cross sections were labeled using an anti-laminin primary antibody (1:1000, Sigma Aldrich, L9393) for 1hr at room temperature and Alexa Fluor 488 secondary antibody (1:400, Life Technologies) for 1hr at room temperature. Images were captured on a Leica SP5 confocal microscope with appropriate filters. Gomori trichrome staining was performed as previously described (Gomori, 1950).

157 *Image processing*

158 Images of sural nerve cross sections from 3 nerves labeled for laminin were used
 159 to calculate the cellular and connective tissue area fractions of the sural nerve. A
 160 randomly selected sub-region from an image of each nerve was cropped and manually
 161 segmented by tracing the inner area of the laminin rings in ImageJ (*fig 4b-d*). The
 162 cumulative areas of the manually segmented regions were divided by the total area of
 163 the cropped region, then normalized to dimensions of the whole nerve as measured by
 164 trichrome staining (*fig 4a*).

165 **Results**

166 Because paclitaxel primarily causes sensory neuropathy, we examined the sural
 167 nerve, a purely sensory nerve branch of the sciatic nerve. As for many tissues, stress-
 168 strain curves showed a toe region in which nerves incurred substantial strains under
 169 minimal tension, followed by a transition region in which the nerve stiffened, and finally,
 170 a linear region with a constant slope. Strain was normalized to two lengths, L_0 , the
 171 length at which the nerve first began to incur detectable passive load, and $L_{\sigma=2}$, the
 172 length at which the nerve stress was equal to 2g-force/mm². This stress was the
 173 minimum stress at which nerves visibly began to lengthen and achieved a consistent
 174 signal to noise ratio. Representative plots for control and paclitaxel-treated samples are
 175 shown (*fig 2a and b*), with the latter also illustrating piece-wise curve fits for paired
 176 nerves. Raw stress-strain curves for all nerves are also provided (*supplementary fig 1*).

177 We first probed paclitaxel-induced changes to the mechanical properties of the
 178 peripheral nerves in the linear regions of stress-strain curves (*fig 2a,b*). ϵ_L , the point

defining the start of the linear region (*fig 2b*), trended ($p = .115$) towards a lower value in paclitaxel-treated nerves compared to contralateral control nerves (*fig 2e*). Paclitaxel-treated nerves were significantly stiffer in their linear region, based on elastic moduli calculated from stress-strain curves normalized both to L_0 and $L_{\sigma=2}$ ($p < 0.05$, *fig 2c,d*). We also examined the maximum stress in 5 separate pairs of nerves stretched to failure. Most paclitaxel treated nerves showed a higher ultimate stress, however no significant difference was observed, potentially due to one paclitaxel treated nerve failing at a much lower stress than all other nerves (*supplementary fig 2*).

To examine biomechanical properties in a more physiologically relevant range of strains (Topp and Boyd, 2006), we then examined the transition region of the stress-strain curve, which preceded the linear region. Tangent moduli were calculated from the exponential curve used to fit the stress-strain curve between $\epsilon = 0$ to ϵ_L , and were significantly higher (stiffer) in paclitaxel treated nerves than contralateral control nerves for low strains ($\epsilon = 1-5\%$).

To better understand how increased stiffness in individual cells could influence biomechanics of the whole nerve, we developed a theoretical composite-tissue model based on the relative moduli and cross-sectional area fractions of connective tissue and cellular components. As expected, as visible by trichrome labeling, sural nerves displayed, from outside in, clear epineurial, perineurial and endoneurial compartments (*fig 4a*). To predict whether and by how much the modulus of the cellular fraction increased after paclitaxel treatment, we required area fractions and moduli of the connective tissue and cell components in untreated nerves. Trichrome labeling revealed the epineurial cross-sectional area to be $24.7 \pm 2.7\%$ ($n=3$) (*fig 4a*). To determine area

fractions of the cell and connective tissue compartments of the inner sheath, we labeled sural nerve sections with antibodies targeting laminin, which sharply demarcated cellular boundaries (*fig 4b-d*). The cellular area fraction of the nerve was $43.8 \pm 0.9 \%$ ($n = 3$) and the inner sheath connective tissue fraction was $31.1 \pm 0.5 \%$ ($n=3$).

We next determined the modulus of the epineurial and inner sheath regions by mechanically testing sural nerves stripped of their epineurium (*figure 5a*). Following peeling, the inner sheath still appeared structurally intact (*figure 5b*), with expected laminin localization and a visible perineurial boundary. Sural nerves lacking their epineurium were ~5x more compliant than intact nerves (elastic moduli: $1.6 \pm .9$ MPa vs. 7.8 ± 2.2 MPa, $p<0.001$).

Finally, an estimated modulus of the cellular fraction was needed. To our knowledge, mechanical properties of axons excised from peripheral nerves have not been measured. However, several studies have estimated axonal stiffness *in vitro* or through simulations (Bernal et al., 2007; Dennerll et al., 1989; Garcia, Pena et al., 2012). We therefore solved our model using axonal moduli ranging from 100 kPa to 1 MPa, based on the range of reported values in these studies. Assuming these moduli, the moduli of the epineurium and inner sheath connective tissue fractions were calculated (*equations 1 and 2*). For paclitaxel-treated nerves, the connective tissue moduli was assumed to stay constant (i.e., no microtubules in this compartment) while the cell fraction modulus was varied. Upon incorporating the modulus of intact nerves following paclitaxel treatment, the modulus of the cellular fraction after paclitaxel treatment was calculated (*fig 6a*). Additionally, we calculated a stiffening factor (ratio of paclitaxel-treated to untreated cell fraction modulus), as a function of initial cellular fraction

225 stiffness. Results show that the cellular fraction must stiffen 115x to 12x, depending on
226 the initial axonal modulus used (*fig 6b*).

227 In an effort to determine a structural basis for biomechanical changes, we next
228 sought to determine the extent by which cytoskeletal structures changed in response to
229 paclitaxel. We therefore treated performed transmission electron microscopy on
230 untreated and paclitaxel-treated rodent sural nerves *in vivo*. Microtubules, other
231 cytoskeletal filaments, and their crosslinks have been suggested to impact cell
232 mechanical properties(Ouyang, Nauman et al.,2013; Peter and Mofrad,2012). Because
233 paclitaxel directly affects microtubules, we quantified their density and spacing with and
234 without paclitaxel treatment in myelinated and unmyelinated axons. Paclitaxel treated
235 axons showed frequent clusters of microtubules not present in control axons (*fig 3 a-d*)
236 with highly consistent spacing between microtubules (34.88 ± 3.83 nm center to center
237 distance, n=10 pairs), suggestive of a crosslink with a characteristic length. In addition
238 to inducing large microtubule clusters, overall microtubule density and spacing also
239 changed after paclitaxel treatment. In both myelinated and unmyelinated axons,
240 paclitaxel treatment caused a marked increase in the microtubule density of the entire
241 axon ($p<.05$) and decreased the nearest neighbor distance between microtubules
242 ($p<.01$, *fig 3e*). The cytoplasmic regions in the paclitaxel-treated nerves appeared
243 darker, with increased cytoskeletal density than the contralateral control. In addition,
244 some paclitaxel treated axons (~9%) appeared to have compacted regions cytoplasm
245 excessively dense with structural proteins (*fig 3d*); such malformations were not
246 observed in the control sural nerve. Interestingly, though some cell bodies of non-
247 neuronal cells were interspersed within the extraneuronal space, no microtubules were

observed in endoneurial or perineurial compartments, suggesting that these regions are not appreciably changed with paclitaxel treatment. Collectively, these results indicate that paclitaxel treatment increased microtubule, and possibly other cytoskeletal protein, content and connectivity in the axonal shaft, providing a potential structural basis for the observed mechanical changes.

Discussion

Mechanical properties of paclitaxel-treated sensory nerves

To our knowledge, mechanical testing of sensory nerves such as the sural nerve has not yet been performed, despite sensory branches often spanning joints and being used as autologous grafts. Interestingly, elastic moduli in rodent sural nerves appear to be an over an order of magnitude greater than those of sciatic nerves(Borschel et al.,2003) and slightly lower than those of the porcine laryngeal nerve(Alexander et al.,2010) and rabbit tibial nerve(Kwan et al.,1992). This could reflect several reasons, including fewer fascicles(Jeronimo et al.,2005), smaller neurons, and a thicker epineurium relative to the total cross-sectional nerve area, leading to differences in the relative contributions of intra-epineurial fractions to whole nerve properties. In addition, while several studies have suggested that paclitaxel increases microtubule bundling in rat sciatic nerves, few studies have analyzed sensory nerve structure after paclitaxel treatment(Flatters and Bennett,2006; Sahenk et al.,1994), despite the fact that paclitaxel primarily produces sensory neuropathies.

In vitro studies show that paclitaxel nearly doubles the compressive modulus of DRG cell bodies(Au, Fang et al.,2014), at a much lower dose than that used in this

270 study. Paclitaxel additionally modifies the tensile response of cultured DRG
271 axons(Dennerll, Lamoureux et al.,1989), and may increase tensile axonal stiffness,
272 though this observation was not discussed by the authors and only reflected a single
273 sample in each group (figures 4 and 11 in(Dennerll, Lamoureux et al.,1989)). Here we
274 show that cellular properties can influence the tensile properties of a nerve (*fig 2*). This
275 finding is consistent with a tensile load-bearing role for intact neurons in nerves of *C.*
276 *elegans*(Hammarlund et al.,2007), which is impaired by destabilizing the spectrin
277 cytoskeleton; however, it contrasts with previous assumptions that the cellular portion of
278 the nerve does not contribute significantly to the stiffness of nerves(Rydevik, Kwan et
279 al.,1990; Sunderland and Bradley,1961; Topp and Boyd,2006; Walbeehm, Afoke et
280 al.,2004).

281   When a nerve is stretched, connective tissue and axons, which initially undulate,
282 unravel and begin to accommodate loads(Rydevik, Kwan et al.,1990). Conventionally,
283 this loading is believed to be primarily borne by the connective tissue, especially the
284 perineurium(Topp and Boyd,2006; Walbeehm, Afoke et al.,2004), with axonal fibers
285 contributing minimally. This argument is primarily supported by two observations: (i)
286 Wallerian degenerated nerve stumps have similar mechanical properties to intact
287 nerves(Sunderland and Bradley,1961); and (ii) rabbit tibial nerves stretched past the
288 point of plastic deformation show ruptured perineurium without observation of nerve
289 fiber or epineurial damage(Rydevik, Kwan et al.,1990). However, conclusions based on
290 these observations could be an oversimplification. In the first case, Wallerian
291 degeneration takes weeks, and could alter the mechanical properties of nerves during
292 that time via extracellular and intracellular remodeling. In the second case, nerve fibers

do not remain in a single image plane through their entire course and thus are often observed as discontinuous in 2D images, making confirmation of fiber rupture difficult to observe. Alternatively, axons could be plastically deformed due to cytoskeletal protein breakage, as opposed to cell membrane rupture.

A limitation of our study is that our methods ignore the viscous response of the tissue, which could provide important information regarding changes in nerve properties. Furthermore, because we are unable to visualize the interior of the nerve during stretch, we cannot reliably interpret the integrity of inner sheath tissue and cells from measurements such as ultimate stress (*supplementary fig 2*). In addition, the method of paclitaxel delivery to the nerve via local soaking is not a clinical delivery mechanism. However, this approach precludes confounding effects on nerve stiffness or intraneural pressure after systemic delivery, including inflammatory or fibrotic influences. Finally, while the concentration of taxol used here (20 μ M) was larger than peak concentrations measured in human subjects (Vasanthan et al., 2011), several animal models for paclitaxel-induced neuropathy use peak plasma paclitaxel concentrations nearly an order of magnitude higher than those used here (Huehnchen et al., 2013; Sparreboom et al., 1996). Thus, though a direct comparison is difficult, due to the metabolism of systemically injected paclitaxel, the fact that electron microscopy showed significant structural changes within 2 hours, and literature reports also show bundled microtubules in sciatic nerves treated with paclitaxel (Roytta and Raine, 1986) suggests that our dosage is appropriate for modeling paclitaxel induced neuropathy.

315   *Structural basis for nerve biomechanics*

316   Structurally, mechanical changes correlate with increased microtubule bundling
317 and density. These structural changes are in agreement with previous observations that
318 paclitaxel increases cytoskeletal connectivity in homogenized brain tissue(Turner and
319 Margolis,1984) and brine shrimp(Campbell et al.,1989). Microtubule bundles seen in
320 paclitaxel-treated axons via electron microscopy showed remarkably uniform spacing
321 with center-to-center distances of 34.88 ± 3.83 nm (*fig 3b*). Given microtubule diameters
322 of ~25 nm, our data suggest a potential cross-linking (bundling) protein of ~10 nm in
323 length. This dimension is close to but slightly smaller than spacings expected for
324 microtubule crosslinking proteins such as tau (20-27 nm(Rosenberg et al.,2008)) or
325 dynein (consisting of a 13-15 nm diameter ring and a 10-15 nm length stalk(Reck-
326 Peterson et al.,2006)), suggesting other microtubule associated proteins may be
327 involved. Neurofilaments also appear to be more densely packed around microtubule
328 bundles (*fig 3b,d*), though their potential role in nerve mechanics remains to be
329 determined. Finally, the perineurium contains densely packed layers of
330 cells(Thomas,1963) – we cannot exclude the possibility that nerve stiffening was due to
331 mechanical changes in the perineurial cells, though electron microscopy (*fig 3*) does not
332 indicate many microtubules in these cells(Thomas,1963).

333   Because the cytoskeleton anchors the cell to the surrounding extracellular matrix,
334 a more networked cytoskeleton could also alter the way axons strain with respect to
335 their surroundings. Stiffer axons or altered adhesion between the cell and its
336 extracellular environment could alter the unraveling of undulating axons, or change the
337 relative extension of cells and their surrounding matrix under asymmetric loads (e.g.,

shear). These less intuitive possibilities could additionally amplify the mechanical effects of paclitaxel.

Model parameters and predictions

Our model of nerve mechanics relies on several parameters relating to the stiffness of connective tissue and cells within the nerve which have not yet been reported. Interestingly, though the perineurium is suggested to be the primary load bearing tissue in nerves(Rydevik, Kwan et al.,1990), peeling of the epineurium dramatically decreased nerve stiffness and tensile modulus (*fig 5*), indicating that the epineurium contributes significantly to the overall stiffness of the sural nerve, and is likely to be engaged during tensile loading.

In addition, values of the tensile modulus of the cellular fraction of untreated nerves were also necessary to determine the stiffening effects of paclitaxel. Several *in vitro* and *in silico* studies have been performed to measure or estimate the stiffness of axons. The Young's modulus of PC12 neurites grown *in vitro* has been experimentally measured to be 12 ± 2 kPa(Bernal, Pullarkat et al.,2007). However, PC12 neurites are ~10 times more compliant than DRG axons(Dennerll, Lamoureux et al.,1989), and so this value was not used. Cultured DRG neurites show a wide range of diameters from .05 to 1.20 μm (Windebank et al.,1985) with a median diameter of 0.24 μm ; using data from figure 4 in(Dennerll, Lamoureux et al.,1989) and the median reported cultured DRG diameter of 0.24 μm , one can roughly estimate the modulus of a cultured DRG axon to be ~90 kPa. Finally, axonal moduli have been determined via modeling to be ~1 MPa(Garcia, Pena et al.,2012). The authors reconcile this difference with the above

papers by noting that *in vitro* studies use short axons where the tensile modulus is calculated based on force measurements taken from the entirety of the axon; the weakest point along the axon therefore dominates stiffness measurements. The axon does not necessarily show a uniform distribution of microtubules along its length(Garcia, Pena et al.,2012; Zhou et al.,2004) and therefore such *in vitro* studies may underestimate stiffness. Our model shows that paclitaxel treated cells must stiffen considerably ($\geq 12x$) to cause the observed results, suggesting that paclitaxel induced changes to tensile mechanical properties of cells are dramatic.

Conclusions

Nerves necessarily experience substantial strains during joint movement, including regional deformations of 20-30%(Aoki, Takasaki et al.,2005; Ochi, Horiuchi et al.,2013; Topp and Boyd,2006). Our results suggest that paclitaxel stiffens sensory nerves, consistent with morphological observations of increased microtubule packing and density. If these changes alter the magnitude or distribution of strains along a nerve or impair axonal unraveling, thus forcing axons to incur larger loads than normal, nerve fibers could be damaged by simple daily movement. Though this idea is currently highly speculative, altered nerve mechanics may thus influence neuropathic progression. Such damage may impact cell function directly, due to microtubule breakage, such as that observed in neurons loaded at high strain rates (Tang-Schomer et al.,2012), or indirectly, by amplifying axonal transport defects(Bober et al.,2015) . Given the possibility of a mechanical contribution to neuropathy, the physical management of patients treated with paclitaxel, including the development of effective post-

382 chemotherapy immobilization or physical therapy protocols, warrants further
383 investigation.

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Figure Legends

Figure 1 – Mechanical testing of sural nerves

A) Sural nerves were exposed in rat cadavers. A ~5 mm segment of each nerve immediately distal to the trifurcation point of the sciatic nerve was sutured *in situ* with 6-0 nylon sutures. Sc and Su indicate the sciatic and sural nerves, respectively. Scale bar = 5mm. B) Each nerve was tied to a force transducer and a linear actuator, and progressively strained over time until visible nerve rupture (arrow). Ink dots along the nerve were used to monitor regional deformation.

Figure 2 – Paclitaxel treatment alters whole nerve mechanical properties

Stress-Strain curves of contralateral nerve pairs soaked in Ringer's solution with and without paclitaxel were generated normalized to L_0 (A) and $L_{\sigma=2}$ (B). Each curve was fit with an exponential function from strain 0 to ϵ_L , the point at which the slope of the function was equal to the slope in the linear region (arrow). The slope of in the linear region was determined. C) In all cases, the tensile modulus of the paclitaxel treated nerve was greater than or equal to that of the contralateral control nerve. D) The mean tensile modulus of paclitaxel treated sural nerves is significantly (*, $P < .05$) greater than that of the contralateral control nerves. E) ϵ_L did not significantly differ between paclitaxel treated and control nerves. F) Tangent modulus calculations in the transition region reveal that paclitaxel significantly increases nerve stiffness (*, $P < .05$) even at low strains. Error bars indicate standard error of the mean.

Figure 3 – Paclitaxel causes bundling of microtubules in sural nerves

Sural nerves were exposed in anesthetized mice. One sural nerve was surrounded in a hydrophobic boundary and soaked in 20 μM paclitaxel for 2 hours. Mice were sacrificing and paclitaxel treated and control nerves were then fixed for processing. A) TEM image of a myelinated axon within control sural nerves showing expected morphology with microtubules (arrows) scattered throughout the axon B) An axon within a paclitaxel -treated sural nerve shows a contraction of the cytoplasm and buildup of electron dense proteins indicated by the overall darker appearance. Microtubules form clusters of parallel arrays (boxes) and increase dramatically in number. A digitally zoomed inset is provided for increased clarity. C) Control sural

nerve axons show intact myelin and axonal structure. D) After paclitaxel treatment, many axons show signs of myelin abnormalities and cytoskeletal collapse. E) Microtubules appear significantly closer together after paclitaxel treatment (*, $P < .05$). F) Overall microtubule density per axon increases significantly after paclitaxel treatment (*, $P < .05$). Error bars indicate standard error of the mean. Scale bars indicate 500 nm (A and B) and 5 μm (C and D).

Figure 4 – Sural Nerve Histology

A) Gomorri Trichrome stain of a rat sural nerve cross-section, used to calculate epineurial area. Traces indicate sample boundaries from which area fractions were calculated for the inner sheath and epineurial compartments. B) Cross-section of a rat sural nerve stained for laminin. C) In order to estimate the area fraction of the cellular components of the inner sheath, smaller regions of laminin staining were digitally cropped and the inner regions of the laminin rings were manually sectioned in ImageJ (D). Area of the inner rings of laminin relative to the entire cropped region was then computed. Scale bars indicate 25 μm .

Figure 5 – Peeled Epineurium Testing

A) Mechanical properties of sural nerve stripped of epineurium with strain normalized to L_0 . B) Cross-section of sural nerve stripped of epineurium labeled with a laminin antibody. Note that the nerve appears structurally intact. Scale bar indicates 100 μm .

Figure 6 – Model of paclitaxel induced stiffening of cells

560 A) Our composite-tissue model was solved to determine the stiffness of the cell
561 fraction after paclitaxel treatment assuming a range of initial cell fraction moduli. B)
562 After paclitaxel treatment, the cell fraction stiffens significantly.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6