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Probing multi-scale mechanics of peripheral nerve collagen and myelin by X-ray diffraction

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ABSTRACT

Peripheral nerves are continuously subjected to mechanical forces, both during everyday movement and as a result of traumatic events. Current mechanical models focus on explaining the macroscopic behaviour of the tissue, but do not investigate how tissue strain translates to deformations at the microstructural level. Predicting the effect of macro-scale loading can help explain changes in nerve function and suggest new strategies for prevention and therapy.

The aim of this study was to determine the relationship between macroscopic tensile loading and micro scale deformation in structures thought to be mechanically active in peripheral nerves: the myelin sheath enveloping axons, and axially aligned epineurial collagen fibrils. The microstructure was probed using X-ray diffraction during *in situ* tensile loading, measuring the micro-scale deformation in collagen and myelin, combined with high definition macroscopic video extensiometry.

At a tissue level, tensile loading elongates nerves axially, whilst simultaneously compressing circumferentially. The non-linear behaviour observed in both directions is evidence, circumferentially, that the nerve core components have the ability to rearrange before bearing load and axially, of a recruitment process in epineurial collagen. At the molecular level, axially aligned epineurial collagen fibrils are strained, whilst the myelin sheath enveloping axons is compressed circumferentially. During induced compression, the myelin sheath shows high circumferential stiffness, indicating a possible role in mechanical protection of axons. The myelin sheath is deformed from low loads, despite the non-linearity of whole tissue compression, indicating more than one mechanism contributing to myelin compression. Epineurial collagen shows similar load-bearing characteristics to those of other collagenous connective tissues.

This new microstructural knowledge is key to understand peripheral nerve mechanical behaviour, and will support new regenerative strategies for traumatic and repetitive injury.

1. Introduction

Peripheral nerves are continuously subjected to mechanical forces, elongation, and compression during everyday movement, without suffering functional losses and damage. Traumatic events and inappropriate continuous mechanical loading, however, are associated with common disabling and painful entrapment, overstretch, or compression neuropathies. Carpal tunnel syndrome, for example, has a prevalence in the United Kingdom of 5–16% annually, varying by age and gender group, with decompression surgery provided for 43–74 people per 100,000, annually (Aroori and Spence, 2008; Burke, 2000). Erb's palsy, caused by excessive stretching of infant heads and arms during birth, induces loss of sensation and abnormal motor function in 0.1% of births in the US (Gilbert et al., 1999). In sciatic nerves and brachial plexus, stretch due to trauma and abnormal limb positioning during operations are widespread causes of debilitating iatrogenic injury, both temporary and chronic (Lalkhen and Bhatia, 2012). Mechanical tension has also been proposed as a regenerative method, with mild stretch inducing axonal and whole-nerve elongation and growth, although the multi-scale effects of this elongation have not been studied (Pfister et al., 2004; Chuang et al., 2013; Saijilafu et al., 2008). A better understanding of the multi-scale link between macroscopic loading and loss of function in peripheral nerve is required for effective prevention and treatment of neuropathies, and to explore tension as a strategy for injury prevention and regeneration (Bueno and Shah, 2008).

Multiple concurrent factors cause functional alterations in

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peripheral nerves. Occlusion of nerve blood supply, leading to hypoxia, occurs at macroscopic strains above 15% (Ogata and Naito, 1986). A reduction in electrical conduction has also been observed as a result of stretch, with Compound Action Potential magnitude decreasing with increasing strain (Li and Shi, 2007; Wall et al., 1992), and completely subsiding at strains of 5–20% (Li and Shi, 2007; Takai et al., 2002). At a cellular level, strains above 10% have been shown to affect axonal transport, responsible for motility of cytoskeletal elements, energy production, and growth factor trafficking (Ikeda et al., 2000; Aomura et al., 2016), and AFM studies on single axons showed that the compression required to block axonal transport was variable, between 65 and 540 Pa (Magdesian et al., 2012). Furthermore, voltage-gated sodium channels clustered at Nodes of Ranvier, fundamental for saltatory impulse conduction, have been shown to disperse with applied nerve elongation (Ichimura et al., 2005). Axonal tensile strains of 5% can cause growth cone collapse (Yap et al., 2017), membrane permeabilisation (Geddes et al., 2003), and morphological changes in neurons (Kilinc et al., 2008), also potentially leading to loss of function.

The mechanical properties of peripheral nerve tissue derive from its complex structural organisation. Substructures in peripheral nerves include the endoneurium, a loose structure of collagenous channels in which myelinated and unmyelinated axons are embedded, surrounded by the perineurium, made up of multiple layers of transversely aligned lamellar collagen. The epineurium, a thick layer of densely packed collagen fibres, envelopes the whole nerve in an axially aligned pattern, showing fibril waviness and crimp (Fig. 1) (Ushiki and Ide, 1990). The properties of these collagenous substructures have been shown to be similar to those of tendon (Mason and Phillips, 2011) and either or both the epineurium and perineurium have been described as the main load bearing structures (Sunderland and Bradley, 1961; Haftek, 1970; Rydevik et al., 1990; Tillett et al., 2004). Here, we analyse the partitioning of strain between whole tissue and collagen molecules, to compare the mechanical properties of axially aligned eipneurial collagen to those of other load-bearing collagenous tissues such as tendon.

Mechanically, peripheral nerves have been modelled as concentric, two-layer composites: a more compliant, water-rich core representing endoneurium, the inner part of the perineurium, and myelinated and unmyelinated axons, and a stiffer outer sheath representing the epineurium and the outer perineurium, connected by a sliding interface layer (Tillett et al., 2004; Georgeu et al., 2005; Walbeehm et al., 2004). Independent mechanical testing characterises the core as significantly softer and more compliant than the sheath, and indicates that, during tensile loading of the nerve, the outer sheath applies a compressive force on the core, which is resisted by a positive endoneurial pressure (Walbeehm et al., 2004; Georgeu et al., 2005). The loose organisation of endoneurial tissue suggests that, at low loads, components within the core rearrange, rather than compress, during whole tissue compression (Millesi et al., 1995). The transverse compression induced by tensile loading has been observed *in vitro*, but its effect on the microstructural elements within the core has not been studied (Topp and Boyd, 2006; Millesi et al., 1995).

Within the nerve core, chemical demyelination has been shown to reduce nerve axial stiffness, implying that myelin contributes to tissue mechanical properties (Shreiber et al., 2009). Additionally, AFM studies have shown that digestion of Schwann cell basal lamina reduces nerve fibre circumferential stiffness and resilience significantly (Rosso et al., 2014), suggesting a protective role for myelin during nerve deformation. However, the mechanical role of myelin is not yet clear, as it has mostly been studied in isolation, rather than within the multi-scale mechanical environment of the whole nerve. Here, we aim to characterise the mechanical properties of the myelin sheath during circumferential compression induced by whole-nerve elongation.

Linking macroscopic loading of peripheral nerves with changes in cell function and damage requires knowledge of the multi-scale mechanical behaviour of nerves. Current knowledge of the micro-scale effects of macroscopic loading is limited, and a better multi-scale understanding is required to successfully prevent and treat mechanically-induced peripheral neuropathy and functionality loss (Bueno and Shah, 2008). X-ray diffraction is an ideal modality for *in situ* strain measurements of micro-scale quasi crystalline structures. In nerve, it has been shown to effectively probe collagen fibres (Inouye and Worthington, 1983) as well as the intra-lamellar spacing in the myelin sheath (Finean, 1960; Inouye et al., 2014; Kurg et al., 1982), but it has not been applied to studying the mechanical properties of these structures during *in situ* loading. Here we investigate the multi-scale mechanical properties of peripheral nerve during tensile loading, by probing the microstructure of peripheral nerve collagen and myelin by X-ray diffraction during macroscopic tensile loading.

2. Materials and methods

2.1. Sciatic nerve harvesting and imaging

Sciatic nerves were harvested from 300 to 350 g (10–12 week old), male Sprague-Dawley rats, sacrificed by cervical dislocation for an unrelated study. Briefly, following skin removal, the sciatic nerves were exposed by dorsal incision of the gluteus muscle. Nerves were excised proximally close to the spinal cord, and distally at the tibial-peroneal branching. Nerve samples were stored in Phosphate Buffered Saline (PBS, Gibco, UK), immediately frozen at −20° until use. This has been previously shown not to not alter the mechanical properties of collagenous tissues (Bruneau et al., 2010; Fessel et al., 2014), as well as

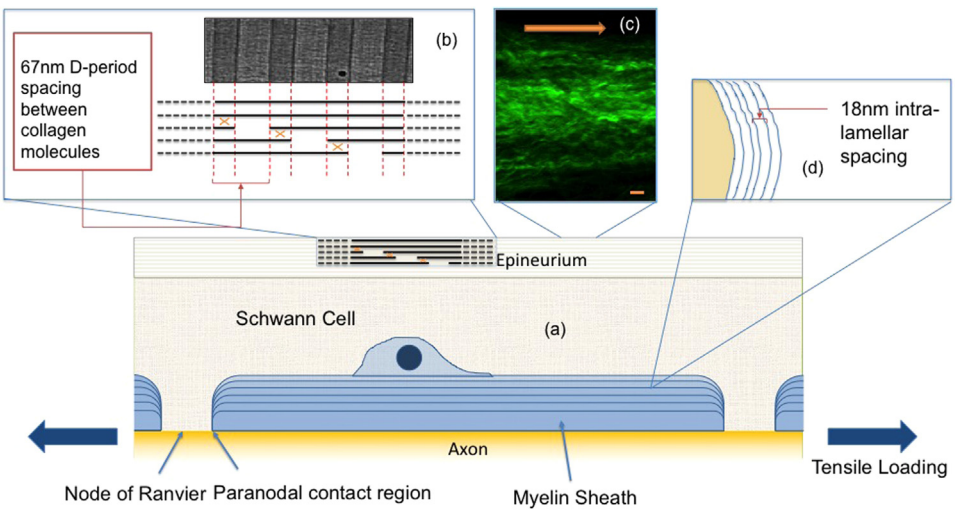

Fig. 1. (a) Peripheral nerves consist of axons projecting from neuron bodies, which can be myelinated by Schwann cells or unmyelinated. The structure of quasi-crystalline elements (collagen and myelin) can be observed by X-ray diffraction. (b) Eipneurial, axially aligned collagen fibril structure, with characteristic 67 nm D-period between molecules. (c) Multi-photon second harmonic microscopy of rat sciatic nerve collagen, showing crimped wavy pattern of axially aligned fibres. Scale bar = 100 µm. Arrow indicates direction of nerve axis. (d) Cross-sectional view of myelin sheath, showing 18 nm spacing between lamellae.