

# Optimisation and validation of immunohistochemical axonal markers for morphological and functional characterisation of equine peripheral nerves

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## Abstract

**Background:** Horses are affected by various peripheral nerve disorders but defining their aetiology and pathophysiology is hampered by limited understanding of associated morphological and pathological changes and involvement of specific axonal types.

**Objectives:** To investigate the hypothesis that selected antibody markers, used in conjunction with various tissue processing methods, would enable identification of axons with different functional modalities within a range of equine peripheral nerves.

**Study design:** Optimisation and validation study.

**Methods:** A range of antibodies were evaluated immunohistochemically via fluorescence confocal microscopy in cadaver equine nerve samples of primary motor, mixed or primary sensory functions (recurrent laryngeal, phrenic and plantar digital) within formalin-fixed paraffin-embedded (FFPE) and formalin-fixed frozen (FFF) tissues subjected to different antigen retrieval protocols.

**Results:** Immunohistochemistry of FFPE-derived nerve samples with selected antibodies and specific antigen retrieval methods enabled identification of myelinated and unmyelinated axons, cholinergic, sympathetic and peptidergic axons. The recurrent laryngeal and phrenic nerves are composed of myelinated cholinergic (motor), myelinated sensory fibres, unmyelinated adrenergic (sympathetic) axons and unmyelinated peptidergic (sensory) axons. In contrast, as expected, the plantar digital nerve had no myelinated motor fibres being mainly composed of myelinated sensory fibres, unmyelinated sympathetic and unmyelinated peptidergic sensory axons.

**Main limitation:** Attempts specifically to label parasympathetic fibres were unsuccessful in any nerve examined in both FFPE and FFF tissues.

**Conclusions:** A panel of antibody markers can be used to reveal morphological and functional properties of equine nerves. Future work should enable better characterisation of morphological changes in equine neuropathies at various stages of disease development.

The abstract is available in German in the Supporting Information section of the online version of this article.

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**KEY WORDS**

peripheral neuropathy, immunohistochemistry, recurrent laryngeal, antibody, horse, nerve

## 1 | INTRODUCTION

Horses are affected by various neuropathies; these include, the axonal degeneration seen in peripheral nerves of horses with equine motor neuron disease,<sup>1</sup> motor neuropathy observed in recurrent laryngeal neuropathy (RLN),<sup>2</sup> sensory disorders such as equine trigeminal-mediated head shaking syndrome<sup>3</sup> and autonomic dysfunction seen in equine grass sickness.<sup>4</sup> The aetiology of many of these disorders remains poorly characterised, due in part to limitations in our understanding of pathophysiology. The latter can be facilitated by detailed characterisation of associated pathological changes but sophisticated methods for evaluating equine nerves are lacking. Typically, the morphology of equine nerves is examined in resin-embedded samples: the method is useful for assessing the severity of axonal loss and other pathological features,<sup>5</sup> but it is somewhat laborious and limited to specialised laboratories; importantly, the method does not enable identification of specific nerve fibres as motor, sensory or autonomic. Therefore, optimising a panel of neuronal markers to label different axonal subtypes might shed light on the pathophysiology of some equine neuropathies.

Nerve fibres are classified according to different criteria based on their size (small, medium or large diameter), their myelination (myelinated or unmyelinated), their function (sensory or motor) and their neurochemical constituents (cholinergic, adrenergic, nitroergic or peptidergic).<sup>6</sup> Study of the structural and functional complexity of the peripheral nervous system components requires validation of neuronal markers to investigate the pattern of tissue innervation, the associated physiological mechanisms as well as their alterations in many neuromuscular diseases. Various antibodies have been validated to label and characterise nerve fibres in dogs<sup>7</sup> and cats.<sup>8</sup> In horses, a few reports have demonstrated the neurochemical characterisation and the nature of innervation of some tissues including the small intestine,<sup>9</sup> nasal mucosa<sup>10</sup> and in equine spinal ganglia,<sup>11</sup> but to our knowledge, no studies have examined the neurochemical characterisation of axons within whole equine nerves by immunohistochemistry.

The aim of this study, therefore, was to optimise and validate a selection of markers, different processing techniques (formalin-fixed frozen [FFF] and formalin-fixed paraffin-embedded [FFPE]) and different antigen retrieval protocols (pressure cooker and microwave) for immunohistochemistry of equine peripheral nerves in order to provide a research tool for (in future) studying the associated pathological changes in certain equine neurological disorders. In particular, we hypothesised that immunohistochemical identification of motor fibres could be achieved by the use of an antibody to choline acetyltransferase (ChAT),<sup>12</sup> and that sensory unmyelinated axons

would be labelled with antibodies to calcitonin gene-related peptide (CGRP) or substance P.<sup>13</sup> In addition, since autonomic sympathetic and parasympathetic fibres are immunoreactive to tyrosine hydroxylase (TH) and vasoactive intestinal polypeptide (VIP), respectively,<sup>14</sup> we hypothesised that antibodies to these proteins would be suitable for detection of autonomic fibres. This manuscript, therefore, describes the optimisation and validation of antibody markers for assessment of individual axonal types in a selection of equine nerves.

## 2 | MATERIALS AND METHODS

### 2.1 | Sample collection

Tissue samples were collected from five adult ponies and Thoroughbred horses (age range 3-8 years; all geldings) that were subjected to euthanasia as part of separate studies approved by the Home Office (PED82E67D) and local Animal Welfare Ethical Review Board. All horses and ponies were determined to be clinically and neurologically normal, and prior to euthanasia, Thoroughbred horses underwent resting and unsedated laryngoscopy<sup>15</sup>; only horses with grade 1 or 2.1 laryngeal function were included as they are normal or minimally (subclinically) affected by RLN.<sup>16</sup> Sample collection was achieved within 20-90 minutes of euthanasia.

Specific nerves were chosen for this study to ensure examination of the wide variety of different axonal types. Whole larynges were carefully removed in their entirety with approximately 20 cm of the rostral trachea. Tissues were further dissected by removing cricopharyngeal and thyropharyngeal muscles and the oesophagus. Left and right distal recurrent laryngeal nerves (RLNs) were identified, 2-3 cm from the larynx, and carefully resected. Branches of other peripheral nerves from various anatomical locations were also sampled; these included the phrenic, vagus and plantar digital nerves, obtained as follows. In brief, after removing the skin over the left jugular furrow, the left vago-sympathetic trunk was identified as it passed parallel to the jugular vein in the caudal third of the neck. Distal left phrenic nerve samples were collected by making a craniocaudal skin incision at the level of costochondral junction, cutting the ribs and displacing the lung laterally to allow visualisation of the nerve approximately 10 cm prior to its ramification and entry to the diaphragm. For dissection of lateral plantar digital nerve, fascia and blood vessels at the abaxial aspect of the metatarsophalangeal (fetlock) joint were initially removed and the nerve identified. Nerve samples (approximately 3 cm) were removed from each sampling site by sharp dissection with a scalpel, taking care to avoid damage to the tissue, and prior to fixing in 10% neutral buffered formalin.

## 2.2 | Tissue processing

Two methods for tissue processing were used to assess optimal staining quality of various antibodies.

### 2.2.1 | 1-Formalin-fixed paraffin-embedded (FFPE) tissue

After fixation in 10% neutral buffered formalin for 24 hours, portions of the fixed nerves were subsequently embedded in wax (to enable transverse sectioning) as is routine for paraffin-embedded sections. Paraffin sections of 4 µm were cut and then dried in 37°C oven overnight before storage at room temperature until used.

Antigen retrieval was performed on paraffin-embedded tissue using two different heat-mediated antigen retrieval protocols: the first method achieved antigen retrieval with a domestic pressure cooker. Briefly, slides were dewaxed in xylene for 20 minutes and rehydrated in ethanol (twice in 100% and then 70% in distilled water

for 2 minutes each). Finally, slides were incubated for 10 minutes in a pressure cooker containing 1 L of 10 mM sodium citrate buffer solution (pH 6.0) (diluted in distilled water) that had been boiling, by being placed on a hot plate at 270°C. Ultimately, this antigen retrieval protocol was adopted for immunohistochemistry of TH and ChAT.

The second antigen retrieval protocol involved the use of a domestic microwave (800 W). In brief, after dewaxing and rehydration, slides were placed in a slide rack, with the slides labelled and back-to-back oriented. After rehydration, slides were transferred to a slide box containing distilled water and left to rinse for 3 minutes, while a slide box containing 300 ml of 10 mM sodium citrate buffer solution (pH 6.0) was heated in the microwave at a power of 100% for 3 minutes. Once heated, the slide rack was added to the sodium citrate solution in the microwave at 50% power for 5 minutes.

Finally, the slides treated with these two heat-mediated antigen retrieval procedures were allowed to cool down to room temperature for 30 minutes in the same sodium citrate buffer. Then slides

Primary antibody	Host species	Product code	Dilution in PBS	Manufacturer
NF200	Rabbit	Ab8135	1:1500	Abcam
Beta III tubulin	Rabbit	Ab18207	1:1500	Abcam
ChAT	Goat	AB144P	1:300	Millipore
TH	Rabbit	Ab152	1:1500	Millipore
VIP	Rabbit	ab22736	1:1000	Abcam
MBP	Rat	Ab7349	1:100	Abcam
CGRP	Rabbit	T-4032	1:2000	Peninsula
Substance P	Mouse	MAB4375	1:2000	R&D
Collagen V	Goat	135001	1:50	BIO RAD

**TABLE 1** Details of primary antibodies used to label peripheral nerve fibre components including anti-neurofilament heavy chain (NF200), beta III tubulin (B3T), choline acetyltransferase (ChAT), tyrosine hydroxylase (TH), myelin basic protein (MBP), calcitonin gene-related peptide (CGRP) and collagen V with their host species, concentration of primary antibodies in phosphate-buffered saline (PBS) and other products details

Staining purpose	Primary antibody mixture	Secondary antibody mixture
Labelling of myelinated and unmyelinated axons	Rabbit anti-NF200 (1:1500) Rabbit anti-B3T (1:1500) Rat anti-MBP (1:100)	Donkey anti-rabbit IgG (1:1000) Donkey anti-rat IgG (1:1000)
Labelling of myelinated motor and unmyelinated sympathetic axons	Goat anti-ChAT (1:300) Rabbit anti-TH (1:1500) Rat anti-MBP (1:100)	Donkey anti-goat IgG (1:1000) Donkey anti-rabbit IgG (1:1000) Donkey anti-rat IgG (1:1000)
Labelling of unmyelinated sensory axons and fascicular outline	Rabbit anti-CGRP (1:2000) Goat anti-collagen V (1:50)	Donkey anti-rabbit IgG (1:1000) Donkey anti-goat IgG (1:1000)
Co-expression of CGRP and substance P proteins	Rabbit anti-CGRP (1:2000) Mouse anti-substance P (1:2000)	Donkey anti-rabbit IgG (1:1000) Donkey anti-mouse IgG (1:1000)

**TABLE 2** Details of primary and secondary antibodies used for double or triple immunofluorescent labelling to detect myelinated and unmyelinated axons, myelinated motor and unmyelinated sympathetic axons, unmyelinated sensory axons (CGRP- and substance P-positive axons) and perineurial area. These antibody mixtures enabled clear morphological and functional identification of equine peripheral nerve constituents

were rinsed in phosphate-buffered saline (PBS) for 2 minutes and immediately transferred to a humidified slide chamber and prepared for blocking and immunohistochemistry steps.

### 2.2.2 | 2- Formalin-fixed frozen tissue (FFF)

Portions of the formalin-fixed nerves were also embedded in optimum cutting temperature (OCT) medium (Agar Scientific Ltd, AGR1180) for cryosectioning. Briefly, the tissues were initially incubated in 50 mL of ascending concentration of sucrose solutions diluted in PBS (10% and 20% for an hour each followed by 30% overnight at 5°C). The following day, the nerve segments were placed in a plastic cryomould (Agar Scientific Ltd, G458) filled with OCT and then slowly frozen in liquid nitrogen by floating the cryomould on the surface. The frozen block was then removed from the plastic cryomould and frozen vertically on another cork disk (Fisher Scientific UK Ltd, 12688566) to enable transverse cryosectioning of the nerves. Fifteen micrometre sections were cryosectioned (Bright) at a temperature of -20°C onto glass slides (Fisher Scientific UK Ltd, 10149870) and allowed to air dry at room temperature for 30 minutes (to improve adherence) before storage at -80°C until used.

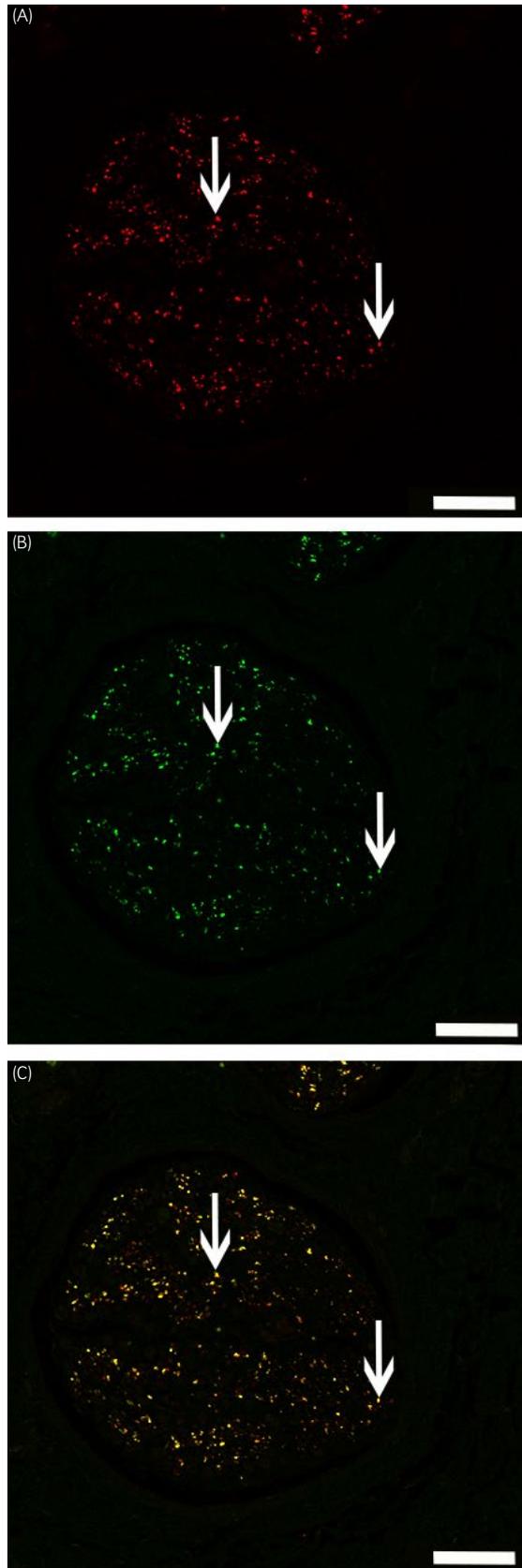
## 2.3 | Immunohistochemistry

Primary antibodies used in this study are summarised in Table 1. All secondary antibodies were IgG species-specific Alexa Fluor conjugates raised in donkey (Invitrogen (<http://www.invitrogen.com>) and Abcam) for wavelengths 488 (green) and 594 nm (red) and 647 nm (far red) and used at a concentration of 1:1000 (diluted in PBS).

The method of double or triple labelling is summarised in Table 2. Briefly, after appropriate antigen retrieval of paraffin-embedded tissue, each section was circled with a hydrophobic pen (ImmEdge™ Hydrophobic Barrier Pen, H-4000) (Vector Laboratories) and blocked with 10% donkey serum in PBS for 1 hour at room temperature. Excess serum was then removed and primary antibodies were applied (as triple or double antibody mixtures) and incubated at 5°C in a cold room as follows: triple labelling with rabbit anti-NF200<sup>5</sup>, rabbit anti-beta III tubulin (B3T) (Abcam) and rat anti-myelin basic protein (MBP) (Abcam) was performed to stain myelinated and unmyelinated nerve fibres. A combination of rabbit anti-NF200 and rabbit anti-beta III tubulin (B3T) was used due to the relative difference in their level of expression in different axons: NF200 is predominantly expressed in large-diameter axons and B3T mainly found in small-diameter axons.<sup>17</sup> The combination improved the staining quality of all fibres with different diameters (results not shown). For detection of myelinated cholinergic motor and unmyelinated adrenergic sympathetic fibres, triple labelling with rat anti-MBP, goat anti-choline acetyltransferase (ChAT) (<http://www.invitrogen.com>) and rabbit anti-tyrosine hydroxylase (TH) (<http://www.invitrogen.com>) was used to functionally characterise nerve fibres. For CGRP detection, double labelling with rabbit anti-CGRP antibody (Peninsula Laboratories International) and goat anti-collagen V (BIO RAD, Watford) was performed to stain sensory unmyelinated C-fibres within detectable endoneurial and perineurial architecture revealed by the collagen stain. A rabbit anti-vasoactive intestinal polypeptide (VIP) (Abcam) was evaluated in order to detect parasympathetic fibres. Double staining with rabbit anti-CGRP antibody and mouse anti-substance P (R&D Systems, Inc.) antibody was also performed to show co-expression of these two proteins in sensory unmyelinated fibres within plantar digital nerve. Positive control tissue sections of dorsal (sensory) and ventral (motor) horn of the spinal cord and cranial cervical sympathetic ganglion were

**TABLE 3** Summary of testing neuronal markers with different tissue preparation and different antigen retrieval protocols. The results of staining of formalin-fixed paraffin-embedded (FFPE) tissue showed specific immunoreactivity for all markers tested. In contrast, some other markers (anti-ChAT, anti-TH and anti-substance P antibodies) did not achieve immunostaining on formalin-fixed frozen (FFF) tissues. Some markers (anti-ChAT and anti-TH antibodies) worked only with pressure cooker retrieval and the remaining markers achieved good staining quality with microwave retrieval

Marker	Modality/tissue localisation	Tissue preparation		Antigen retrieval (10% sodium citrate)	
		FFPE	FFF	Pressure cooker (10 min)	Microwave (5 min)
NF200	Axonal marker (large diameter)	✓	✓	✓	✓
Beta III tubulin	Axonal marker (small diameter)	✓	✓	✓	✓
ChAT	Motor axons	✓	×	✓	×
TH	Sympathetic axons	✓	✓	✓	×
MBP	Myelin	✓	×	✓	✓
CGRP	Sensory unmyelinated fibres	✓	✓	×	✓
Substance P	Sensory unmyelinated fibres	✓	×	×	✓
Collagen V	Endo/peri/epineurium	✓	✓	×	✓



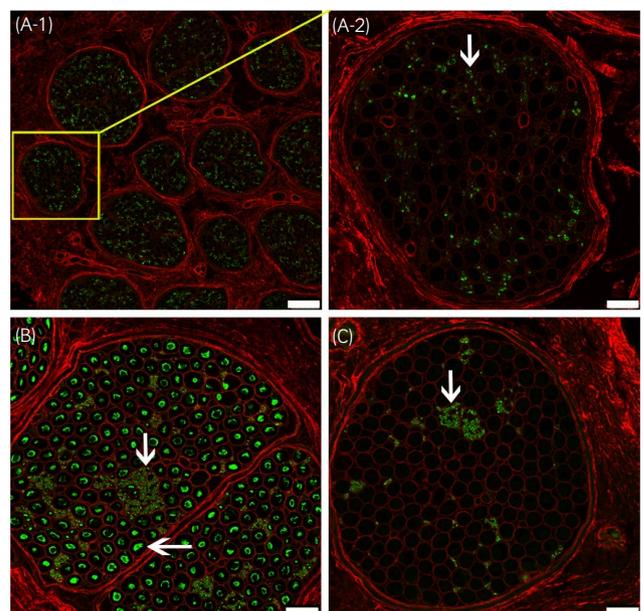
**FIGURE 1** Double staining on paraffin-embedded section showing co-localisation of CGRP (red staining in A) and substance P (green staining in B) in equine plantar digital nerve. There is extensive co-expression of CGRP and substance P (C). Scale bar: 50  $\mu$ m

initially used to confirm the specificity of antibodies to CGRP and substance P, ChAT and TH respectively (results not shown). Double and triple antibody labelling reactions were initially tested independently also to ensure the specificity of these markers; further, all primary antibodies were omitted in negative control reactions to ensure no nonspecific binding of the secondary antibodies (results not shown).

Tissue sections were incubated overnight with the primary antibody mixtures, and then washed three times in PBS over 10 minutes. Secondary antibody mixtures were applied for 1 hour in a humidified chamber at room temperature, followed by three washes with PBS before adding a Slow fade diamond antifade Mountant (Invitrogen, S36963) (<http://www.invitrogen.com>). Slides were coverslipped and then examined by confocal microscope (Leica SP8) with at least three fascicles scanned from each nerve.

### 3 | RESULTS

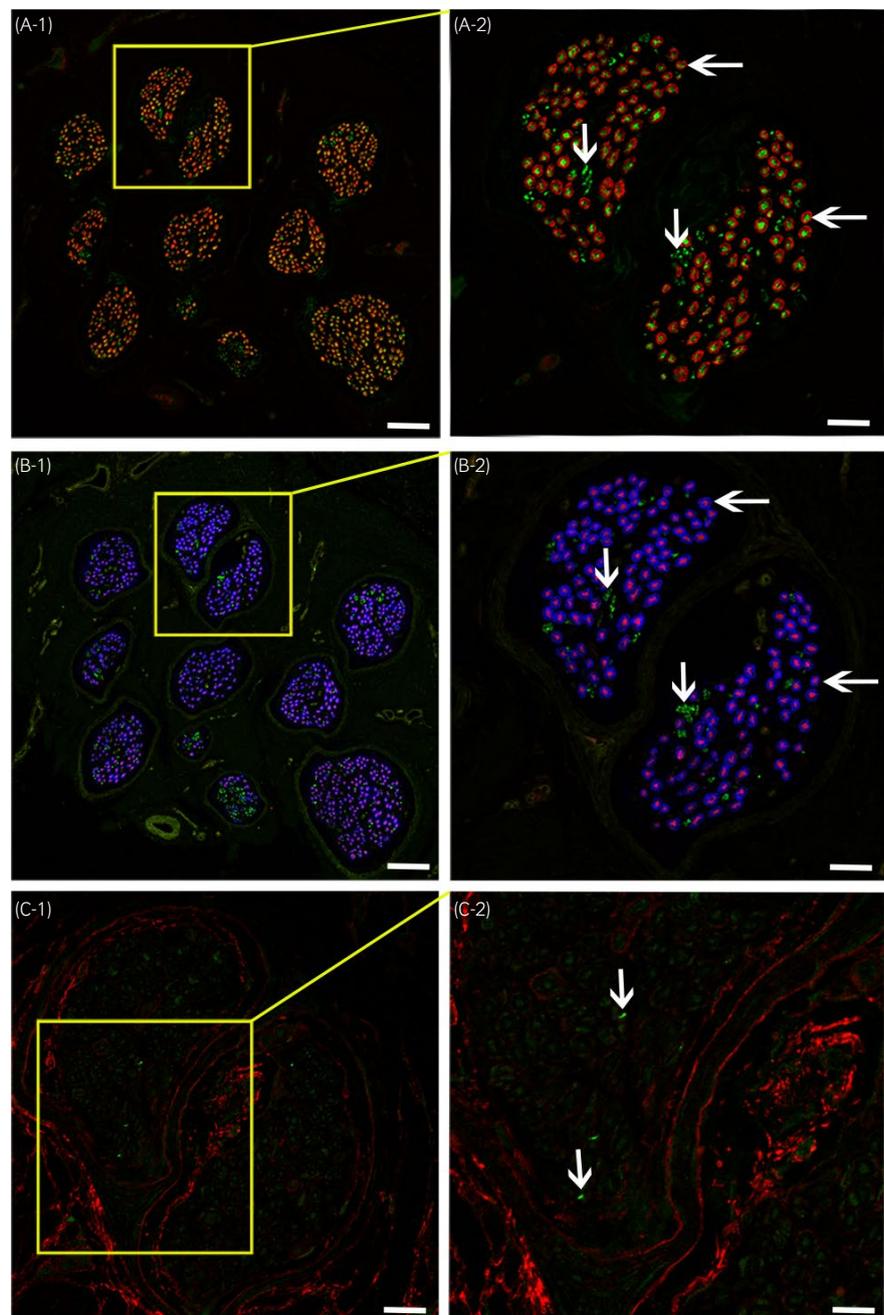
The same pattern of staining for each marker was observed in each nerve from all horses/ponies examined ( $n = 5$ ). The comparative results of different nerve preparations and antigen retrieval



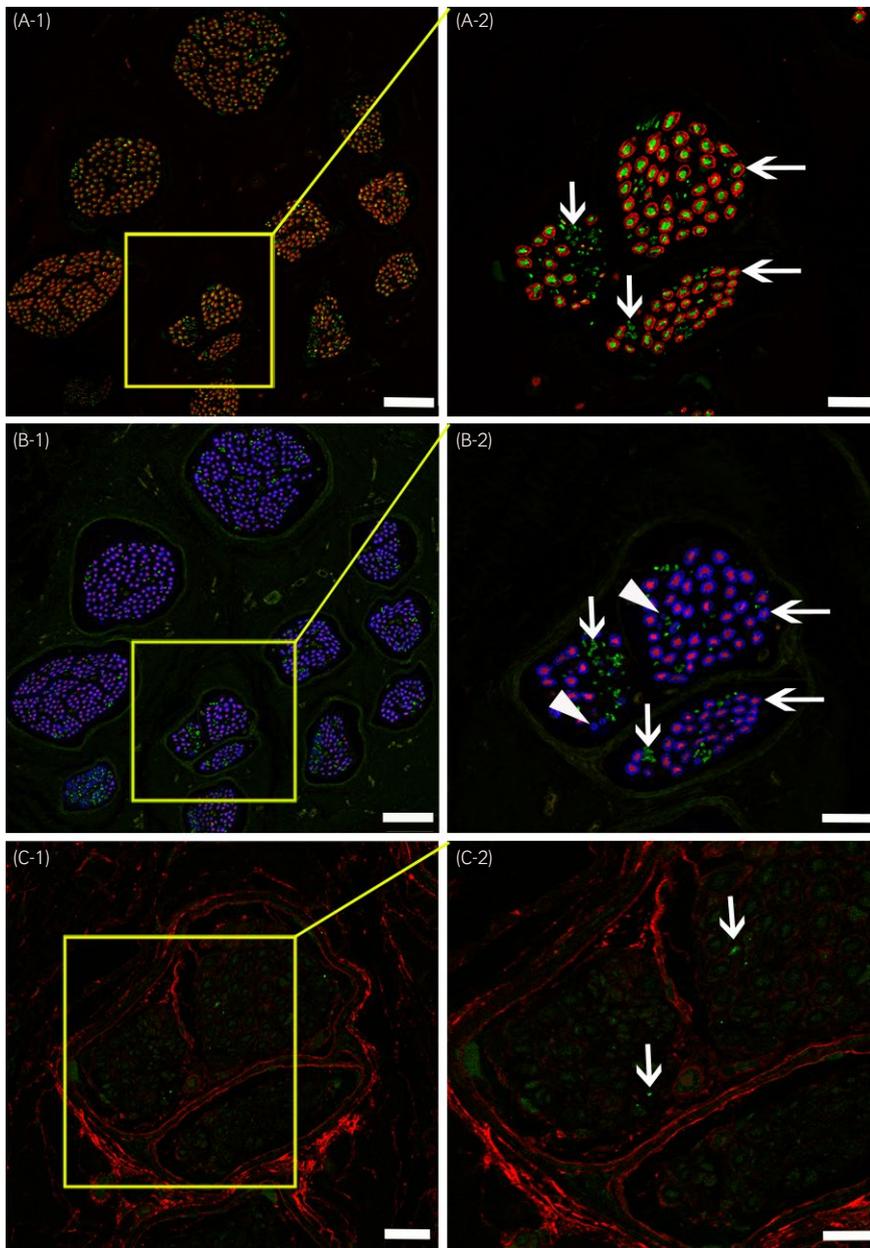
**FIGURE 2** Cryosections of formalin-fixed frozen nerve tissue from a pony stained for (A-1 and A-2; plantar digital nerve) CGRP (green) and collagen V (red); (B; vagus nerve) NF200 and B3T (green) and collagen V (red) and (C; vagus nerve) TH (green) and collagen V (red). (A-1) shows several fascicles of plantar digital nerve with numerous CGRP-positive sensory fibres at low magnification. (A-2) shows a fascicle from the same nerve at higher magnification showing diffuse small-diameter CGRP-positive fibres (green) (white arrow). Labelling with NF200 and B3T in (B) reveals large clusters of small-diameter axons (vertical arrow) among numerous large-diameter axons (horizontal arrow). The clusters of small-diameter axons (C) are TH-positive (sympathetic) fibres (vertical arrow). Scale bar is 100  $\mu$ m in A-1 and 25  $\mu$ m in A-2, B and C

protocols are summarised in Table 3. Some antibodies (NF200 and BIIIIT, TH, CGRP and collagen V) achieved clear, unambiguous staining with low background on both FFPE and FFF tissues. In contrast, detection of ChAT, MBP and substance P was only achieved in paraffin-embedded tissue. Some markers (NF200, BIIIIT and collagen V) yielded reliable staining quality using either pressure cooker or microwave antigen retrieval protocols. Staining with anti-ChAT and anti-TH antibodies did not show positive immunoreactivity following microwave retrieval, whereas pressure cooker retrieval consistently enabled detection of ChAT and TH immunoreactive fibres in each nerve examined. Finally, CGRP and substance P-positive C-fibres were most readily identified after microwave antigen retrieval.

In general, immunohistochemistry of FFF tissue enabled clear labelling of axons with minimum or no fascicular distortion. However, not all markers were detected on FFF tissues, and it was noted that some axonal markers (especially in left RLn) had a smeared appearance under fluorescence. Immunohistochemical staining with rabbit anti-VIP antibody failed to achieve reliable immunoreactivity in any nerve examined neither in FFPE nor in FFF tissues. Further, transverse section orientation of all fascicles could not always be obtained even after treatment with 30% sucrose (which helps hardening the tissue to facilitate cryosectioning) due to nerve displacement during sectioning. In addition, FFF tissue often failed to adhere to the slides throughout the staining steps. For these reasons, and since all the antibodies



**FIGURE 3** Serial sections of formalin-fixed paraffin-embedded tissues from the left recurrent laryngeal nerve of a Thoroughbred horse stained for NF200 and B3T (green) and MBP (red) (A-1 and A-2); TH (green), ChAT (red) and MBP (blue) (B-1 and B-2); CGRP (green) and collagen V (red) (C-1 and C-2). In (A-2), vertical arrows show loosely arranged small-diameter unmyelinated axons and horizontal arrows indicate large myelinated fibres. Unmyelinated axons indicated by vertical arrows in (B-2) are TH-positive (sympathetic), whereas horizontal arrows show myelinated fibres positive for ChAT (motor). (C-2) reveals low numbers of CGRP-positive fibres (sensory). This panel shows that the L-RLn is composed of myelinated motor and unmyelinated sympathetic and a few sensory axons. Scale bar measurement is 100  $\mu$ m in (A-1 and B-1); 25  $\mu$ m in (A-2 and B-2) and (C-1); 15  $\mu$ m in (C-2)



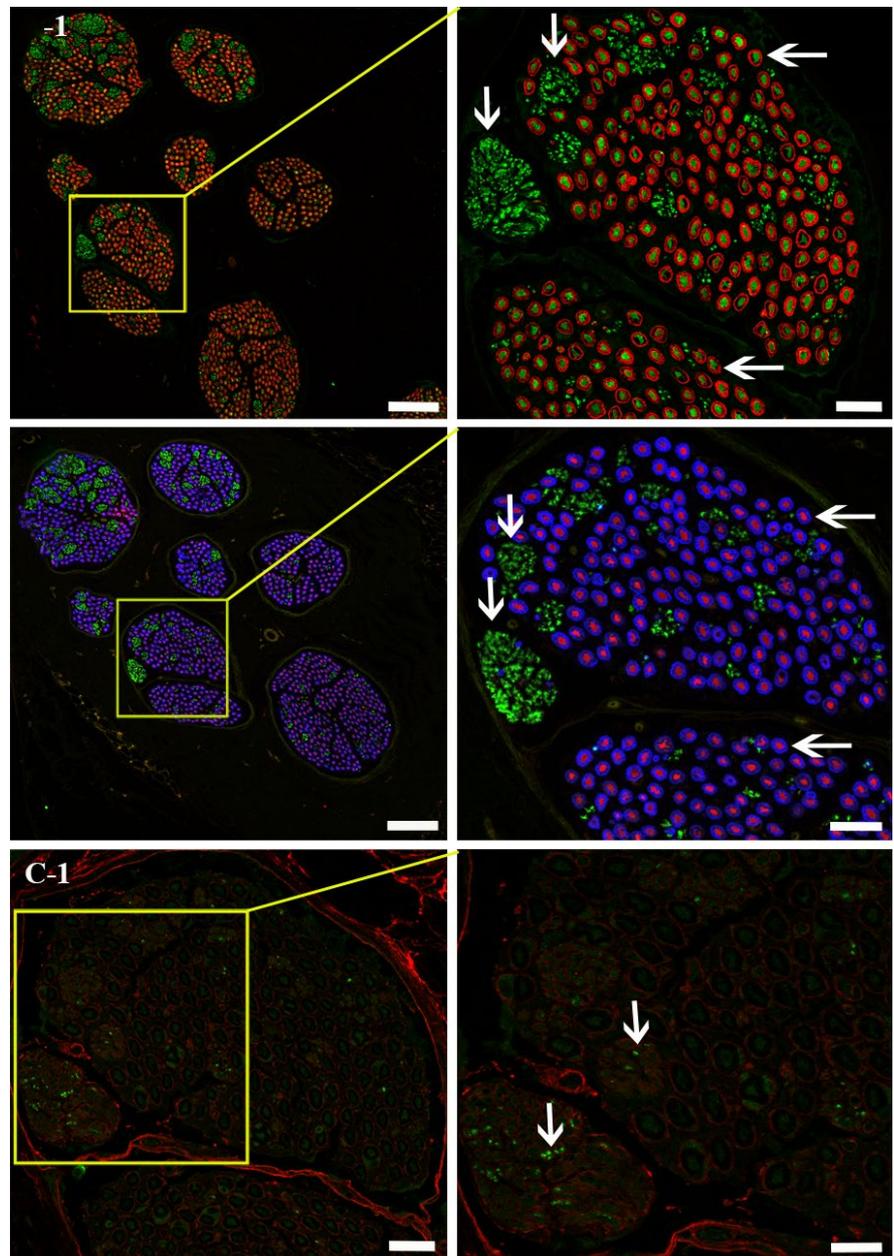
**FIGURE 4** Serial sections of formalin-fixed paraffin-embedded right recurrent laryngeal nerve of a Thoroughbred horse stained for NF200 and B3T (green) and MBP (red) (A-1 and A-2); TH (green), ChAT (red) and MBP (blue) (B-1 and B-2); CGRP (green) and collagen V (red) (C-1 and C-2). In (A-2), vertical arrows show discrete small-diameter unmyelinated axons and horizontal arrows indicate large myelinated fibres. Unmyelinated axons indicated by vertical arrows in (B-2) are TH-positive (sympathetic), whereas horizontal arrows show myelinated fibres positive for ChAT (motor) and arrowheads indicate ChAT-negative axons (most likely myelinated sensory axons). (C-2) shows occasional detectable CGRP-positive (sensory) fibres. This panel reveals that the composition of axonal subtypes in the R-RLn is similar to that of L-RLn. Scale bar is 100  $\mu\text{m}$  in (A-1 and B-1); 25  $\mu\text{m}$  in (A-2, B-2 and C-1); 15  $\mu\text{m}$  in (C-2)

were optimised in FFPE, this method of tissue processing was selected for further studies of the various selected equine peripheral nerves.

The neurochemical characterisation of all equine nerve samples investigated in this study revealed that they contain both myelinated and unmyelinated axons as expected. Sensory unmyelinated fibres in plantar digital samples showed co-expression of CGRP and substance P as shown in Figure 1. Representative images of markers that provided positive staining in FFF tissue are shown in Figure 2. Triple labelling with anti-NF200 IgG, anti-beta III tubulin IgG and anti-MBP IgG showed that myelinated fibres were large in diameter compared with the unmyelinated fibres. In the left and right RLns, unmyelinated fibres were scattered between myelinated axons. Also, although the vast majority of the myelinated fibres in many fascicles in the RLns were

ChAT-positive (horizontal arrows in Figures 3 and 4, B-2), some ChAT-negative myelinated fibres were also seen (arrowheads in Figure 4, B-2). In addition, unmyelinated fibres were either TH- (Figures 3 and 4, B-2) or CGRP-positive (Figures 3 and 4, C-2). In the phrenic nerve, the pattern of fibre composition was similar to that of the RLns; however, unmyelinated axons were arranged in clusters (Figure 5, A-1 and A-2). TH-positive fibres were arranged in large clusters (Figure 5, B-2) and CGRP-positive fibres (Figure 5, C-2) were frequently seen in each fascicle and in greater numbers when compared with those in the RLns. In contrast to the RLns and phrenic nerve branches, all myelinated fibres in the plantar digital nerve were ChAT-negative (Figure 6, B-2) as expected. TH- and CGRP-positive fibres were abundant in each fascicle (Figure 6, B-2 and C-2) of the plantar digital nerves.

**FIGURE 5** Serial sections of formalin-fixed paraffin-embedded phrenic nerve of a Thoroughbred horse immunolabelled for NF200 and B3T (green) and MBP (red) (A-1 and A-2); TH (green), ChAT (red) and MBP (blue) (B-1 and B-2); CGRP (green) and collagen V (red) (C-1 and C-2). In (A-2), vertical arrows show large clusters of unmyelinated axons and horizontal arrows indicate large myelinated fibres. Unmyelinated clusters of axons indicated by vertical arrows in (B-2) are TH-positive (sympathetic), whereas horizontal arrows show myelinated fibres positive for ChAT (motor). (C-2) shows some CGRP-positive fibres (sensory). This panel shows that this nerve branch contains myelinated motor axons, largely clustered sympathetic axons and sensory fibres found in higher frequency compared with the RLNs. Scale bar measurement is 100  $\mu\text{m}$  in (A-1 and B-1); 25  $\mu\text{m}$  in figures (A-2, B-2 and C-1); 15  $\mu\text{m}$  in (C-2)



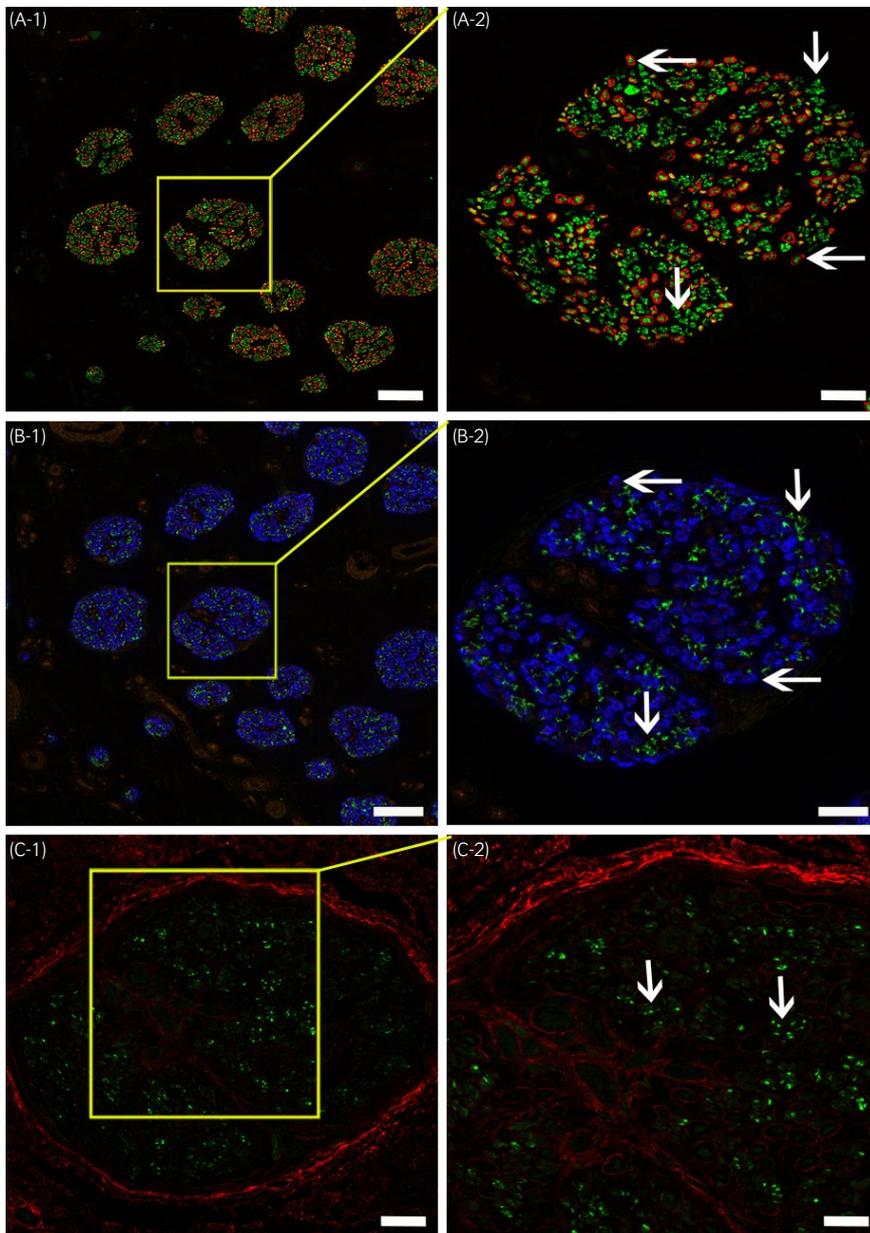
## 4 | DISCUSSION

We envisage that determining the neurochemical composition of axons in equine peripheral nerves might be important for assessment of various pathophysiological processes associated with equine neuropathies. Consequently, in this study, we evaluated different tissue processing and antigen retrieval protocols and methods for optimisation of a range of antibodies for identification of nerve fibre constituents within selected peripheral nerves with differing function. These methods, combined with confocal microscopy, enabled unambiguous and reliable detection of motor, sensory and sympathetic axons.

Immunohistochemistry of FFF tissue enabled clear labelling of axons with minimal distortion, although with some nerves, we encountered smearing of the fluorescent signal. Nevertheless, this

well-maintained fascicular morphology allowed identification of clearly defined sympathetic and sensory axons. However, some other antibody markers (ChAT and substance P) did not show immunoreactivity with this technique. The difficulty in detecting substance P-positive fibres in frozen preparations has been previously reported in nasal mucosal tissue of horses [10; 13]. In contrast, confocal microscopy in our study enabled identification of well-defined substance P-positive fibres in FFPE tissue without background staining.

The equine RLNs innervate the laryngeal muscles ipsilaterally.<sup>18</sup> Morphometric analysis of equine RLNs in healthy young horses revealed that they contain large-diameter (mean of 9.5  $\mu\text{m}$ ) myelinated fibres<sup>19</sup>; the authors did not describe the presence of unmyelinated fibres. However, unmyelinated fibres have previously been confirmed in the RLNs by electron microscopy.<sup>20</sup> Several studies have demonstrated that in horses with RLN, there is a loss of large-diameter



**FIGURE 6** Serial sections of formalin-fixed paraffin-embedded plantar digital nerve of a Thoroughbred horse stained with antibodies specific for NF200 and B3T (green) and MBP (red) (A-1 and A-2); TH (green), ChAT (absent red staining) and MBP (blue) (B-1 and B-2); CGRP (green) and collagen V (red) (C-1 and C-2). In (A-2), vertical arrows show large clusters of unmyelinated axons and horizontal arrows indicate large myelinated fibres. Unmyelinated clusters of axons indicated by vertical arrows in (B-2) are TH-positive (sympathetic), whereas horizontal arrows show myelinated fibres with no immunoreactivity for ChAT. (C-2) shows numerous CGRP-positive (sensory) fibres. In contrast to the RLNs and phrenic nerve branches, plantar digital nerves that did not contain motor axons and sensory axons were abundant. Scale bar measurement is 100  $\mu\text{m}$  in (A-1 and B-1); 25  $\mu\text{m}$  in (A-2, B-2 and C-1); 15  $\mu\text{m}$  in (C-2)

myelinated fibres innervating the laryngeal muscles, predominantly on the left side<sup>20-27</sup> (summarised by Draper and Piercy<sup>28</sup>). Our current results reveal that the myelinated fibres are (ChAT-positive) motor axons and that unmyelinated fibres are predominantly sympathetic, with occasional interspersed unmyelinated sensory axons. Presence of some ChAT-negative myelinated axons in the RLNs raises the possibility that such fibres might be of either motor or sensory origin. Motor fibres devoid of ChAT might suggest that loss of ChAT expression might precede motor neuron degeneration in RLN. Alternatively, myelinated ChAT-negative axons might be sensory fibres innervating the larynx, oesophagus and trachea as previously proposed.<sup>29</sup> We are, however, unaware of specific antibody markers for myelinated sensory axons. Future studies on diseased horses will be necessary to determine whether RLN involves preferential loss of just the large-diameter (motor) axons or whether there is simultaneous or associated loss of additional myelinated sensory axons and unmyelinated (TH- and

CGRP-positive) axonal components of these nerves. Such analysis might help shed light on the pathophysiology of RLN.

Our results also demonstrated that the fibre composition of the phrenic nerve is similar to the RLNs: it contains myelinated motor and unmyelinated sympathetic and sensory fibres. The afferent innervation of the diaphragm is thought to provide sensory perception of breathing, which has a powerful impact on the regulation of motor output.<sup>30</sup> The extensive expression of TH-positive sympathetic fibres is mainly found in larger clusters between the myelinated fibres of the phrenic nerve in contrast with the more dispersed distribution in the RLNs. This morphology might indicate that these fibres run in parallel throughout the entire length of the phrenic nerve and branch off distally within different regions of the diaphragm. While the sympathetic innervation of equine skeletal muscles has not been studied, in humans, sympathetic innervation regulates the tonicity of blood vessels in skeletal muscles,<sup>31</sup> and there is strong evidence

of sympathetic innervation of intrafusal and extrafusal muscle spindle receptors in human skeletal muscles.<sup>32</sup>

The immunostaining in the plantar digital nerve showed that all myelinated fibres in this nerve were ChAT-negative, and those that were unmyelinated are TH- or CGRP-positive. The absence of ChAT in this nerve branch supports the specificity of this marker. Here, TH- and CGRP-positive axons confirm the presence of sympathetic and sensory axons in this nerve branch respectively. We postulate that ChAT-negative myelinated fibres in this nerve branch are sensory myelinated fibres that innervate sensory receptors within the hoof. The release of CGRP from sensory fibres has a powerful vasodilator effect on digital arteries and veins.<sup>33</sup> Researchers<sup>34</sup> revealed that degeneration of both myelinated and unmyelinated C-fibres occurs in horses with laminitis (detected by electron microscopy), but they did not reveal the specific modality of the degenerating axons in this disorder. Consequently, application of IHC on digital nerves from horses with laminitis might help clarify the pathogenesis of this common disorder in the future.

In summary, this study provides a validation of neuronal antibody markers that could be used to study the normal composition of equine peripheral nerves and their pathological changes in various disorders, including RLN and laminitis.

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#### CONFLICT OF INTERESTS

No competing interests have been declared.

#### AUTHOR CONTRIBUTIONS

All authors contributed to sample collection and preparation of the manuscript; A. Lane, S. Cahalan and D. Goodwin contributed to sample preparation and experimental procedures; A. Almuhanna performed the experiments and with R. Piercy, wrote the manuscript which was approved by all other authors. R. Piercy obtained funding and with J. Perkins, provided overall project guidance.

#### ETHICAL ANIMAL RESEARCH

Animal procedures and tissue samples were obtained according to studies approved by the Home Office within the Animal Scientific Procedures Act 1986 (UK) and with approval of the local Animal Welfare Ethical Review Board.

#### INFORMED CONSENT

Not applicable.

#### DATA ACCESSIBILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analysed in this study.

#### PEER REVIEW

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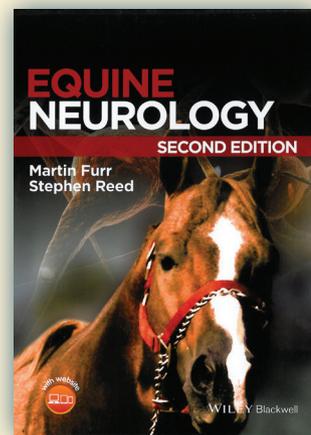
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