

REVIEW

Pathological classification of equine recurrent laryngeal neuropathy

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Funding information

Horseshoe Betting Levy Board, United Kingdom

Recurrent Laryngeal Neuropathy (RLN) is a highly prevalent and predominantly left-sided, degenerative disorder of the recurrent laryngeal nerves (RLn) of tall horses, that causes inspiratory stridor at exercise because of intrinsic laryngeal muscle paresis. The associated laryngeal dysfunction and exercise intolerance in athletic horses commonly leads to surgical intervention, retirement or euthanasia with associated financial and welfare implications. Despite speculation, there is a lack of consensus and conflicting evidence supporting the primary classification of RLN, as either a distal ("dying back") axonopathy or as a primary myelinopathy and as either a (bilateral) mononeuropathy or a polyneuropathy; this uncertainty hinders etiological and pathophysiological research. In this review, we discuss the neuropathological changes and electrophysiological deficits reported in the RLn of affected horses, and the evidence for correct classification of the disorder. In so doing, we summarize and reveal the limitations of much historical research on RLN and propose future directions that might best help identify the etiology and pathophysiology of this enigmatic disorder.

KEYWORDS

axonopathy, bilateral mononeuropathy, horse, larynx, myelinopathy, polyneuropathy, recurrent laryngeal neuropathy

1 | INTRODUCTION

Recurrent laryngeal neuropathy (RLN), is an equine degenerative disorder of the recurrent laryngeal nerves (RLn) of (particularly) tall breeds such as Thoroughbreds and Drafts.^{1,2} The disease is characterized by preferential degeneration of the left RLn over the right RLn^{3,4} that causes paresis or, in severe cases, paralysis of the left intrinsic laryngeal muscles preventing arytenoid movement, which is vital for enabling unimpeded inspiratory airflow. Recurrent laryngeal neuropathy (RLN) is performance limiting in horses performing at their greatest exertion, in

particular, in racehorses.⁵ Many studies have identified RLN-associated neuropathological changes in both RLns (left more so than right) in clinically unaffected horses, leading authors to postulate whether the majority of unaffected horses are in fact subclinical cases.^{3,4,6,7} The mechanisms that lead to the varying severity (from subclinical to severe) in horses are largely unknown, however, the identification of many subclinical cases means that case selection for "unaffected" controls for research is challenging. Amongst the apparent risk factors, horse height is reported as a significant contributor to RLN-status, with taller horses being at greater risk^{1,2}; it is unclear whether height itself is the principal risk factor or simply a covariate for nerve length. Males are more commonly affected than females.^{2,8} Genetic risk factors are plausible given that the heritability of RLN ranges from 8 to 40% depending on the breed affected^{9,10}; however, as of yet no specific risk alleles have been reported despite many published studies.^{1,11,12} Indeed, very little is known about the cause of RLN¹³; the array of etiologies proposed includes mechanical stress¹⁴ or ischemic nerve

Abbreviations: RLN, recurrent laryngeal neuropathy; CMT, Charcot-Marie-Tooth disease; RLn, recurrent laryngeal nerve; EM, electron microscopy; EDL, extensor digitorum longus muscle; PMP22, peripheral myelin protein 22 gene; MFN2, mitofuscin 2; Cx-32, connexin 32; SARM1, sterile alpha and TIR motif containing 1; SCG-10, stathmin-2; NMNAT2, nicotinamide nucleotide adenyltransferase 2; NAD⁺, nicotinamide adenine dinucleotide; ATP, adenosine triphosphate; CMAP, compound motor action potential; NCV, nerve conduction velocity..

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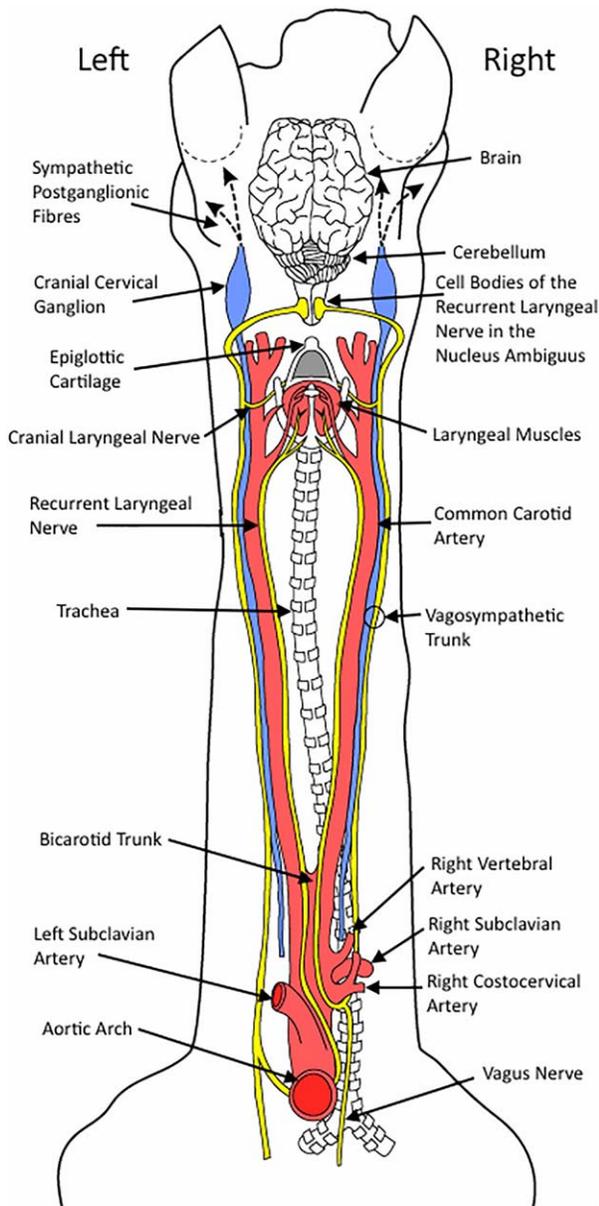


FIGURE 1 Anatomical representation of the dorsal view of the course taken by the RLn (yellow) in the horse, including both vagosympathetic trunks, vagus nerves, and important vasculature. Both RLn originate in the medulla oblongata where their cell bodies are located within the nucleus ambiguus. The left RLn courses around the aortic arch before innervating the muscles of the larynx and the right RLn courses around the right subclavian artery before it courses rostrally

damage,⁴ pressure damage,⁴ an infectious etiology,¹³ vitamin deficiencies,¹⁵ and toxic insults.^{16–19}

RLn is consistently described as a peripheral neuropathy but without a more detailed classification describing the primary defect.^{20,21} Conflicting data is available supporting RLn as affecting just the RLn (ie, a mononeuropathy) or whether it affects other peripheral nerves (a polyneuropathy).^{3,4,7,20,22–24} Also unanswered is whether the degeneration begins within the axon or the myelin sheath. Providing answers to these key questions would likely expedite the search for the etiology,

and potential therapies by helping focus research. For instance, molecular therapies are in development for similar human peripheral neuropathies, such as the group of peripheral neuropathies known as the Charcot-Marie-Tooth (CMT) diseases²⁵ but a key element to developing these therapies is the understanding of where the degeneration begins (ie, in the nerve cell body, axon, synapse, myelin sheath or glia), to enable focused treatment. Potential issues that have hindered research in these areas for RLn include the selection of adequate control groups (as outlined previously), the trend for studies to evaluate subjective histopathological features in unblinded, uncontrolled fashion and that many RLn-studies are typically statistically underpowered.

This review article discusses the current literature (veterinary and human) pertaining to the pathophysiology underlying RLn, addressing the issues relating to characterizing RLn as an axon- versus myelinopathy and a mononeuropathy or polyneuropathy.

2 | EQUINE RLn

The RLn contains the longest motor axons in the horse, measuring up to 2.5 m in length in Draft and Thoroughbred horses, with the left nerve being ~30 cm longer than the right in tall breeds⁶ (Figure 1). Their cell bodies are situated within the nucleus ambiguus in the brainstem and they project their axons caudally within the vagus nerves before RLn fibers exit and, on the left, traverse medially around the aorta and on the right, medially around the right subclavian artery.²⁶ The nerves then follow an identical bilateral course, moving rostrally, adjacent to, and on either side of the trachea and carotid arteries. Their long axonal course is established during embryonic development: in the growing embryo, the RLn branch from the vagus nerve at the level of the sixth aortic arch, whereby they innervate the developing intrinsic laryngeal muscles. As the neck of the embryo elongates, the larynx migrates rostrally (relative to the aortic arches) resulting in elongation of the RLn axons as they are held distally by the aortic arches.²⁷ At the level of the aorta, the equine RLn is ~1.5 mm in diameter and the epineurium contains between 6 and 29 perineuronal bundles (fascicles), (generally between 11 and 15; Figure 2).²⁸ Several authors have

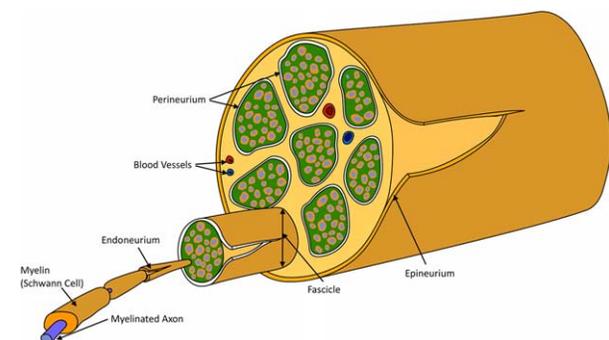


FIGURE 2 Schematic representative of a peripheral nerve. The main connective tissue structures include the epineurium (surrounding the entire nerve), the perineurium (surrounding each fascicle within the nerve) and the endoneurium (located around an individual axon). The perineurium also contains blood vessels and lymphatics

reported structural variations at the hairpin turn of the left RLn, as it courses around the aorta (eg, Haslam's Anomaly and Renault bodies) although these appear with the same frequency in horses with and without RLN.^{4,28–30}

The RLn provide general somatic efferent innervation to the laryngeal and cranial esophageal muscles^{26,31} and are comprised of wide diameter, alpha, myelinated nerve fibers of 12–20 μm diameter.²⁸ Interspersed between the large myelinated fibers are less numerous, narrower myelinated fibers (that are speculated to be beta and gamma fibers) and small unmyelinated fibers.²⁶ A study of 9 mixed-breed horses under 2 years old with no evidence of laryngeal disease, revealed that the total number of myelinated fibers, mean fiber diameter and percentage of fibers over 9.5 μm in diameter were significantly lower in the left RLn compared with the right ($p < 0.05$).³² In other species where greater numbers of myelinated nerve fibers have been reported in the right RLn compared with the left (humans, dog, and cats) typically the myelinated nerve fiber diameter is larger in the left RLn, which is believed to compensate for the greater length of the left RLn, thus maintaining comparable conduction properties since wider axons have faster conduction speeds.^{33–37} Unequal fiber numbers are not found in all species: indeed equal (or near equal) fiber numbers have also been reported in humans, rats, and giraffes.^{37,38} The greater numbers of myelinated fibers in the right RLn might reflect the presence of afferent (sensory) fibers, however the presence of sensory axons within equine RLn has not been investigated. In humans, these sensory axons receive input from the mucosa in the region of the vocal folds, and in cats, the pharyngeal swallow reflex occurs in part, from esophageal pressure increases transmitted through the RLn.³⁹ However, staining for afferent branches in the rat RLn did not show any sensory branches⁴⁰ suggesting that there are species differences in RLn-associated sensory pathways.

2.1 | The importance of the classification of neuropathies

Historically, human peripheral neuropathies were classified according to the site of the primary pathology—as a myelinopathy or axonopathy—caused by degeneration of the myelin sheath or axon respectively, and by the type of neuron affected: sensory or motor.⁴¹ In addition, the involvement of a single nerve or many different nerves in the disease process is described as a mononeuropathy or polyneuropathy, respectively.⁴¹ These classifications were critical for accurate diagnoses, and they underpinned all investigations into the diseases' etiopathogenesis. However, with advanced genomics, many inherited neuropathies are now classified by their causative genetic mutations. The genetic era has highlighted that a single disease phenotype can result from mutations in different genes, and further, that subtle differences in a disease phenotype can be explained by different mutations within the same gene.

The characterization of a peripheral neuropathy as either an axonopathy or a myelinopathy is achieved by amalgamating data from nerve conduction velocity (NCV) testing, pathological observations (from nerve biopsy specimen or postmortem examination) and genetic

studies.^{42–44} Nerve conduction velocities (NCVs) are markedly slowed when there is extensive demyelination, and normal to partially slowed, with reduced compound muscle or sensory action potentials, in an axonopathy.⁴² In the diagnosis of human CMT disorders, genetic testing has largely superseded nerve biopsy⁴³: when a causative genetic mutation is found, differentiating an axonopathy from a myelinopathy is simplified by the mutated protein's normal localized expression. However, mutation of an axonally localized protein can lead to a disease phenotype typically attributed to a myelinopathy and vice versa (see below). As a genetic cause of RLN has not yet been identified, the disease's classification as an axonopathy or myelinopathy can only be based on pathological and electrophysiological studies.

2.2 | Charcot Marie tooth disease complex

The CMT diseases are inherited human peripheral polyneuropathies that affect either sensory or motor axons, or both, and are arranged into categories depending upon their primary site of pathology.⁴³ Where possible, a definitive diagnosis of a specific form of CMT is made after genetic testing, however where a mutation is not found, pathological classifications provide the framework for the diagnosis and importantly, prognostic information.⁴³

Demyelinating forms of CMT are classified as CMT type 1 (median motor NCV < 38 m/s), with subgroups labeled according to the underlying genotype; CMT1a is a common form of CMT1 caused by a duplication in peripheral myelin protein 22 gene (PMP22).⁴³ Human PMP22 duplications were discovered after observation that a mouse model (Trembler) with similar neuropathological changes to those found in CMT1a (peripheral hypomyelination) had a point mutation within PMP22.^{45–47} highlighting the importance of accurate and detailed histopathological disease descriptions. The axonal forms of CMT are classified as type 2 (median motor NCV > 38 m/s), with dominant mutations in the mitofusin 2 (MFN2) gene being the most common (CMT2a).⁴⁸

An intermediate form of CMT disease (CMTX1) that produces axonal and myelin-related pathological changes, as it results from mutations within Connexin-32 (Cx-32).⁴⁹ Cx-32 protein is expressed by Schwann cells in the peripheral nervous system and is involved in tight junction formation between the myelin lamella at the paranode and Schmidt-Lantermann incisions.^{50,51} CMTX1 accounts for $\sim 10\%$ of all human CMT cases, and as a result of the paranodal abnormalities, NCVs are usually slower than CMT type 2 (axonal), ranging from 18 to 60 m/s, but not as slow as the hypomyelinated forms of CMT (eg, CMT type 1).⁴²

3 | CLASSIFICATION OF RLN

3.1 | Neuropathology of RLN

The majority of pathological studies investigating RLN have been conducted by a handful of groups.^{3,4,6,7,20,23,24,29,30,52–56} Their research has demonstrated loss of large, alpha myelinated nerve fibers in the left distal RLn (Figure 3), and in some severely affected horses, within the

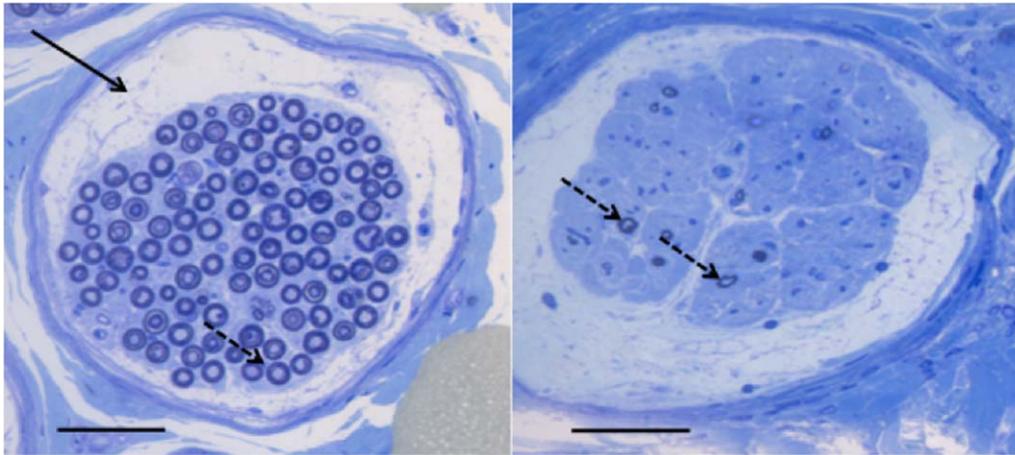


FIGURE 3 Distal Left RLn individual fascicle sections from a horse with Havemeyer grade I/IV (unaffected) RLN (left) and a horse with Havemeyer grade IV/IV (severely affected) RLN (right). There is obvious loss of large myelinated nerve fibers (dotted arrow) in the affected horse (right) compared with the unaffected horse (left), and also note the increased subperineurial space and endoneurial connective tissue present in the section from the affected horse. A Renaut Body is highlighted by a solid black arrow in the normal horse. Black bar: 50 μ m

proximal left RLn, and the distal right RLn.^{3,4,7,20,23} Consequently, RLN has been classified as a distal axonopathy by some authors.^{3,4,20} However, preferential loss of large myelinated nerve fibers in a distal to proximal manner does not per se indicate either an axonopathy or myelinopathy. For example, distal to proximal axonal degeneration is typically seen with toxic or metabolic “dying-back” or “central-peripheral” distal axonopathies (ascending destruction of the distal axon of both central and peripheral nerves).⁵⁷ Likewise, humans with end-stage CMT type 1 (with primary demyelination), present primarily with length-dependent axonal degeneration, with the distal appendages displaying muscle atrophy and the foot deformity, pes cavus.^{58–60}

Clues as to the primary definition of RLN’s status as an axonopathy or myelinopathy might then be possible from other pathological features. Common additional pathological changes affecting the distal left RLn in severely affected cases (Havemeyer grade III/IV or IV/IV⁶¹) include high numbers of Büngner bands and clusters of regenerating fibers.^{3,4} These changes are also found in the proximal left RLn and (less commonly) in the distal right RLn in severely affected horses.^{3,4} Büngner bands are formed from Schwann cell aggregates within the original basal lamina of the Schwann cell-axon unit, and result from proliferation of that Schwann cell after axonal injury⁴¹; they are believed to serve as scaffolding to allow for axonal regeneration.⁶² Regenerating clusters are composed of axonal sprouts that form within the basal lamina as a result of axonal degeneration.^{41,62} The presence of both Büngner Bands and regenerating clusters are suggestive of primary axonal degeneration.⁶²

Other common pathological features seen in RLN are onion bulb formation and segmental demyelination/remyelination. The development of these structures cannot immediately be attributed to that of a primary axonopathy or demyelinating disorder. Onion bulbs are classically seen with repeated cycles of demyelination/remyelination: they are composed of concentric layers of remyelinating Schwann cells (that macrophages have failed to clear) before a successive demyelination episode.⁴¹ Repeated demyelination/remyelination is most commonly

reported in primary myelinopathies⁴¹ but does occur in primary axonopathies, (whence they are termed pseudo-onion bulbs).^{63,64} Segmental demyelination/remyelination manifests as inconsistent internodal lengths and myelin thicknesses, typically within the same fiber, and is a common feature of CMT type 1 (demyelinating form).^{41,58,60} However, segmental myelin loss also occurs secondarily in primary axonopathies when there is loss of communication with the axon terminal^{65,66}; this is a key feature of RLN.²⁰

As both onion bulbs and segmental demyelination/remyelination can occur with either primary myelinopathies or axonopathies, clues as to the primary site of degeneration might be evident from other associated localized pathological changes. In subclinical RLN cases, onion bulbs and regions of demyelination/remyelination occur most commonly at prominent sites of axonal histopathological change.^{3,53} This is consistent with secondary demyelination that occurs as a direct result of axonal degeneration.⁴¹ These axonal histopathological changes included paranodal axoplasmic outpouchings (see below),⁵³ organelle accumulations within swollen axons and active axonal degeneration at sites of onion bulb formation.^{3,7} Specifically, Cahill and Goulden³ remarked that swollen fibers with dense axoplasm and attenuated myelin sheaths were observed in the RLn; often these swollen fibers were at the center of onion bulb formations. Duncan and Hammang⁵³ stated at [paranodal] areas there was evidence of paranodal demyelination and remyelination, and as in all the other similarly swollen axons studied, that there was notable evidence of concentric Schwann cell proliferation leading to typical onion bulb formation. They concluded that the myelin attenuation occurring around axonal spheroids (axonal degeneration) resulted from mechanical slippage of the paranodal myelin.⁵³ The paranode is the main anchorage point of the myelin lamellae to the axon, and mechanical myelin slippage is a well-recognized consequence of paranodal pathology.^{67–69}

When sural nerve biopsy specimen from human patients with a demyelinating polyneuropathy were compared to those with a primary axonal polyneuropathy (diagnosed on electrophysiological tests), there

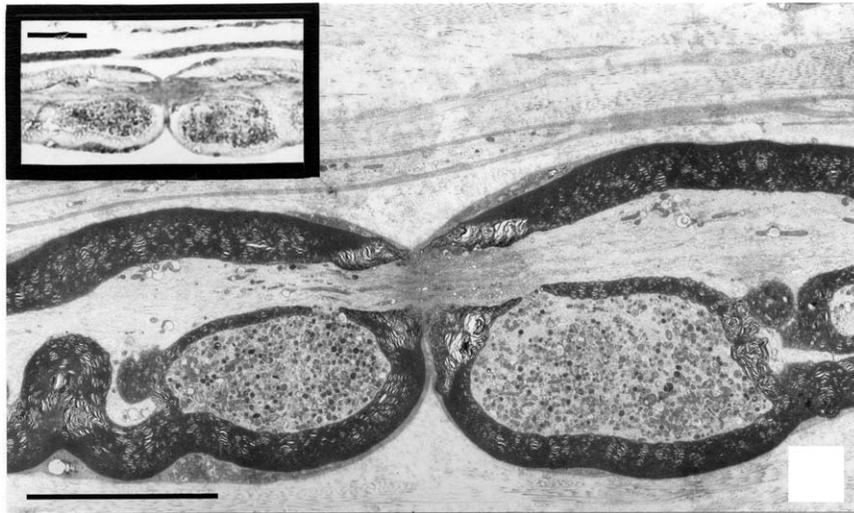


FIGURE 4 Electron microscopic image from longitudinal sections of the left RLn from a subclinically RLN-affected horse. The node of Ranvier, with both surrounding paranodes containing large organelle accumulations (mitochondria and lysosomes) within splits to the myelin sheath can be easily appreciated. The image in the upper left corner is an etched section (1 μm) showing positive staining for anti-cathepsin D in the region of the paranodal evaginations. Cathepsin D is found ubiquitously distributed in lysosomes, confirming their presence within the organelle accumulations. Both black bars correspond to 10 μm . Reprinted by permission from RightsLink: Springer Link, Journal of Neurocytology, Duncan and Hammang⁵³ Copyright 1987

was no difference in the number of onion bulbs between the two conditions.⁶⁴ The authors explained the lack of difference in onion bulb numbers resulted from the fact that onion bulbs identified in patients with the primary axonopathy had been misclassified: they were instead, pseudo-onion bulbs resulting from axonal degeneration/regeneration.⁶⁶ The differentiation of pseudo-onion bulbs from onion bulbs can be difficult, and is typically made by an experienced pathologist who will assimilate the clinical history, results from other diagnostic tests and the type and distribution of the pathology present in a nerve biopsy specimen (sometimes with electron microscopy [EM] and immunohistochemistry); where the concentric Schwann cell processes mainly contain axons or axonal sprouts, this is more indicative of axonal degeneration and regeneration, and these Schwann cell processes are therefore termed pseudo-onion bulbs.⁷⁰ As Bosboom et al⁶⁴ shows, correctly classifying these Schwann Cell proliferations as onion bulbs or as pseudo-onions relies on knowing the underlying pathological process at work, so it is entirely feasible that the demyelination/remyelination reported in RLN results from axonal pathology and not primary demyelination.

Defining the primary site of disease pathology is likely harder in end-stage disease tissue; study of tissue from individuals early in the disease course to identify active pathological processes might be more informative. In the RLn from subclinical cases (equivalent to grade II-IIa⁶¹) organelle accumulations were seen in the paranodes (Figure 4) and along some internodal regions^{3,4,7,53} in the most distal 40 cm of the left RLn and became more numerous in the most distal regions; they were only found in the distal 10 cm of the right RLn. The majority of the paranodal swellings were located proximal to the nearest Node of Ranvier. These swellings were further identified as axoplasmic evaginations that protruded into the myelin sheath, containing mitochondria (normal and degenerate) and dense lamellar bodies (lysosomes).⁵³ Distal

to the swellings, the neurofilaments aggregated at the axon center, causing the mitochondria to be displaced peripherally. Duncan and Hammang⁵³ hypothesized that deficits in axonal transport were most likely the cause of the paranodal evaginations, as they occurred at the bracelet of Nageotte, the site of myelin attachment at the paranode which is a point of axolemmal weakness; they speculated that if axonal transportation is perturbed, cargo will accumulate and cause bulging of the axolemma at its weakest point.⁷¹ Axonal transportation deficits are typically seen in primary axonopathies; in particular, certain forms of CMT type 2 (2a, 2e, 2f) all exhibit transportation deficits whereas different forms of CMT type 1 (myelinopathies) do not (reviewed by Millecamps and Julien⁷²).

Further evidence that these paranodal evaginations result from pathology arising in the axon can be found when looking for their replication in other diseases. Duncan and Hammang⁵³ commented that the paranodal evaginations appear to be a hallmark of RLN; however, they are almost identically replicated in certain toxic polyneuropathies (acrylamide and 2,5-hexane-diol) and were also identified in a rat neuroma study.⁷³⁻⁷⁶ Acrylamide- and hexane-associated polyneuropathies share many other similarities to RLN. They both cause a "dying-back" central-peripheral neuropathy, preferentially affecting long motor neurons.^{77,78} Pathological studies of these toxic neuropathies identified mitochondria (and neurofilaments) located within the paranodal evaginations, typically located proximal to the node.⁷⁹ In addition the evaginations precede classical signs of axonal degeneration in acute models of intoxication.^{71,74,76,80} The exact etiopathogenesis of these toxins has not yet been fully accepted,^{81,82} but both bind to microtubule-associated proteins and neurofilaments,⁸¹ disrupting their maintenance and function, leading to long term deficits in the cytoskeleton and axonal transportation. Both anterograde and retrograde axonal transportation deficits have been recorded in acute acrylamide and hexane neurotoxicity.^{79,83-86} Another important observation

made from models of acrylamide and hexane neurotoxicity is that these paranodal evaginations disrupt axoplasmic flow leading to defective organelle transportation^{79,82} that could contribute to further neurodegeneration. Acrylamide and hexane neurotoxicity are classified as primary axonopathies, which lends support to classification of RLN also as an axonopathy.^{57,73,79,81,82}

Axonal paranodal evaginations were also detected in the proximal sciatic nerve axons of rats, 1 month after the formation of a neuroma, after crushing and ligation.⁷⁶ The evaginations contained similar membrane-bound organelles and dense laminar bodies seen in subclinical RLN cases and in neurons from acrylamide- or hexane-treated animals^{53,71,73,74,80}; they were most numerous in the large myelinated fibers.⁴¹ These findings suggest that loss of trophic support from the muscle could be another important factor leading to generation of these paranodal evaginations,⁷⁶ or it could simply reflect that disruption to both anterograde and retrograde axonal transportation systems can cause evagination.

Other histopathological features reported to occur in the RLN of subclinical RLN cases are axonal spheroids and axonal atrophy though they have not been quantified.^{3,4,7,53} Axonal spheroids are the hallmark pathological sign of axonal dystrophy, a process that is distinct from axonal degeneration, and associated with synaptic dysplasia, abnormal axonal regeneration, or an imbalance with axonal transportation.^{87,88} The timing of axonal spheroid development can serve as a clue for a primary (before degeneration signs occurring) versus a secondary (development of end-bulbs after degeneration) axonal transportation deficit.⁸⁹ End-bulb formation (where the axon terminal swells) has not been reported specifically in RLN, and with the reported paranodal swellings, it is likely that axonal transportation deficits arise in the RLN (left more than right) before the loss of axonal integrity. Axonal atrophy is detectable in end-stage axonal degeneration of various types.⁴¹ These pathological changes, although associated with many different etiologies, are all interpreted as an axonal pathology, supporting primary axon degeneration in RLN.

4 | AXONAL DEGENERATION

For many years, axonal degeneration, studied in humans and mice, was thought simply to encompass Wallerian degeneration and apoptosis via two separate pathways. Wallerian degeneration is seen in the axon distal to a site of injury and involves activation of calpain, resulting from increased axonal calcium influx that leads to orderly degradation of the cytoskeleton and membrane proteins.⁹⁰ The influx of calcium is hypothesized to result from the reversed action of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger as the Na^+/K^+ ATPase pump fails.⁹¹ The axonal debris generated by the proteolysis of the structural proteins and axonal fragmentation is then removed in a coordinated glial response,⁹² including phagocytosis by infiltrating macrophages.⁹³

In 2002, Raff et al⁹⁰ proposed a different classification for types of axonal degeneration:

1. the classical example of *Wallerian degeneration* of the distal axon after axotomy, whereby the proximal axon and cell body degenerate via apoptosis

2. dying-back degeneration of the distal axon following:

- a chronic insult to the cell body, resulting in the distal axon degeneration extending proximally which can, eventually, result in the death of the cell body
- b pathology at the axon terminal (eg, removal of a neurotrophic factor), that maintains the cell body's integrity (eg, axonal pruning during metamorphosis, whereby excessive or redundant neurons are refined in the central nervous system during development)

These observations of 3 different axonal degeneration scenarios were first made from in vitro experimental observations and mouse models⁹⁴ but there was very little understanding of the relevant cellular pathways that contributed to them. Coleman⁸⁹ expanded upon the consequences of the classical "transecting" lesion inducing Wallerian degeneration, by hypothesizing that one or more focal lesions, not necessarily transecting the axon, trigger degeneration of the whole, distal axon. This would explain why proximal axonal numbers remain nearly normal in many peripheral neurodegenerative diseases.⁴¹ Indeed, current evidence suggests that neuronal cell body numbers in the nucleus ambiguus are unaffected in RLN, providing further supportive evidence that RLN is a distal axonopathy.^{22,55,95} Raff et al⁹⁰ speculated that degeneration of only the distal axon could convey a distinct advantage to neurons if they become disconnected from their target cells: it would allow conservation of neuronal resources enabling the axon to reconnect with its terminal when conditions become more favorable. Coleman surmised that Wallerian degeneration could be the final convergent pathway of axonal degeneration in dying-back and distal axonopathies,⁸⁹ which would explain this key pathological feature of RLN.⁶

Defining RLN as a distal axonopathy and the confirmation experimentally, of deficits in axonal transportation, would provide an opportunity for extrapolation from related research in other species that might help define novel therapeutic targets. In recent years, significant strides have been made towards unraveling the molecular signaling of different forms of axonal degeneration⁹⁶ (reviewed by Conforti et al⁹⁷) and identifying pathways that converge on the common Wallerian degeneration pathway, proposed by Coleman⁸⁹ (Figure 5). A key upstream event is the activation of SARM1, which occurs after axotomy,⁹⁸ traumatic brain injury,⁹⁹ mitochondrial potential loss,¹⁰⁰ and excitotoxicity,¹⁰¹ that is, both in disease and with injury, leading to MAP kinase pathway initiation¹⁰² and SCG10 (Stathmin-2; neuronal associated growth protein) loss.¹⁰³ Overexpression of SARM1 alone does not result in axonal degeneration; an additional signal (such as an axotomy) is essential.¹⁰⁴ The other key upstream event that initiates axonal degeneration is down regulation of the enzyme NMNAT2 (a nicotinamide mononucleotide adenyltransferase that undergoes bidirectional axonal transport). NMNAT2 converts nicotinamide mononucleotide to NAD⁺, and its overexpression in experimental models delays Wallerian degeneration.⁹⁷ NMNAT2 is also essential for axonal survival and growth.^{105,106} The exact mechanism behind the protective effects of NMNAT2 overexpression are undetermined, but it does not appear simply to be mediated through an increase in

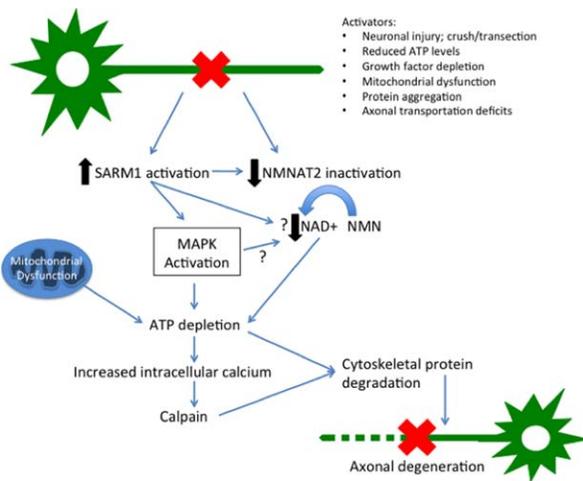


FIGURE 5 Working model of an integrated axon degeneration signaling cascade. Injury leads to SARM1 activation and NMNAT2 depletion. Energetic failure promotes ionic imbalance including intra-axonal calcium accumulation, leading to calpain activation and proteolysis of intermediate filaments in the axonal cytoskeleton. Cumulative structural damage leads to irreversible fragmentation of the damaged axon. Arrows with question marks reflect postulated interactions

NAD⁺; the basal levels of NAD⁺ are not increased by this overexpression, and supplementation of neurons with NAD⁺ *in vitro*, does not protect against degeneration.^{107–109} However, SARM1 overexpression does trigger rapid NAD⁺ consumption,⁹⁶ supporting the idea that changes in NAD⁺ concentrations might play a role in this model of axonal degeneration. SCG10 loss accelerates axonal degeneration in a similar fashion to NMNAT2,¹⁰³ however its protective effects are less pronounced. SARM1 activation and NMNAT2 inactivation both lead to MAPK activation, and in turn, ATP levels are depleted (causing failure of the Na⁺/K⁺ ATPase pump), and intracellular calcium concentrations will increase leading to cytoskeletal disassembly.⁹⁷ The increased calcium concentration activates calpain and in turn, degrades calpastatin, both of which are well-established events in the latter phases of Wallerian degeneration.^{110,111} It is clear that mitochondria play a key role in Wallerian degeneration, but whether their roles are limited to the initiation or downstream execution of axonal degeneration (or both) is not yet established. Axonal degeneration is a key feature of many neurological conditions caused by mutations in mitochondrial proteins (eg, CMT type 2K caused by mutations in *GDAP1*),¹¹² implying that mitochondrial dysfunction itself can initiate axonal degeneration under certain circumstances.

Although two key elements in this pathway of axonal degeneration (SARM1 and NMNAT2) have been identified (Figure 5), their exact actions are, as yet, unknown. It will be crucial to identify disease-specific degeneration pathways associated with distal axon loss to ensure that targeted, efficacious therapies can be developed in RLN.

4.1 | Bilateral mononeuropathy or polyneuropathy?

When the severity of RLN is sufficient to cause clinical signs, the typical neuropathological changes identifiable in the left RLN, are also

detected (with less severity) in the right^{6,20,23,55,56,113,114}; a disparity that has been explained by the right RLN's shorter length compared with that of the left.²¹ If true, this feature alone implicates length-dependency as a key feature in this disease. Although degeneration of the right RLN is consistently reported in pathological studies, development of right arytenoid dysfunction has been reported in only one horse with RLN¹¹⁵ (RLN cases typically present with left laryngeal dysfunction in isolation^{20,115,116}). However, these pathological studies confirm the disorder's bilateral occurrence and reveal that the disorder cannot solely be explained by the physical course of the left RLN.

Bilateral degeneration of one pair of peripheral nerves has been reported in humans as a component of the mononeuropathy multiplex syndrome (defined as the isolated and differential involvement of separate nerves) often because of a generalized systemic disorder and most commonly diabetes mellitus.¹¹⁷ Signs of nerve dysfunction relate to the specific nerve affected: frequently they are initially asymmetrical, but they progress to symmetrical nerve dysfunction and then to other nerve groups.^{41,117} This suggests that systemic disorders (at least initially) can have localized nerve involvement. Indeed, length dependency is a common feature of many peripheral nerve disorders with a genetic basis: even though defective protein expression is a generalized feature, the signs are most readily apparent in the longest nerves.^{60,118} In humans, the longest nerves are to the distal extremities and length dependency is revealed by many of the CMT disorders that have distal sensory loss, or distal neurogenic muscle atrophy (leading to pes cavus deformity).^{60,118} Length dependency is also a feature of some acquired neuropathies such as diabetic neuropathy (reviewed by Kazamel and Dyck¹¹⁹).

Determining whether this equine neuropathy affects only the RLN or in addition, other long nerves, has been a key objective in several studies over past decades^{3,6,7,20,23,113} because the answer has major implications for research into the etiopathogenesis. In particular, certain acquired causes (eg, trauma from neck stretch or thoracic vessel pulsation) would be ruled out if other long nerves are affected, whereas toxic or genetic causes might be more likely if the disease is found to be a polyneuropathy. Polyneuropathies typically produce diverse clinical signs according to the nerves that are diseased¹²⁰: for example, equine polyneuropathies include those with a toxic etiology (such as Australian Stringhalt, lead, and possibly haloxon) result in laryngeal dysfunction, dysphagia, proprioceptive deficits and gait abnormalities.^{121–127} Similarly, disorders such as equine motor neuron disease, are associated with widespread, generalized and symmetrical peripheral motor nerve involvement.¹²⁸

Clinical signs associated with involvement of other nerves is not a consistent feature of RLN, but the sub-clinical involvement of the right RLN, suggests that other nerves might also have subclinical involvement. Consequently, some researchers investigating RLN's pathology, have included the histological examination of other long peripheral nerves in horses, such as the phrenic, lateral palmar, ulnar, tibial, medial plantar, median, and peroneal nerves.^{3,4,7,20,23,24} Unfortunately, many of these studies were underpowered, unblinded, subjective or poorly controlled. Some authors report a lack of involvement of other nerves in this disorder. For example, Duncan et al⁴ examined multiple

additional peripheral nerves from 2 Thoroughbreds with RLN, but with no significant pathological findings. Hahn et al²⁰ examined median, peroneal and phrenic nerves of 3 horses (a Thoroughbred, Warmblood, and a Clydesdale) with severe RLN, concluding that there were no significant neuropathological findings in nonlaryngeal nerves, even though one of the horses was reported with moderate loss of large myelinated fibers, multiple onion bulbs, and an increased frequency of hypomyelinated internodes in semi-thin sections of peroneal and median nerves.

In contrast, several reports detail the possible significance of RLN-associated pathological features in other long, nonlaryngeal nerves.^{3,7,23} Indeed, Duncan et al (14 years after their study described above) examined the deep peroneal nerves of 14 male Draft horse foals, reporting the presence of Büngner bands and onion bulbs in nerves from 6 month old foals.²⁴ The authors made no comment about clinical signs associated with peroneal nerve dysfunction being present in any foal, nor was there any investigation into pathological changes within the peroneal nerve-innervated muscles.⁵⁴ Cahill and Goulden examined long limb motor nerves (median, peroneal and tibial) in 2 of 4 male Thoroughbred horses with clinically relevant RLN^{3,7,23,56} that ranged in age from 2 to 22 years; unfortunately the ages of the 2 cases chosen for limb nerve analysis were not disclosed^{3,7,23} and no control horses were included. The pathology was graded as the number of pathological changes (regenerating clusters, onion bulbs, demyelination/remyelination and myelin debris in the Schwann cell cytoplasm) per fascicle. The 2 cases had occasional (1–2 changes per fascicle) or moderate (5–10 changes per fascicle) pathological changes in the extensor digitorum longus (EDL) peroneal branch, superficial and common peroneal, median and the tibial nerves, by light microscopy.³ EM and teased fiber studies on the peroneal nerves revealed that the type and degree of pathology in the peroneal nerves from RLN-affected horses was similar to that present in the proximal left and distal right RLN.^{7,23}

Some authors have additionally, or instead, searched for histopathological, neuropathic changes in selected limb muscles because RLN-associated intrinsic laryngeal muscle pathology is readily identified.^{20,56} Cahill and Goulden⁵⁶ found no significant histopathological changes in the tibialis cranialis, gastrocnemius or biceps femoris muscles of 4 horses with clinically relevant RLN. In contrast, presumed neurogenic muscle fiber atrophy was detected in the EDL muscle of 3 of the 4 horses, although in only one of these animals was the key feature of neuronal regeneration (seen in RLN), known as fiber type grouping detected.⁵⁶ It is noteworthy that these apparent EDL muscle changes occurred despite the lack of nerve pathological changes in previous studies of the deep peroneal nerve by the same and other authors^{3,23,56} suggesting either that muscle histopathological assessment for neurogenic change has greater sensitivity than nerve histology, or that the detected muscle changes were not neurogenic in origin.

Conflicting results obtained by these various groups and between examination of nerves and muscles, reveal the problems associated with unblinded, uncontrolled, subjective histopathological assessment. In particular, including greater numbers of height-, sex-, and breed-matched control horses would have enabled more robust conclusions to be drawn, given that each is associated with RLN development.^{1,2,8,14,129}

Furthermore, typically authors have failed to consider the influence of age, even though age-related neuronal degenerative changes occur in many peripheral nerves of various species.^{67,130–133} Indeed, neurodegenerative changes reported in the median and peroneal nerves of a horse with clinically relevant RLN, might have been age-related (the horse was 16 years old)²⁰ rather than associated with underlying disease. In a study looking at the lateral palmar (sensory) nerve in horses free from detectable neuromuscular disease (unknown breeds) horses aged between 5 and 7 years old had evidence of demyelination/remyelination in up to 20% of myelinated nerve fibers.¹³³ Other than the RLN (and the paired phrenic nerves), the longest peripheral nerves in horses are sensory rather than motor; however, no studies have evaluated clinical or histopathological sensory nerve involvement in horses with RLN.

In certain neuropathies in other species, both the peripheral and central nervous systems can be involved.¹³⁴ For example, in horses with equine motor neuron disease, there is prominent degeneration of the spinal cord ventral horn cell bodies of the peripheral motor nerves.¹²⁸ Cahill and Goulden^{22,55} examined the nucleus ambiguus of clinically affected or subclinically affected RLN horses⁵⁵ but identified no relevant pathological features. However, a search for central involvement in RLN (ie, in a length-dependent neuropathy) might most logically be made in the longest central nerve tracts: these would be the sensory proprioceptive tracts from the thoracic limbs (funiculus cuneate) and pelvic limbs (funiculus gracilis), with the latter containing the longest axons. The same authors reported significantly higher numbers of spheroids in the lateral cuneate nuclei (which receives sensory input from the cervicothoracic spinal nerves) in subclinical and clinical RLN cases when compared with the control group,²² but despite evaluating the gracilis nucleus for increased spheroids, none was reported.⁵⁵ Since spheroid formation is seen with aging, the apparent increased spheroidal numbers in the diseased groups could be explained by the lack of age matching between the control and diseased groups: the horses in the control group were all <3 years old, whereas the diseased group's horses ranged from 2 to 22 years old.¹³⁵

Perhaps then, RLN represents the localized involvement of two nerves (the left and right RLN) associated with a systemic disorder that has subclinical involvement of other long nerves in only a few (eg, the most severely affected, or tallest) horses. Unfortunately, despite the previous work, no clear conclusion regarding the possible involvement of other nerves in RLN can be made. In particular, the trend for studies to evaluate subjective histopathological features in unblinded, uncontrolled fashion and with low power, means that this crucial question remains unanswered such that RLN cannot yet be defined as bilateral mononeuropathy or a polyneuropathy with certainty.

5 | OBJECTIVE FUNCTIONAL TESTING OF THE RLN

Nerve function testing for a suspected neuropathy helps define each tested nerve's involvement but also the type of pathology (axonal or myelin-based).⁴⁴ Function is assessed either directly, by examining the speed of conduction (NCV) or, indirectly, via the measure of latency

(delay from nerve stimulation to muscle contraction) and through functionality of the innervated structure (ie, the larynx in RLN). Testing of the paired RLN functionality has included direct measurement of NCV and nerve conduction latency measurements.¹³⁶⁻¹³⁹ Of these techniques, accurate measurement of the NCV in the RLN (and others) in affected horses might best help define if RLN is an axonopathy or myelinopathy and whether other nerves are also involved. This routine technique is used diagnostically in human peripheral neuropathy patients to help define the precise phenotypic features of the disease (distinguishing between CMT types or sensorimotor peripheral neuropathies for example).¹⁴⁰ The characteristic electrophysiological features of an axonopathy include a reduction in the amplitude of a compound motor action potential (CMAP) and a normal NCV, whereas a myelinopathy is characterized by a normal CMAP, with a slowed NCV.⁴⁴ Conduction blocks have historically been recognized as signifying demyelination, especially segmental demyelination, however more recently this phenomenon has also been detected in distal axonopathies.¹⁴¹ Presumably, conduction blocks could occur in grade 4 RLN horses when the loss of myelinated axons in the left RLN is great enough to stop the propagation of the action potential to the laryngeal muscles. In this situation, pathological evaluation of the RLN would be necessary to differentiate axonal loss from demyelination as the cause for the conduction block. Unfortunately, NCVs are influenced by many factors such as the individual's age, sex, height, and skin temperature: for instance, in humans, every 1 cm increase in height, accounts for a 0.2 m/s decrease in NCV^{142,143} and women have slower NCVs in the RLN than men.¹⁴⁴ The negative correlation between height and NCV has been attributed to the abrupt tapering of the nerve fiber diameter with increasing nerve length (which is mainly caused by a reduction in the axonal diameter) rather than to any effect on internodal distance.¹⁴⁵⁻¹⁴⁷

Likely, because of practical constraints, very few researchers have evaluated the NCV of the RLN in horses. The latency (using electrolaryngography) of the RLN in Clydesdale horses with varying grades of RLN was reported by Have et al (2001). They reported no significant difference between the Clydesdales' size-adjusted latency values and their RLN endoscopic grade.¹³⁸ There was no information included in the study regarding the amplitude of the CMAPs produced. Recurrent laryngeal nerves (RLN) NCVs were reported directly by Cheetham et al¹³⁷ in horses with normal (Havemeyer grade 1) arytenoid function: the NCV of the left RLN (39.1 ± 4.4 m/s; mean \pm SD) was significantly slower than that of the right RLN (50 ± 4.2 m/s). The authors concluded that this data suggests that the primary pathology associated with RLN might be demyelination rather than axonal loss, although significant demyelination has not been reported in horses with (grade 1) normal arytenoid function.^{3,4,7} Finally, a slower left RLN NCV compared with the right was reported in one horse with subclinical RLN by Duncan et al,¹¹³ although the velocities were not included.

Steiss et al reported the latency of a CMAP, as a measure of the NCV, in both the RLN in 5 normal horses and 7 ponies. This revealed a longer conduction latency in the proximal left RLN compared with the proximal right nerve (no left-right differences were detected in the distal RLN).¹³⁹ However, no correction was made for differences in nerve

length in our study, which likely explains the longer latency of the left RLN. Nevertheless, the similar left and right RLN conduction latencies reported in dogs and humans^{144,148} were explained by the left side's wider diameter (and therefore faster conducting) axons when compared to the right, compensating for the longer path.³⁷ The left distal RLN of young (<2 year old) horses that lacked histopathological features of RLN, had significantly fewer myelinated nerve fibers, and smaller diameter myelinated nerve fibers than those in the right distal RLN. Furthermore, the proportion of the widest myelinated fibers (>9.5 μ m) was also always lower in the left RLN compared with the right.¹⁴⁹ These findings imply that the equine RLN and their NCVs are unusual. Nerve conduction velocities (NCVs) in the left and right RLN of horses have been shown to differ between the two nerves in several studies; this has been recapitulated in normal ponies and horses, as well as in RLN cases.^{113,137-139,150} The interpretation of this finding differs between authors: is RLN simply ubiquitous or does it result from deterioration of 'normal' equine anatomy?

Despite this previous research, the correct classification of RLN's primary basis based on objective electrophysiological recordings remains unclear. This might largely be because of differences between methodologies, low numbers of animals, and differences in signalment in the animals examined. It also very likely relates to the practical difficulties encountered when performing these sorts of tests in horses.

6 | CONCLUSIONS

The majority of the histopathological changes (Büngner bands, regenerating clusters, paranodal evaginations, and spheroids) reported in RLN-affected horses are associated with primary axonal dysfunction.^{3,4,6,7,20,30,52,53,113,114} Further, we have discussed how pathological features traditionally associated with primary myelinopathies (ie, onion bulbs and demyelination and remyelination) also occur in primary axonopathies. In RLN, these histopathological changes occur simultaneously with other pathological features that are exclusively reported in axonopathies such as paranodal evaginations (that occur in toxic axonopathies and axotomies) and at sites of axonal degeneration.^{4,53} In addition, we have highlighted evidence that suggests axonal transportation deficits might play a role in the etiopathogenesis of RLN.

While there is compelling evidence that RLN affects both the left and (to a lesser extent) right RLN, it is not yet clear whether in some horses, other long nerves are also affected. There is some pathological evidence to suggest subclinical involvement of other long peripheral nerves in cases of RLN (Büngner bands, and onion bulbs in nonlaryngeal peripheral nerves),^{3,4,7,20,23,24} nevertheless these findings are not found in all studies; typically, and unfortunately, most of these studies suffer from the limitations discussed throughout this review article and a comprehensive, objective, blinded study that evaluates other long peripheral nerves, and long spinal tracts of the CNS, with age, height, breed, and sex matched controls is required.

In summary, in our opinion RLN is best currently classified as a distal axonopathy with clear evidence of involvement of both RLN. Further, from a functional and clinical perspective, this disease can best be

regarded as a mononeuropathy of the left RLn: however, when considering the etiology and pathophysiology of this disorder, there is insufficient evidence to classify the disease as either a bilateral mononeuropathy or polyneuropathy, a classification that remains fundamentally important for the study of this enigmatic, and highly prevalent equine neurodegenerative disease.

ACKNOWLEDGMENTS

The authors acknowledge the many discussions had over the course of several years with colleagues that have helped inform content within this review, and in particular, those with Robert Adalbert, Julian Blake, Jon Cheetham, Michael Coleman, Norm Ducharme, Ian Duncan, Rosalind King, Joe Mayhew, Vince Molony, Justin Perkins, Mary Reilly, Ed Robinson, Giampietro Schiavo, and Laura Tulloch. Figures 1 and 2 were kindly generated by Brian Cox. Alex Draper is a PhD scholar at the Royal Veterinary College supported by a grant from the Horserace Betting Levy Board of the United Kingdom. The manuscript was approved by the Royal Veterinary College's research office and assigned the following unique reference number: CSS_01735.

CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Ethic approval for all work conducted by Alex Draper, during her PhD was received from the Ethics Committee, Royal Veterinary College. Ref No. 2015 1381.

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How to cite this article: Draper ACE, Piercy RJ. Pathological classification of equine recurrent laryngeal neuropathy. *J Vet Intern Med*. 2018;00:1–13. <https://doi.org/10.1111/jvim.15142>