Received: 26 September 2023 | Accepted: 4 January 2024

DOI: 10.1111/evj.14063

ORIGINAL ARTICLE

Equine Veterinary Journal WILEY

Equine grass sickness is associated with major abnormalities in the ultrastructure of skeletal neuromuscular junctions

Bruce C. McGorum[1] | Tracey Davey[2] | Miranda C. M. Dosi[1] | John A. Keen[1] | Linda R. Morrison[1] | R. Scott Pirie[1] | Darren J. Shaw[1] | John B. Harris[3]

[1]Royal (Dick) School of Veterinary Studies and The Roslin Institute, University of Edinburgh, Roslin, UK

[2]Electron Microscopy Research Services, Newcastle University, Faculty of Medical Sciences, Newcastle upon Tyne, UK

[3]Medical Toxicology Centre and Institute of Neuroscience, Newcastle University, Faculty of Medical Sciences, Newcastle upon Tyne, UK

Correspondence
Bruce C. McGorum, Royal (Dick) School of Veterinary Studies and The Roslin Institute, University of Edinburgh, Roslin, UK.
Email: bruce.mcgorum@ed.ac.uk

Funding information
Royal College of Veterinary Surgeons Trust Blue Sky Research Fund; The Equine Grass Sickness Fund

Abstract

Background: Equine grass sickness (EGS) is a frequently fatal multisystem neuropathy of equids. The aetiology is unknown; proposed causes include toxicoinfection with *Clostridium botulinum* and a mycotoxicosis. The effect of EGS on the organisation and structural integrity of the skeletal neuromuscular junction (NMJ), the target of botulinum neurotoxins (BoNTs), is unknown.

Objectives: To compare the organisation and structural integrity of skeletal NMJs from EGS horses, control horses and one horse with a presumptive diagnosis of botulism.

Study design: Blinded, retrospective case control.

Methods: NMJs in samples of diaphragm or intercostal muscle from six EGS horses, three control horses and one equine botulism case were compared using electron microscopy, morphometry and confocal light microscopy.

Results: A significantly higher percentage of EGS NMJs had abnormal morphology (EGS 72.2%, 95% CI 55.6–84.4; Controls 6.9%, 1.7–23.8; OR 35.1, 8.47–244.8; $p < 0.001$). EGS NMJs had a significantly lower mean volume fraction occupied by synaptic vesicles (SVs) (EGS 18.7%, 12.6–28.0; Controls 36.3%, 20.8–63.4; $p = 0.024$). EGS NMJs had evidence of accelerated SV exocytosis and SV depletion, accumulation of neurofilament-like material in terminal boutons and/or bouton degeneration. NMJs from the botulism horse had dense packing of SVs towards the presynaptic membrane active zone, consistent with BoNT intoxication, but had absence of the abnormalities identified in EGS NMJs.

Main limitations: Group sizes were limited by difficulties obtaining suitably processed samples. Ages of control and EGS horses differed. Botulism was diagnosed based on clinical and post mortem findings.

Conclusions: EGS is associated with major changes in skeletal NMJ ultrastructure that are inconsistent with the effects of BoNTs. SV depletion may reflect increased exocytosis coupled with reduced repopulation of SVs via anterograde axonal transport and endocytosis, consistent with the action of an excitatory presynaptic toxin

and/or neurotransmitter reuptake inhibitor. Skeletal NMJs represent a previously unrecognised target for the toxin that causes EGS.

KEYWORDS
botulism, equine grass sickness, horse, multisystem neuropathy, neuromuscular junction

1 | INTRODUCTION

Equine grass sickness (EGS) is an acute, predominantly fatal, multiple system neuropathy of grazing horses.[1,2] An apparently identical disease occurs in cats, dogs, hares, rabbits, llamas and, possibly, sheep.[3–9] EGS is associated with chromatolysis of sympathetic and parasympathetic postsynaptic neurons, particularly in the enteric nervous system, as well as autonomic presynaptic and somatic lower motor neurons in the brainstem and spinal cord.[1,10] The aetiology of EGS is unknown; current hypotheses include an ingested pasture-derived neurotoxic mycotoxin or toxicoinfection with *Clostridium botulinum* types C or D.[2,11–14]

The effect of EGS on the skeletal neuromuscular junction (NMJ), the target of botulinum neurotoxins (BoNTs), has not been previously investigated, despite EGS being associated with chromatolysis of spinal cord lower motor neurons,[10] evidence of neuromuscular dysfunction on quantitative electromyography[15] and clinical signs suggestive of diffuse skeletal muscle weakness.[16,17] Consistent with the effects of diffuse skeletal myasthenia, EGS horses spend increasing time recumbent or stand with a characteristic base-narrow stance, with low head and neck carriage, commonly supporting their hind-quarters against the stable wall, and attempting to relieve weak postural muscles by frequently shifting weight among all four limbs.

To determine whether skeletal NMJs represent a previously unrecognised target for the toxin that causes EGS, the ultrastructure of skeletal NMJs of EGS and control horses was compared using electron microscopy, morphometry and confocal light microscopy. Furthermore, to determine whether any abnormalities identified in EGS NMJs were consistent with the actions of BoNTs, comparisons were made of the ultrastructure of EGS NMJs, NMJs from a horse with a presumptive diagnosis of botulism, and NMJs from laboratory animals in previously published experimental studies of BoNT intoxication.

2 | MATERIALS AND METHODS

2.1 | Horses

The study used samples from 10 mixed-breed and mixed-gender horses, comprising 6 EGS horses, 3 control horses, and 1 horse with a presumptive diagnosis of botulism (metadata in Table S1). All horses were euthanised on humane clinical grounds by intravenous administration of barbiturates. EGS horses had a mean age of 4 years (range 2–7) and comprised four acute and two sub-acute cases, categorised according to reported clinical criteria.[18] EGS was confirmed by gross necropsy findings and demonstration of characteristic neuropathological features upon histopathological examination of autonomic ganglia.[19,20] Control horses had a mean age of 20 years (range 8–24) and had no clinical evidence of gastrointestinal or neurological disease. The animal with a presumptive diagnosis of botulism was a 14-year-old Shetland pony mare that had clinical examination findings consistent with those of botulism.[21] Full details of this case are presented in Text S1. The pony was euthanised 6 days after onset of clinical signs. Consistent with the presumptive diagnosis, post mortem examination did not identify any significant abnormalities to account for the myasthenia and other clinical abnormalities. Histopathological examination of the pony's cranial cervical ganglion ruled out EGS.

2.2 | Sample collection

Small segments (~2–5 mm^3) of diaphragm with the distal phrenic nerve attached (first EGS and first control horse) or external intercostal muscle (all other horses), were collected from all horses within 60 min of euthanasia.

2.3 | Electron microscopic examination of NMJs

Samples from all 10 horses were processed for ultrastructural analyses as described previously.[22] Briefly, samples were immersed in saline and teased into segments of 5–10 muscle fibres. These were immersed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (Sigma-Aldrich) for 30 min at room temperature, trimmed to produce blocks approximating 1 × 1 × 2 mm and fixed overnight at 4°C. Fixed samples were rinsed in 0.1 M sodium cacodylate buffer, post-fixed in 1% OsO4 (Agar Scientific) for 1 h, dehydrated in sequential steps of acetone (25%, 50%, 75% and 100%) and impregnated in increasing concentrations (25%, 50%, 75%) of resin (TAAB Laboratories Equipment) in acetone followed by 100% resin changed 3 times, placed in moulds, orientated transversely and polymerised at 60°C for 24 h. Survey sections (1 μm) were cut using an ultramicrotome (EM UC7, Leica) and stained with 1% toluidine blue in 0.5% borax to identify areas containing NMJs at light microscopy level. Ultrathin sections at a thickness of 50–70 nm (silver/gold in colour) were subsequently cut using a diamond knife. Sections were stretched with chloroform to eliminate compression and mounted on Pioloform filmed copper washer style grids (Gilder Grids) prior to staining with 1% aqueous uranyl acetate and lead citrate (Leica). Sections were examined under a transmission electron microscope (Phillips CM100) and images of NMJs photographed using an AMT CCD camera (Deben). Images were coded and analysed blindly by one examiner

(JBH). Images were selected for morphometric analysis if the terminal bouton was located in a clearly defined synaptic trough that was lined by synaptic folds and covered by a Schwann cell process. Images distorted by grid bars or folds were excluded. Standardised morphometric and stereological techniques were used to measure terminal bouton cross-sectional area, width of the synaptic cleft between the pre- and post-synaptic membranes (synaptic cleft width) and volume fraction occupied by synaptic vesicles (SVs) populating boutons (SV volume fraction).[22–24] In addition, NMJs were classified as normal, activated, markedly depleted or degenerating. Normal NMJs had a terminal bouton that sat snugly within the synaptic cleft clearly delineated by the presence of deep synaptic folds and plentiful SVs. Activated NMJs had SVs of variable size, clear signs of fusion with the presynaptic membrane and a deeply infolded presynaptic membrane. Markedly depleted NMJs had severe depletion of SVs. Degenerating NMJs had apparent loss of structural integrity of terminal boutons.

2.4 | Confocal light microscopic examination of NMJs

Samples of diaphragm or intercostal muscles from EGS and control horses were processed for confocal light microscopic examination as described previously.[22] Samples were dissected into blocks of tissue approximating 2 × 2 × 5 mm and snap-frozen in isopentane cooled in liquid nitrogen. Transverse and longitudinal cryosections (6 and 20 μm, respectively) were collected onto coated glass slides, permeabilised in ethanol then methanol (both −20°C, 10 min) and then in 0.1% Triton X-100 in phosphate buffered saline, pH 7.4 (PBS; 10 min, ambient temperature). Sections were rinsed in PBS and labelled with primary antibodies overnight at 4°C. Neurofilament protein in terminal axons was labelled using chicken anti-neurofilament antibody (AB5539, Chemicon International) followed by FITC-conjugated donkey anti-chicken IgG (03095155, Jacksons ImmunoResearch). Post-junctional acetylcholine receptors were labelled for 1 h using TRITC-conjugated α-bungarotoxin (10 μg/mL) (1175, Molecular Probes). Secondary antibodies were incubated with rat serum (Dako) and centrifuged to yield a clear supernatant before use. Antibodies were diluted 1:100 in PBS containing 3% w/v bovine serum albumin and 0.1 M lysine. Sections were rinsed again in PBS, mounted in Vectashield (Vector Laboratories) and the intensity of labelling assessed subjectively under a Bio-Rad MRC confocal scanning laser microscope by an assessor (JBH) blinded to group.

2.5 | Data analysis

To investigate inter-group differences in terminal bouton area, SV volume fraction and synaptic cleft width, linear mixed-effect models were run with sample ID entered as the random effect to take account of the multiple samples obtained per horse. Models were run twice, with tissue (diaphragm or intercostal muscle) added in the second model as a potential confounder. Prior to analysis, terminal bouton area and SV volume fraction were log transformed to normalise the residuals. Data are presented as estimated marginal geometric mean (terminal bouton area and SV volume fraction) or mean (synaptic cleft width) ± associated standard error and 95% CI. $p < 0.05$ was considered to indicate statistical significance. A general linear model with binomial errors was used for inter-group comparison of the proportion of abnormal NMJs (including activated, markedly depleted and degenerating NMJs).

3 | RESULTS

3.1 | Confocal light microscopy

EGS was not associated with alteration in the intensity of α-bungarotoxin labelling or its distribution at the site of innervation (Figure 1). EGS was not associated with alteration in the labelling of terminal axonal neurofilament and there was no evidence of neurofilament degeneration in the motor innervation or in intramuscular axon bundles (Figure 1).

3.2 | Electron microscopic examination of NMJs

Data were available for 29 NMJs from the 3 control horses (range 6–12/horse), 56 from the 6 EGS horses (range 3–16/horse) and 11 from the botulism horse (Table 1). Representative ultrastructural features of NMJs from control (Figure 2A), EGS (Figures 2B–6) and botulism (Figure 8) horses are presented.

3.3 | EGS and control horses

Control and EGS boutons, respectively, were classified as normal (93% vs. 28%), activated (3% vs. 28%), markedly depleted (3% vs. 39%) or degenerating (0% vs. 6%). Control NMJs had tightly opposed terminal and junctional membranes and numerous SVs of similar size (Figure 2A). EGS NMJs had reduced SV numbers (Figures 2B and 5), variable SV sizes (Figure 3), variable SV densities across the respective terminals (Figures 3 and 4), sites of SV fusion to terminal membranes (Figures 3 and 4), deeply folded terminal membranes (Figure 4) and/or bouton degeneration, with areas of withdrawal of the terminal from the synaptic cleft and a breakdown of membrane integrity (Figure 5). Loose accumulations of fine filamentous material resembling neurofilaments were present in 22% EGS boutons (Figure 6) but were absent from controls. Clathrin coated SVs and larger than usual vesicular structures were present relatively infrequently in EGS and control boutons (Figure 2). These larger vesicles could not be formally identified but may have been lysosomes or endosomes. Mitochondria were damaged in control and EGS boutons, but this was considered artefactual, reflecting delayed sample preparation and fixation. Occasional myelin whorls within intramuscular myelinated axons of control and EGS horses were considered to be non-specific changes of unknown origin. Post-junctional folds and skeletal muscle fibre ultrastructure of EGS and control horses were indistinguishable.

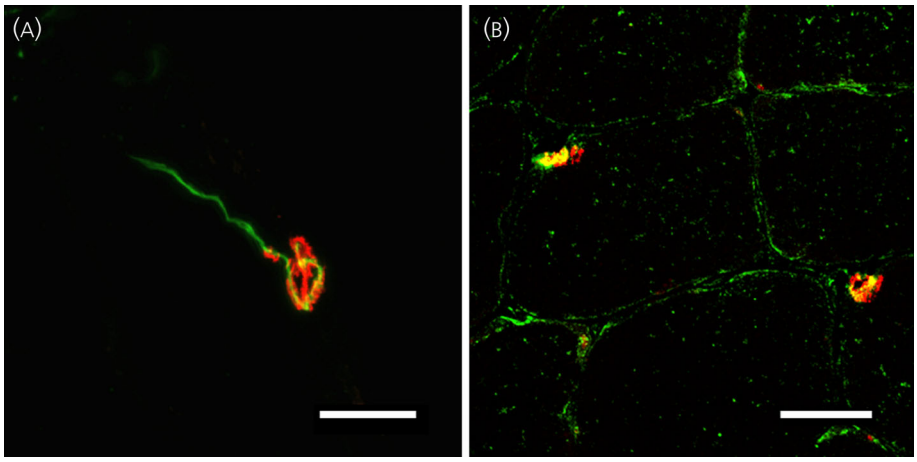

FIGURE 1 Longitudinal (A) and transverse (B) confocal light microscopy images showing neuromuscular junctions in equine grass sickness (EGS) diaphragm (Horse EGS 1). Images demonstrate axons labelled with primary antibodies to neurofilament protein and a secondary fluorescent antibody (green), and AChRs labelled with rhodamine conjugated α-bungarotoxin (red). Motor axons show no evidence of degeneration or withdrawal from receptor sites on muscle fibres. There is no evidence of distal axonal neurofilament degeneration. Scale bar 20 μm.

TABLE 1 Morphometric data for skeletal neuromuscular junctions (geometric mean [terminal bouton area and synaptic vesicle volume fraction] or mean [synaptic cleft width] ± associated SE; 95% CI in brackets).

Horse	Muscle	Terminal boutons analysed (*N*)	Terminal bouton cross sectional area (μm^2)	Synaptic vesicle volume fraction (%)	Synaptic cleft width (μm)
Control 1	Diaphragm	12	4.67 ± 1.21 (3.17–6.87)	33.1 ± 1.2 (24.5–44.8)	0.050 ± 0.002 (0.045–0.055)
Control 2	Intercostal	11	2.53 ± 1.22 (1.69–3.78)	43.9 ± 1.2 (32.0–60.2)	0.066 ± 0.003 (0.061–0.071)
Control 3	Intercostal	6	4.18 ± 1.32 (2.42–7.22)	32.5 ± 1.2 (21.2–49.8)	0.066 ± 0.004 (0.058–0.075)
EGS 1	Diaphragm	16	3.46 ± 1.18 (2.47–4.83)	25.8 ± 1.1 (19.9–33.6)	0.070 ± 0.002 (0.066–0.075)
EGS 2	Intercostal	11	3.80 ± 1.22 (2.54–5.69)	23.2 ± 1.2 (16.9–31.8)	0.070 ± 0.003 (0.065–0.076)
EGS 3	Intercostal	10	2.65 ± 1.24 (1.74–4.05)	21.1 ± 1.2 (14.9–30.0)	0.074 ± 0.005 (0.064–0.084)
EGS 4	Intercostal	11	7.49 ± 1.22 (5.00–11.22)	8.4 ± 1.2 (6.2–11.6)	0.055 ± 0.003 (0.050–0.061)
EGS 5	Intercostal	3	2.47 ± 1.47 (1.14–5.35)	13.8 ± 1.4 (7.6–25.3)	0.055 ± 0.005 (0.045–0.064)
EGS 6	Intercostal	5	2.48 ± 1.35 (1.36–4.52)	28.3 ± 1.3 (17.7–45.1)	0.074 ± 0.004 (0.065–0.082)
Botulism	Intercostal	11	4.2 ± 1.16 (3.01–5.86)	39.2 ± 1.1 (29.4–52.2)	0.069 ± 0.001 (0.067–0.071)

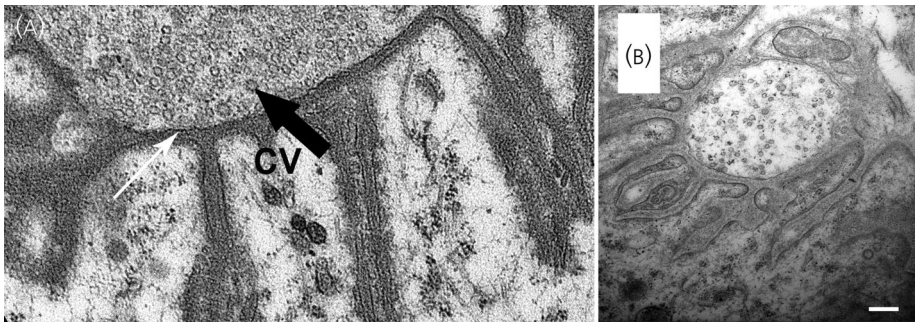

FIGURE 2 Transmission electron micrographs. (A) Neuromuscular junction (NMJ) from a control horse showing tightly opposed terminal (white arrow) and junctional membranes, numerous synaptic vesicles (SVs) of similar size and several clathrin coated vesicles (dark arrow, CV). (B) NMJ from an equine grass sickness horse showing normal terminus morphology but reduced number of SVs. Scale bar 500 nm.

Compared with controls, EGS boutons had a significantly lower SV volume fraction whether tissue (diaphragm vs. intercostal) was excluded (control 36.3% [95% CI 20.8–63.4]; EGS 18.7% [12.6–28.0]; $p = 0.024$; Figure 7) or included (control 37.2% [19.7–70.2]; EGS 19.7% [11.6–33.4]; $p = 0.044$; Table 1). This represented a mean 48% reduction in mean SV volume fraction in EGS boutons compared with

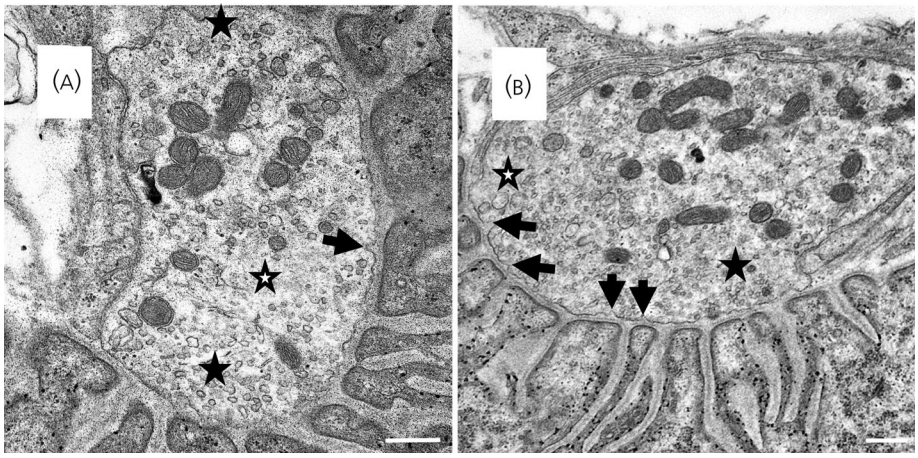

FIGURE 3 Neuromuscular junctions from two equine grass sickness horses, showing varying synaptic vesicle (SV) densities across the respective terminals, with areas of high (solid star) and low (open star) SV density highlighted. There are occasional sites of SV fusion to terminal membranes (arrows) and variable SV sizes. Scale bar 500 nm.

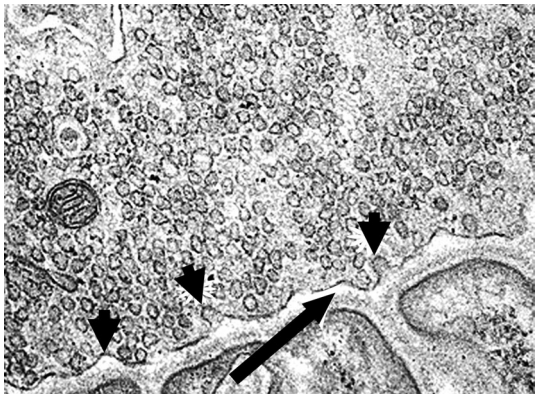

FIGURE 4 Neuromuscular junction from an equine grass sickness horse showing deeply folded terminal membranes (long arrow; compare with normal terminal membrane morphology in Figure 2A), numerous synaptic vesicle (SV) fusion sites (short arrows), and variable SV densities, features that are consistent with accelerated exocytosis and limited endocytosis.

controls. There was no statistically significant inter-group difference in terminal bouton area (control 3.64 μm^2 [2.08–6.37]; EGS 3.56 [2.38–5.34]; $p = 0.938$) or synaptic cleft width (control 0.061 µm [0.048–0.073]; EGS 0.066 [0.057–0.075]; $p = 0.364$). Because NMJs in diaphragm and intercostal muscle preparations from each group were indistinguishable in all respects, with NMJs in the two tissues from EGS horses having identical pathology, data from the two muscle sources were not further differentiated.

3.4 | Equine botulism

The presumptive diagnosis of botulism was supported by the identification of changes in NMJ ultrastructure consistent with those reported for laboratory animals following experimental BoNT/A and BoNT/B intoxication.[25–28] There was a characteristic abnormal distribution of SVs within terminal boutons, with dense packing of SVs towards the active zone of the presynaptic membrane (Figure 8). Morphology of NMJs from the botulism pony was otherwise normal. In contrast to NMJs from EGS horses, NMJs from this horse had no reduction in SV volume fraction, accumulation of neurofilament-like filaments within terminal boutons or bouton degeneration.

4 | DISCUSSION

This study has demonstrated that the skeletal NMJ represents a previously unrecognised target for the toxin that causes EGS. Ultrastructural abnormalities, identified in 72% of EGS NMJs, included a mean 48% reduction in mean SV volume fraction, activated NMJs, accumulation of neurofilament-like material in terminal boutons and/or bouton degeneration. EGS was not associated with a significant alteration in terminal bouton cross sectional area or synaptic cleft width. These data suggest that there is an initial phase of activated SV mobilisation, migration of SVs towards the synaptic cleft, and fusion of the SV membranes with the presynaptic membrane of the terminal bouton. The deep infolding of the terminal membrane and the preservation of profiles of fused vesicles in EGS terminals suggests that endocytosis is impaired. This phase appears to lead to a second phase of SV depletion possibly reflecting the ultimate recycling and degradation of the fused SVs and the inhibition of the more usual process of SV replenishment via anterograde axonal transport and endocytosis, giving rise to the appearance of empty boutons. Ultimately, the nerve terminal appears to degenerate leading to a state of denervation. It was notable that, despite the abnormalities observed in the terminal boutons, intramuscular axonal degeneration was absent suggesting that the terminal bouton is a primary target of the presumed toxin that causes EGS.

These findings are consistent with the almost total depletion of SVs, particularly those immunoreactive for vasoactive intestinal peptide and substance P, from terminal boutons of enteric neurons within the ileum of EGS horses.[29] SV depletion was attributed to massive release of peptides from the nerve endings coupled with a cessation of peptide production within the neuron. The observation that peptide containing SVs were lost from the proximal ileum of EGS horses

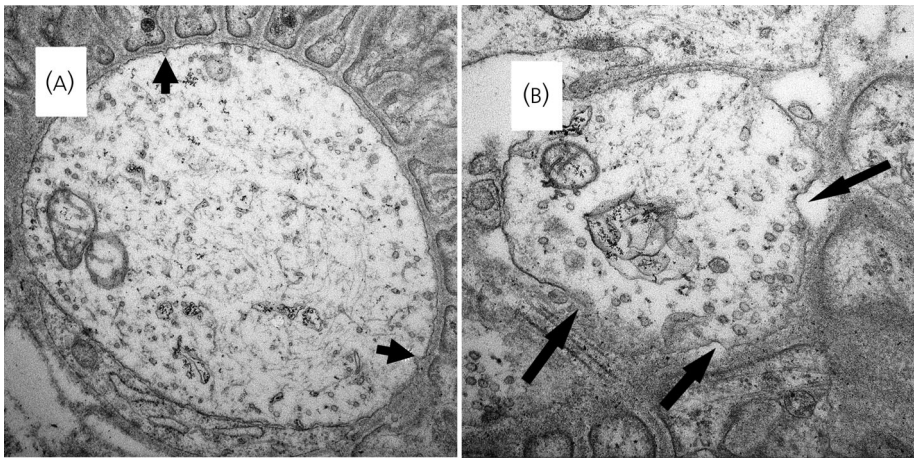

FIGURE 5 Neuromuscular junction from equine grass sickness horses showing (A) depletion of synaptic vesicles, and fusion sites (arrows), and (B) early-stage bouton degeneration, with areas of withdrawal of the terminal membrane from the synaptic cleft (thin arrow) and a breakdown of membrane integrity (see the region between the two thicker arrows).

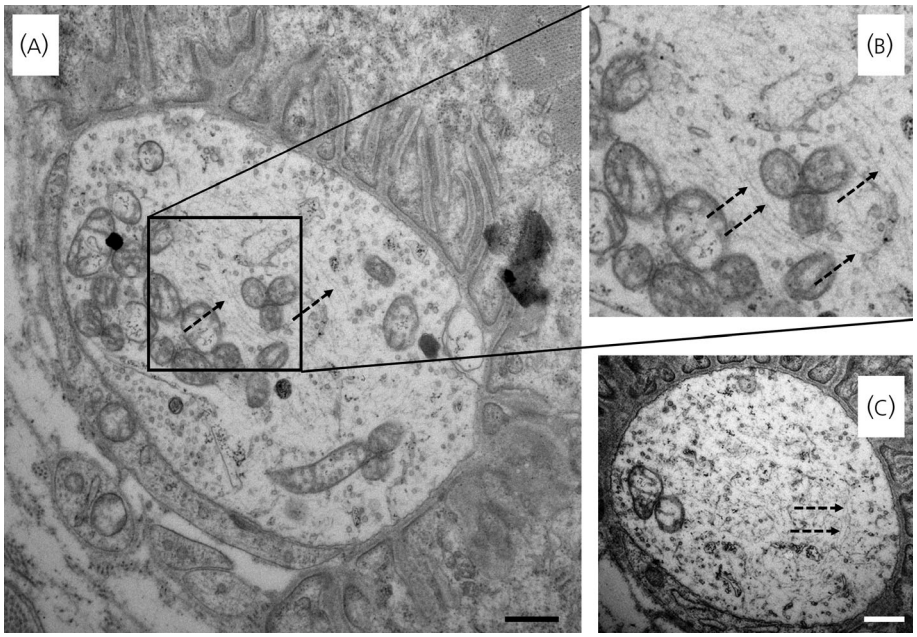

FIGURE 6 Terminal boutons from equine grass sickness horses showing accumulations of fine filaments resembling disorganised neurofilaments (arrows). (B) Higher power image of A to highlight the filaments [arrows]. Scale bar 500 nm.

without any other significant neurodegenerative changes suggested that the release of peptides from nerves is one of the earliest morphologically identifiable events in EGS.[29] Taken together with the findings of the present study, this suggests that the terminal boutons of both enteric and somatic neurons are primary targets of the presumed toxin that causes EGS.

While there was no apparent disruption of terminal axonal neurofilament, fine filamentous material resembling neurofilaments was present in terminal boutons of 22% of EGS NMJs, but not in control boutons. Neurofilaments are synthesised and assembled in neuronal soma before being transported via anterograde axonal transport to the terminal boutons where they are normally degraded in a process involving calcium-activated proteases and the ubiquitin proteasome system.[30–32] Consequently, axonal neurofilaments rarely extend into nerve terminals. Accumulations of neurofilament-like material in terminal boutons of EGS horses could potentially reflect a failure of neurofilament proteolysis at terminal boutons, due to the abnormalities in the ubiquitin proteasome system that occur in EGS.[33,34] Neurofilaments may also accumulate in terminal boutons of neurons undergoing chromatolysis.[35,36] While many spinal cord somatic neurons are chromatolytic in EGS,[10] it is not known whether motor neurons innervating the diaphragm and intercostal muscles are also chromatolytic. Neurofilament accumulation also occurs in mice with ubiquitin depletion[36] and in murine spinal muscular atrophy.[33] Interestingly, as demonstrated in EGS horses, terminal boutons from mice with spinal muscular atrophy also have reduced numbers of SVs.[37] Accumulation of neurofilaments at motor end plates differentiates EGS from those toxic neurofilamentous axonopathies that are characterised by accumulations of neurofilaments within proximal axons and diminished neurofilaments at end plates.[38]

The cause of the ultrastructural abnormalities in EGS NMJs is unknown. The NMJ is vulnerable to attack by a wide range of toxins, in part because it is not protected by a blood/axon barrier and is therefore vulnerable to haematogenous toxins.[39] Numerous toxins target the NMJ including botulinum and tetanus toxins, toxins found in snake, spider and fish venoms, and toxins produced by algae and

fungi.[40–45] Toxins act at single or multiple sites of the neuromuscular apparatus, interfering with voltage-gated ion channels, acetylcholine release, depolarisation of the postsynaptic membrane, or generation and spread of the muscle action potential. The ultrastructural changes observed in EGS NMJs, and in particular the marked SV depletion, resemble those induced by the presynaptic excitatory toxin α-latrotoxin which is present in venom from black widow spiders (*Latrodectus* spp.).[42–45] While the epidemiology of EGS is inconsistent with involvement of those presynaptic excitatory neurotoxins that are present in snake, spider and fish venoms,[2,46] it is possible that EGS is caused by another presynaptic toxin that has α-latrotoxin-like activity.

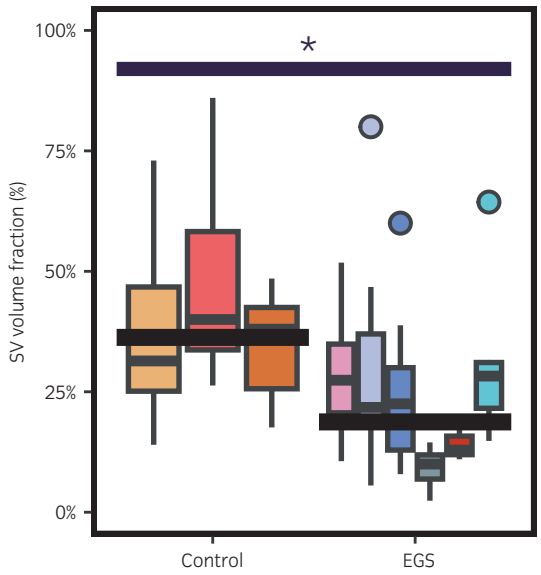

FIGURE 7 Box and whiskers plot showing synaptic vesicle (SV) volume fraction (%) for equine grass sickness (EGS) ($n = 6$) and control ($n = 3$) horse neuromuscular junctions. Compared with control boutons, EGS boutons had a mean 48% reduction in mean SV volume fraction. Solid horizontal black lines are the back transformed least square means from the linear mixed-effect model analyses of $\log_{10}$ transformed SV volume fraction comparing control and EGS boutons. Asterisk (*) and associated dark purple line $p < 0.05$ are from the linear mixed-effect model.

The ultrastructural changes observed in NMJs from EGS horses appear inconsistent with the hypothesis that the disease is caused by BoNT intoxication. BoNTs are inhibitory presynaptic toxins that act by blocking SV exocytosis.[47] BoNT intoxication does not alter SV volume fraction, but consistently results in altered SV distribution, with dense packing of SVs towards the active zone of the presynaptic membrane, an otherwise normal overall NMJ morphology, and absence of degenerative changes.[25–28,47,48] In addition, BoNTs do not cause the accumulation of neurofilament-like material in terminal boutons and/or bouton degeneration that occurs in EGS. The ultrastructure of NMJs from the horse with a presumptive diagnosis of botulism was consistent with that of NMJs from experimental animals following BoNT intoxication and was clearly distinguishable from that of NMJs from EGS horses. A potential limitation of this study is that a definitive diagnosis of botulism could not be made. The presumptive diagnosis of botulism was based solely on the findings of clinical and pathological examinations and the clinical progression. It is well recognised that obtaining a definitive diagnosis of equine botulism is extremely difficult, particularly in the United Kingdom, due to lack of available laboratory testing.[21] However, the presence in this pony of abnormalities in NMJ ultrastructure that are consistent with those of BoNT intoxication provides strong support for the diagnosis of botulism. A further limitation of the study is the small group sizes which reflected the considerable practical difficulties obtaining suitably processed samples, particularly from horses with botulism. While control horses were older than EGS horses, age difference was not considered to account for the inter-group ultrastructural differences. Ageing is associated with NMJ degeneration and decreased SV numbers,[49] features which were observed in the younger EGS horses rather than the older control horses.

While all BoNTs block SV exocytosis leading to accumulation of SVs at the active site of the presynaptic membrane, BoNT/C and BoNT/E can also induce massive disruption of axons and neurotoxicity, likely by disrupting exocytosis of essential dynamin-dependent recycling processes.[35,47,50] However, as the threshold concentrations

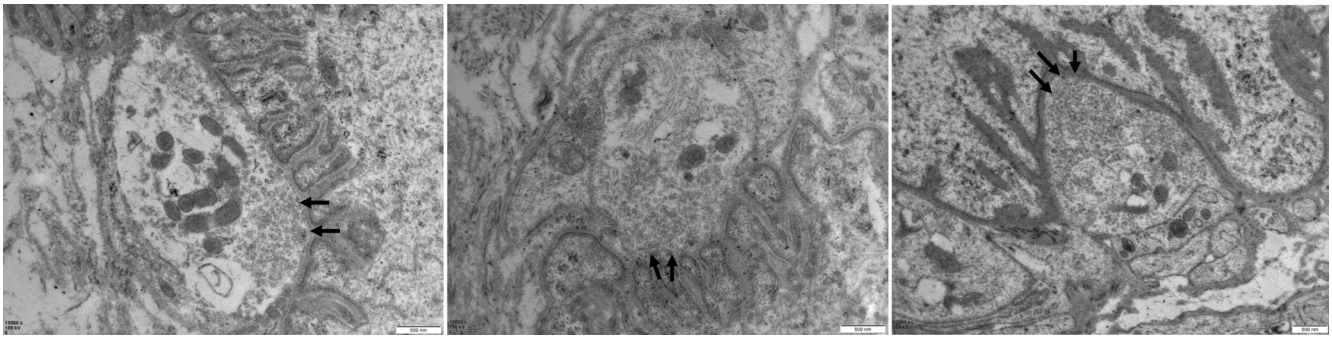

FIGURE 8 Three neuromuscular junctions (NMJs) from the horse with botulism. Consistent with the effect of botulinum neurotoxins, the synaptic vesicles (SVs) are unevenly distributed within the bouton, with SVs being more densely packed towards the active site on the presynaptic membrane (arrows). Terminus morphology is otherwise normal. In contrast to equine grass sickness NMJs, there is no SV depletion, accumulation of neurofilament-like material in terminal boutons or bouton degeneration. Scale bar 500 nm.

of BoNT/C and BoNT/E which cause neurotoxicity far exceed the lethal dose for humans and animals, it is considered unlikely that neurocytotoxicity has a significant role in the pathogenesis of naturally occurring BoNT/C and BoNT/E intoxication.[50] Consequently, it is unlikely that the bouton degeneration observed in EGS is due to the cytotoxic effects of BoNT/C or BoNT/E. The findings of this study therefore do not support the hypothesis that EGS is caused by toxicoinfection with BoNT/C.[11–13] This conclusion is supported by the occurrence of autonomic and enteric neurodegeneration, and increased expression of SNARE proteins within neuronal perikarya, in EGS but not botulism.[51]

Further investigation is warranted to determine whether the EGS-associated abnormalities in diaphragmatic and intercostal muscle NMJ ultrastructure have clinical consequences. However, given the severity of SV depletion and bouton degeneration, it is likely that EGS horses will have diaphragm and intercostal muscle dysfunction. Further study is also needed to determine whether other skeletal NMJs, including those of postural and locomotor muscles, are similarly affected in EGS, and whether this contributes to the lower motor neuron dysfunction identified in EGS horses by quantitative electromyography,[15] and to the apparent diffuse skeletal myasthenia which characterises EGS.[16,17] Comparison of NMJ ultrastructure changes in horses with acute versus chronic EGS is also warranted.

5 | CONCLUSION

Skeletal NMJs represent a previously unrecognised target for the toxin that causes EGS. EGS is associated with major changes in the ultrastructure of skeletal NMJs including evidence of accelerated SV exocytosis and SV depletion, accumulation of neurofilament-like material in terminal boutons and/or bouton degeneration. These changes appear inconsistent with the effects of BoNTs. Instead, they are consistent with the actions of an excitatory presynaptic toxin and/or neurotransmitter reuptake inhibitor.

AUTHOR CONTRIBUTIONS

Bruce C. McGorum: Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; resources; supervision; validation; writing – original draft; writing – review and editing. **Tracey Davey:** Data curation; investigation; methodology; writing – review and editing. **Miranda C. M. Dosi:** Investigation; methodology; writing – review and editing. **John A. Keen:** Investigation; writing – review and editing. **Linda R. Morrison:** Investigation; writing – review and editing. **R. Scott Pirie:** Investigation; writing – review and editing. **Darren J. Shaw:** Formal analysis; software; writing – review and editing. **John B. Harris:** Conceptualization; formal analysis; investigation; methodology; project administration; supervision; writing – original draft; writing – review and editing.

ACKNOWLEDGEMENTS

We thank Daniel Magill, Digitalab, Newcastle upon Tyne, for assistance with digital image processing.

FUNDING INFORMATION

This project was funded by the Royal College of Veterinary Surgeons Trust Blue Sky Research Fund and The Equine Grass Sickness Fund.

CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

DATA INTEGRITY STATEMENT

Bruce C. McGorum had full access to all data and takes responsibility for the integrity of the data and accuracy of the data analysis.

ETHICAL ANIMAL RESEARCH

Tissue samples were collected at necropsy from horses that were euthanised on humane grounds, for unmanageable clinical conditions, with the horse owners' consent. The study was approved by the University of Edinburgh Ethical Review Committee (80.22).

INFORMED CONSENT

Consent was obtained from owners of all horses included in the study.

PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/evj.14063.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Data sharing exemption granted by the editor for this retrospective case control study.

ORCID

Bruce C. McGorum https://orcid.org/0000-0002-6977-6101
Miranda C. M. Dosi https://orcid.org/0000-0002-1094-1184
John A. Keen https://orcid.org/0000-0002-7862-5321
Linda R. Morrison https://orcid.org/0000-0003-0495-4592
R. Scott Pirie https://orcid.org/0000-0003-4299-4077
Darren J. Shaw https://orcid.org/0000-0003-2016-1541

REFERENCES

1. Pirie RS. Grass sickness. Clin Tech Equine Pract. 2006;5:30–6. https://doi.org/10.1053/j.ctep.2006.01.007
2. Pirie RS, Jago RC, Hudson NP. Equine grass sickness. Equine Vet J. 2005;46:545–53. https://doi.org/10.1111/evj.12254
3. Sharp NJH, Nash AS, Griffiths IR. Feline dysautonomia (the Key-Gaskell syndrome): a clinical and pathological study of forty cases. J Small Anim Pract. 1984;5:599–615. https://doi.org/10.1111/j.1748-5827.1984.tb03372.x
4. Whitwell KE. Do hares suffer from grass sickness? Vet Rec. 1991; 128:395–6. https://doi.org/10.1136/vr.128.17.395
5. Longshore RC, O'Brien DP, Johnson GC, Grooters AM, Kroll RA. Dysautonomia in dogs: a retrospective study. J Vet Intern Med. 1996;10: 103–9. https://doi.org/10.1111/j.1939-1676.1996.tb02040.x
6. Kik MJ, van der Hage MH. Cecal impaction due to dysautonomia in a llama (*Lama glama*). J Zoo Wildl Med. 1999;30:435–8.
7. Pruden SJ, McAllister MM, Schultheiss PC, O'Toole D, Christensen DE. Abomasal emptying defect of sheep may be an

acquired form of dysautonomia. Vet Pathol. 2004;41:164–9. https://doi.org/10.1354/vp.41-2-164
8. Hahn CN, Whitwell KE, Mayhew IG. Neuropathological lesions resembling equine grass sickness in rabbits. Vet Rec. 2005;156:778–9. https://doi.org/10.1136/vr.156.24.778
9. Lewis CA, Bozynski CC, Johnson GC, Harral CM, Williams F 3rd, Tyler JW. Colonic impaction due to dysautonomia in an alpaca. J Vet Intern Med. 2009;23:1117–22. https://doi.org/10.1111/j.1939-1676.2009.0351.x
10. Hahn CN, Mayhew IG, de Lahunta A. Central neuropathology of equine grass sickness. Acta Neuropathol. 2001;102:153–9. https://doi.org/10.1007/s004010000289
11. Hunter LC, Miller JK, Poxton IR. The association of *Clostridium botulinum* type C with equine grass sickness: a toxicoinfection? Equine Vet J. 1999;31:492–9. https://doi.org/10.1111/j.2042-3306.1999.tb03857.x
12. Hunter LC, Poxton IR. Systemic antibodies to *Clostridium botulinum* type C: do they protect horses from grass sickness (dysautonomia)? Equine Vet J. 2001;33:547–53. https://doi.org/10.2746/042516401776563418
13. McCarthy HE, French NP, Edwards GB, Poxton IR, Kelly DF, Payne-Johnson CE, et al. Equine grass sickness is associated with low antibody levels to *Clostridium botulinum*: a matched case-control study. Equine Vet J. 2010;36:123–9. https://doi.org/10.2746/0425164044868611
14. McGorum BC, Chen Z, Glendinning L, Gweon HS, Hunt L, Ivens A, et al. Equine grass sickness (a multiple systems neuropathy) is associated with alterations in the gastrointestinal mycobiome. Anim Microbiome. 2021;3:70. https://doi.org/10.1186/s42523-021-00131-2
15. Wijnberg ID, Franssen H, Jansen GH, van den Ingh THSG, van der Harst MR, van der Kolk JH. The role of quantitative electromyography (EMG) in horses suspected of acute and chronic grass sickness. Equine Vet J. 2006;38:230–7. https://doi.org/10.2746/042516406776866309
16. McGorum BC. Diffuse skeletal muscle weakness. In: Robinson NE, editor. Current therapy in equine medicine 5. London: WB Saunders; 2003. p. 740–5.
17. Lyle C, Pirie RS. Equine grass sickness. In Pract. 2009;31:26–32. https://doi.org/10.1136/inpract.31.1.26
18. McGorum BC, Kirk J. Equine dysautonomia (grass sickness) is associated with alterations in plasma amino acid concentrations, and depletion in plasma sulphur amino acids. Equine Vet J. 2001;33:473–7. https://doi.org/10.2746/042516401776254763
19. Scholes SFE, Vaillant C, Peacock P, Edwards GB, Kelly DF. Enteric neuropathy in horses with grass sickness. Vet Rec. 1993;132:647–51. https://doi.org/10.1136/vr.132.26.647
20. Doxey DL, Pogson DM, Milne EM, Gilmour JS, Chisholm HK. Clinical equine dysautonomia and autonomic neuron damage. Res Vet Sci. 1992;53:106–9. https://doi.org/10.1016/0034-5288(92)90093-h
21. Stratford CH, Mayhew IG, Hudson NPH. Equine botulism – a clinical approach to diagnosis and management. Equine Vet Educ. 2014;26:441–8. https://doi.org/10.1111/eve.12198
22. Logonder U, Krizaj I, Rowan EG, Harris JB. Neurotoxicity of ammodytoxin A in the envenoming bites of *Vipera ammodytes ammodytes*. J Neuropath Exp Neurol. 2008;67:1011–9. https://doi.org/10.1097/NEN.0b013e318188c2d7
23. Weibel ER. Stereological methods. Practical methods for biological morphometry. London: Academic Press; 1979.
24. White KE, Bilous RW. Estimation of podocyte number: a comparison of methods. Kidney Int. 2004;66:663–7. https://doi.org/10.1111/j.1523-1755.2004.00787.x
25. Pécot-Dechavassine M, Molgo J, Thesleff S. Ultrastructure of botulinum type-A poisoned frog motor nerve terminals after enhanced quantal transmitter release caused by carbonyl cyanide m-chlorophenylhydrazone. Neurosci Lett. 1991;2:1305–8. https://doi.org/10.1016/0304-3940(91)90214-e
26. Bowden REM, Duchen LW. The anatomy and pathology of the neuromuscular junction. In: Zaimis E, editor. Neuromuscular junction. Berlin: Springer; 1976. p. 22–97.
27. Hirokawa N, Heuser JE. Structural evidence that botulinum toxin blocks neuromuscular transmission by impairing the calcium influx that normally accompanies nerve depolarization. J Cell Biol. 1981;88:160–71. https://doi.org/10.1083/jcb.88.1.160
28. Thesleff S. Supersensitivity of skeletal muscle produced by botulinum toxin. J Physiol. 1960;151:598–607. https://doi.org/10.1113/jphysiol.1960.sp006463
29. Bishop AE, Hodson NP, Major JH, Probert L, Yeats J, Edwards GB, et al. The regulatory peptide system of the large bowel in equine grass sickness. Experientia. 1984;40:801–6. https://doi.org/10.1007/BF01951962
30. Schlaeafer WW, Micko S. Chemical and structural changes of neurofilaments in transected rat sciatic nerve. J Cell Biol. 1978;78:369–78. https://doi.org/10.1083/jcb.78.2.369
31. Fasani F, Bocquet A, Peterson RA, Eyer J. The amount of neurofilaments aggregated in the cell body is controlled by their increased sensitivity to trypsin-like proteases. J Cell Sci. 2004;117:861–9. https://doi.org/10.1242/jcs.00940
32. Chen PC, Qin LN, Li XM, Walters BJ, Wilson JA, Mei L, et al. The proteasome-associated deubiquitinating enzyme Usp14 is essential for the maintenance of synaptic ubiquitin levels and the development of neuromuscular junctions. J Neurosci. 2009;29:10909–19. https://doi.org/10.1523/JNEUROSCI.2635-09.2009
33. Shotton HR, Lincoln J, McGorum BC. Effects of equine grass sickness on sympathetic neurons in prevertebral and paravertebral ganglia. J Comp Pathol. 2011;145:35–44. https://doi.org/10.1016/j.jcpa.2010.11.003
34. McGorum BC, Pirie RS, Eaton SL, Keen JA, Cumyn EM, Arnott DM, et al. Proteomic profiling of cranial (superior) cervical ganglia reveals Beta-amyloid and ubiquitin proteasome system perturbations in an equine multiple system neuropathy. Mol Cell Proteomics. 2015;14(11):3072–86. https://doi.org/10.1074/mcp.M115.05463
35. Osen-Sand A, Staple JK, Naldi E, Schiavo G, Rossetto O, Petitpierre S, et al. Common and distinct fusion proteins in axonal growth and transmitter release. J Comp Neurol. 1996;367:222–34. https://doi.org/10.1002/(SICI)1096-9861(19960401)367:2<222::AID-CNE5>3.0.CO;2-7
36. Marshall AG, Watson JA, Hallengren JJ, Walters BJ, Dobrunz LE, Francillon L, et al. Genetic background alters the severity and onset of neuromuscular disease caused by the loss of ubiquitin-specific protease 14 (Usp14). PloS One. 2013;8:e84042. https://doi.org/10.1371/journal.pone.0084042
37. Cifuentes-Diaz C, Nicole S, Velasco ME, Bora-Cebrian C, Panozzo C, Frugier T, et al. Neurofilament accumulation at the motor endplate and lack of axonal spouting in a spinal muscular atrophy mouse model. Hum Mol Genet. 2002;11:1439–47. https://doi.org/10.1093/hmg/11.12.1439
38. Llorens J. Toxic neurofilamentous axonopathies: accumulation of neurofilaments and axonal degeneration. J Intern Med. 2013;273:478–89. https://doi.org/10.1111/joim.12030
39. Lang B, Vincent A. Autoimmune disorders of the neuromuscular junction. Curr Opin Pharmacol. 2009;9:336–40. https://doi.org/10.1016/j.coph.2009.04.005
40. Senanayake N, Román GC. Disorders of neuromuscular transmission due to natural environmental toxins. J Neurol Sci. 1992;107:1–13. https://doi.org/10.1016/0022-510x(92)90202-v
41. Zhou K, Luo W, Liu T, Ni Y, Qin Z. Neurotoxins acting at synaptic sites: a brief review on mechanisms and clinical applications. Toxins. 2023;15:18. https://doi.org/10.3390/toxins15010018
42. Clark AW, Hurlbut WP, Mauro A. Changes in the fine structure of the neuromuscular junction of the frog caused by Black Widow Spider venom. J Cell Biol. 1972;52:1–14. https://doi.org/10.1083/jcb.52.1.1

43. Longenecker HE Jr, Hurlbut WP, Mauro A, Clark AW. Effects of black widow spider venom on the frog neuromuscular junction. Effects on end-plate potential, miniature end-plate potential and nerve terminal spike. Nature. 1970;225:701–3. https://doi.org/10.1038/225701a0
44. Duchen LW, Gomez LS, Quiroz LS. The neuromuscular junction of the mouse after black widow spider venom. J Physiol. 1980;316:279–91. https://doi.org/10.1113/jphysiol.1981.sp013787
45. Tzeng MC, Cohen RS, Siekevitz P. Release of neurotransmitters and depletion of synaptic vesicles in cerebral cortex slices by alpha-latrotoxin from black widow spider venom. Proc Natl Acad Sci U S A. 1978;75:4016–20. https://doi.org/10.1073/pnas.75.8.4016
46. Newton JR, Hedderson EJ, Adams VJ, McGorum BC, Proudman CJ, Wood JL. An epidemiological study of risk factors associated with the recurrence of equine grass sickness (dysautonomia) on previously affected premises. Equine Vet J. 2004;36:105–12. https://doi.org/10.2746/0425164044868639
47. Schiavo G, Matteoli M, Montecucco C. Neurotoxins affecting neuroexocytosis. Physiol Rev. 2000;80:717–66. https://doi.org/10.1152/physrev.2000.80.2.717
48. Duchen LW. An electron microscopic study of the changes induced by botulinum toxin in the motor end-plates of slow and fast skeletal muscle fibres of the mouse. J Neurol Sci. 1971;14:47–60. https://doi.org/10.1016/0022-510x(71)90129-8
49. Khosa S, Trikamji B, Khosa GS, Khanli HM, Mishra SK. An overview of neuromuscular junction aging findings in human and animal studies. Curr Aging Sci. 2019;12:28–34. https://doi.org/10.2174/1874609812666190603165746
50. Peng L, Liu H, Ruan H, Tepp WH, Stoothoff WH, Brown RH, et al. Cytotoxicity of botulinum neurotoxins reveals a direct role of syntaxin 1 and SNAP-25 in neuron survival. Nat Commun. 2013;4:1472. https://doi.org/10.1038/ncomms24622
51. McGorum BC, Scholes S, Milne EM, Eaton SL, Wishart TM, Poxton IR, et al. Equine grass sickness, but not botulism, causes autonomic and enteric neurodegeneration and increases soluble N-ethylmaleimide-sensitive factor attachment receptor protein expression within neuronal perikarya. Equine Vet J. 2016;48:786–91. https://doi.org/10.1111/evj.12543

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: McGorum BC, Davey T, Dosi MCM, Keen JA, Morrison LR, Pirie RS, et al. Equine grass sickness is associated with major abnormalities in the ultrastructure of skeletal neuromuscular junctions. Equine Vet J. 2024. https://doi.org/10.1111/evj.14063