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Neuropathic changes in equine laminitis pain

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Abstract

Laminitis is a common debilitating disease in horses that involves painful disruption of the lamellar dermo-epidermal junction within the hoof. This condition is often refractory to conventional anti-inflammatory analgesia and results in unremitting pain, which in severe cases requires euthanasia. The mechanisms underlying pain in laminitis were investigated using quantification of behavioural pain indicators in conjunction with histological studies of peripheral nerves innervating the hoof. Laminitic horses displayed consistently altered or abnormal behaviours such as increased forelimb lifting and an increased proportion of time spent at the back of the box compared to normal horses. Electron micrographic analysis of the digital nerve of laminitic horses showed peripheral nerve morphology to be abnormal, as well as having reduced numbers of unmyelinated (43.2%) and myelinated fibers (34.6%) compared to normal horses. Sensory nerve cell bodies innervating the hoof, in cervical, C8 dorsal root ganglia (DRG), showed an upregulated expression of the neuronal injury marker, activating transcription factor-3 (ATF3) in both large NF-200-immunopositive neurons and small neurons that were either peripherin- or IB4-positive. A significantly increased expression of neuropeptide Y (NPY) was also observed in myelinated afferent neurons. These changes are similar to those reported in other neuropathic pain states and were not observed in the C4 DRG of laminitic horses, which is not associated with innervation of the forelimb. This study provides novel evidence for a neuropathic component to the chronic pain state associated with equine laminitis, indicating that anti-neuropathic analgesic treatment may well have a role in the management of this condition.

Keywords: Neuropathic pain, Dorsal root ganglion, Equine laminitis, neuronal injury marker, ATF3

1. Introduction

Laminitis is a common cause of equine lameness involving one or more feet (Dyson, 2003). It is characterised by disruption of the dermo-epidermal laminar bond within the hoof (Fig. 1b, c) and subsequent structural weakness that can result in displacement of the pedal bone within the hoof capsule (Pollitt et al., 1998). The pathogenesis of this disease is poorly understood but it is generally thought that vascular disturbances leading to ischemia-reperfusion injury of the lamellar structures are involved in the pathophysiology of laminitis (Hood, 1999). Currently, no therapeutic regime is able to arrest or prevent its onset (Pollitt, 2003). Moreover, laminitic pain can be difficult to control using traditional anti-inflammatory agents and euthanasia on welfare grounds is not uncommon (Herthel and Hood, 1999; Swanson, 1999; Pollitt, 2003). Therefore, improved understanding of laminitis is much needed.

We hypothesized that the pathological inflammatory processes affecting the hoof laminae during laminitis also damage the sensory neurons innervating this region. Peripheral nerve injury can be associated with the generation of a neuropathic pain state characterised by allodynia (the perception of normally innocuous stimuli as painful), hyperalgesia (a heightened response to painful stimuli), spontaneous pain and a lack of response to conventional analgesics. A key factor in the neural plasticity underlying neuropathic (compared to inflammatory) pain is altered gene expression in sensory DRG neurons (Hökfelt et al., 1994; Cummins et al., 2000; Woolf and Salter, 2000; Xiao et al., 2002). This can be demonstrated by an increase in expression of the neuronal injury marker ATF3, a member of the activating transcription factor/cAMP-responsive element binding protein (ATF/CREB) family, in sensory DRG cells (Hai et al., 1999; Tsujino et al., 2000). Furthermore, phenotypic changes occur in primary afferent DRG neurons after peripheral nerve damage, resulting in altered expression of neuropeptides, including neuropeptide Y (NPY), the expression of which is induced from normally low levels in large diameter, neurofilament-200 (NF-200)-positive neurons following axotomy (Wakisaka et al., 1991; Hokfelt et al., 1994), nerve injury

(Ma and Bisby, 1998; Munglani et al., 1995), demyelination (Wallace et al., 2003) and streptozotocin-induced diabetes (Rittenhouse et al., 1996).

Injury to sensory nerves induces neurochemical, physiological and anatomical modifications to afferent and central neurons that are likely to contribute to chronic, sensitised neuropathic pain responses (Woolf and Salter, 2000). Such changes to the sensory neurons innervating the equine foot could lead to a clinically relevant component of chronic pain as it would explain the limited effectiveness of conventional analgesics in the treatment of laminitic pain (Herthel and Hood, 1999).

Therefore, the aims of this study were to identify and quantify equine laminitic pain using objective behavioural assessment, characterise peripheral nerve damage in the lateral digital nerve and demonstrate potential nerve injury-associated alterations in protein expression in DRG sensory neurons innervating the feet of horses with laminitis.

2. Methods

2.1. Behavioural observations in laminitic and clinically normal horses

In order to define and quantify the behavioural characteristics of equine laminitis, we carried out continuous video monitoring over 3 days to compare behaviours in laminitic and normal horses.

Seven horses admitted for management of refractory laminitis were selected using the following clinical criteria: animals must have displayed clinical signs consistent with this disease including multi-limb lameness, increased amplitude of the digital pulses, warmth across the dorsal hoof wall and a laminitic gait (Stashak, 2002). For details of all laminitic horses used in this study see Table 1.

Latero-medial radiographs of the fore limb digits were obtained from each of these horses (Butler et al, 2000). The position of the pedal bone within the hoof capsule was evaluated both subjectively and objectively by an experienced equine clinician using standard measures (Fig.1a).

Informed client consent was obtained in writing prior to the onset of data collection. Laminitic horses received phenylbutazone (PBZ) twice daily at 08:00h and 20:00h (Equipalazone Arnolds, UK; 2.2-4.0 $mg.kg^{-1}$) and intramuscular acepromazine three times daily at 08:00h, 16:00h and 24:00h (ACP Novartis, UK; 0.02-0.04 $mg.kg^{-1}$). On the day of admission to hospital the timing of drug administration varied between individuals. Pedal bone support (Styrofoam Solar Support System™/Lilypads™) was provided at the clinician's discretion. Subjects participated in the study for a maximum of 3 days. Seven age, type and sex-matched horses, which were considered 'pain free' (control group) were stabled directly opposite the laminitic horses and recorded simultaneously in order to account for extraneous effects on behaviour. All horses were maintained on shavings and had free access to water. Laminitic animals were fed restricted rations of soaked hay, as is standard procedure, whereas control animals received haylage *ad libitum*.

24-hour time-lapse video equipment (AG-6124, Panasonic) was used to record undisturbed behaviour in each stable. Point samples of 1 hour duration were taken at 8 hour-intervals, at 06:00h, 14:00h and 22:00h, during 3 days starting at 14.00h on Day 1. Samples were analysed continuously for duration of state and frequency of event behaviour (The Observer™ vs. 4.1, Noldus Information Technology, The Netherlands).

Two behaviours were selected for statistical analysis as being representative of the behaviours where changes were most likely to be observed (Price et al., 2003; Reitmann et al., 2004). Frequency of 'forelimb lifting' (as lifts min^{-1}) was defined as the raising and lowering of a forelimb, without locomotion and was adjusted for total time spent standing. 'Proportion of time spent at the back of the box' was defined as time spent positioned in the furthest 50% of the box, away from the entrance to the stable.

On a repeated dose regime, peak PBZ concentrations occur between 2 and 6 hours following administration, although individual variation is high (Gerring et al., 1981). In the present study a 12-hour dosing regime was used, minimising variation and increasing the probability of the maintenance of a 'steady state'. For sample point analysis of behaviours, data were

collected at three different time points to reflect an expected minimum plasma PBZ concentration (06:00h) and shortest (2 hours post-administration – 22:00h) and longest (6 hours post administration – 14:00h) times for peak PBZ concentrations were chosen. In addition, to evaluate some of the possible effects of drug accumulation, analyses were repeated just using data from day 1, days 1 and 2 and days 1, 2 and 3.

2.2. Morphological investigations

Lateral digital nerves were obtained from five horses euthanised on clinical grounds due to laminitis which was either recurrent or refractory to therapy and also from four horses which had no history of forelimb lameness that were euthanised for clinical reasons other than forelimb pathology (control group). The lateral digital nerves were removed from the forelimb (3 cm long segments) at the level of the proximal sesamoid bone and fixed for 4 hours in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.3, post-fixed in OsO_4, and embedded in Araldite. For light microscopy, 1 μm resin sections of the nerve were stained with Toluidine blue and three fascicles were chosen at random by bright-field microscopy. Ultra-thin (80 nm) sections were stained with uranyl acetate and lead citrate and examined on a Phillips BioTwin electron microscope (FEI, UK Ltd, Cambridge, UK). Electron microscope (EM) images of cross sections of fascicles within each nerve (areas ranging between 6732 and 47215 μm^2) were analysed by eye by a trained, blinded observer using Image Tool 3.0 (UTHSCSA, USA). The total area of the nerve sections and the percentage of the nerve area occupied by nerve fascicles were calculated in order to investigate any differences between normal and laminitic digital nerves that might reflect oedema and therefore affect the quantification of axon density. The number and axon diameter of intact myelinated fibers was calculated as well as the percentage of damaged myelinated fibers, defined as those with a severe disruption of the myelin sheath and/or axonal degeneration. Myelin sheath thickness was measured and G-ratio of axons was calculated by dividing the axonal diameter by the total diameter of axon plus myelin sheath.

166 The proportion of A-fibers with continuous Schwann cell cytoplasm (an abnormal
167 morphological feature previously described by Court et al., 2004) was also determined. C-
168 fibers were identified as small-diameter unmyelinated fibers, surrounded by Schwann cell
169 cytoplasm. The total number of C-fibers was calculated as well as the percentage of solitary
170 unmyelinated fibers and the number of unmyelinated fibers per Remak bundle. All analysis
171 was carried out on identity-concealed samples.
172
173 **2.3. Immunohistochemistry**
174 DRG from cervical segments 8 (forelimb innervation) and 4 (non-forelimb innervation) from
175 the same horse were obtained post-mortem from the five laminitic horses and four control
176 horses. The tissue was snap frozen and embedded in OCT embedding matrix (Cell Path plc.
177 Powys. Wales, UK). Cryostat sections of C8 DRGs (15 μm) were thaw-mounted on poly-L-
178 lysine slides (Merck-BDH).

179 DRG sections were pre-incubated for 1h at room temperature in 0.1 M PBS, pH 7.4, buffer
180 containing 0.2% Triton X-100, 2% fish skin gelatin and 10% normal goat serum; and then
181 incubated overnight at 4 °C with primary antibodies diluted in the same buffer. For co-
182 localisation of the peptide NPY or ATF3 with the myelinated cell marker neurofilament 200
183 kDa (NF-200) (Lawson and Waddell, 1991; Michael et al., 1999), or either of the
184 unmyelinated cell markers, peripherin or isolectin B4 (IB4) (Goldstein et al., 1991; Michael
185 and Priestley, 1999), antisera/lectin were used at the following concentrations: rabbit anti-
186 NPY (1:250; Peninsula Laboratories Inc, Belmont, CA, USA); rabbit anti-ATF3 (1:300;
187 Santa Cruz Biotechnology, Santa Cruz, CA, USA); mouse monoclonal anti-NF-200 (1:400;
188 clone N52; Sigma); mouse monoclonal anti-peripherin (1:250; Chemicon International,
189 Harlow, UK); IB4 from *Bandeiraea simplicifolia* (1:400; Sigma). Sections were then washed
190 in buffer and incubated at room temperature for 2 hours with Alexafluor 488-labeled goat
191 anti-mouse IgG (1:500; Molecular Probes Europe BV, The Netherlands), Alexafluor 568-
192 labeled goat anti-rabbit IgG (1:1000; Molecular Probes Europe BV, The Netherlands) or

193 Alexa Fluor 488–labeled streptavidin (1:200). Three washes in 0.1M PBS were performed
194 before the addition of To-Pro3 cyanine nucleic acid stain (Molecular Probes Europe BV, The
195 Netherlands). Three final washes in 0.1 M PBS were conducted before cover-slipping with
196 Vecta-Shield (Vector Laboratories, Burlingame, CA, USA). Control sections were processed
197 as above omitting the primary reagents.

198 Observations were made and sections photographed on an Olympus microscope equipped for
199 epifluorescence. All counts of profiles labelled for immunopositive cells were performed by
200 the same observer (who was blinded to sample treatment) on randomly selected, 15 μm
201 sections of DRG from each of the animals in each group. Every sixth section was selected to
202 ensure that measurements were taken only once for each cell. Results were expressed as the
203 proportion of labelled profiles per total number of single or double-labelled profiles from all
204 sections, 95% confidence intervals (CI) are indicated.

205

206 **2.4. Western blots**

207 C4 and C8 DRG were taken from laminitic horses (n=3). Whole lysate preparations were
208 prepared by homogenising tissue in 20 volumes of Laemmli lysis buffer (Tris (tris-
209 hydroxymethylaminoethane, 50 mM, pH 7.4), 5% mercaptoethanol and 2% sodium dodecyl
210 sulphate (SDS)), boiled for 5 min and frozen. Western blotting was carried out as described
211 previously (Garry et al., 2005). Blots were incubated with rabbit polyclonal primary
212 antibodies to ATF3 (1:200 Santa Cruz Biotechnology, Santa Cruz, CA, USA) and detected by
213 peroxidase-linked secondary antibody and enhanced chemiluminescence. The ubiquitous
214 housekeeping enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:750,
215 Chemicon) was monitored as a control for protein level normalisation. Quantitative
216 densitometry analysis of protein bands was performed using the ScanAnalysis (Elsevier)
217 program.

218

219 **2.5 Statistical analyses**

Linear mixed-effect models were used to determine any differences between laminitic and controls horses in the frequency of lifting the forelimb and time spent at the back of the box, in order to account for the repeated sampling of the same horses (Pinheiro and Bates, 2000). The ID of the horse that the samples came from was entered as a random effect. Laminitic/control, time point in experiment and time of day were entered as fixed effects. Prior to analysis of the forelimb lifting results, the data were square root-transformed to achieve normalisation of the residuals.

For the analysis of percentages of damaged A-fibers, A-fibers with Schwann cell cytoplasm and solitary unmyelinated fibers, only one meaned value per horse was obtained, and therefore repeated sampling has not taken place. Repeated measures of mean axon diameters in myelinated and unmyelinated fibers and thickness of myelin sheath were taken in both control and laminitic horses. Therefore, linear-mixed effect models were also used to determine any differences between (i) mean axon diameters in myelinated and unmyelinated fibers; (ii) thickness of myelin sheath from laminitic and control horses. Multiple measurements per horse were also taken of the number of fibers per Remak bundle but as the data were integers, differences in the number of fibers per Remak bundle were analysed using generalised linear mixed-effect models with Poisson errors. Only a single measurement per horse of the percentage of damaged A fibers; A fibers with continuous Schwann cell cytoplasm and solitary unmyelinated fibers were taken, therefore simple logistic regressions were employed to determine the differences between control and laminitic horses.

Differences in total nerve area occupied by fascicles between normal and laminitic horses were investigated using a Student's t-test, and differences in the percentage of nerve area occupied by fascicles between normal and laminitic horses by general linear models with binomial errors. Any differences in the proportion of labelled profiles were assessed by χ^2 analysis. Mann-Whitney non-parametric tests were used to analyse fiber density. Immunoblot data were analysed using a matched pair t-test. All analyses were carried out in S-PLUS 6.0 (Insightful, Seattle, USA) and SigmaStat 2.03 (SPSS Inc., USA). In all cases $p<0.05$ was

taken to indicate statistical significance, and degrees of freedom associated with any tests are denoted by subscripts.

3. Results

3.1. Radiographic abnormalities associated with laminitis were seen in all the laminitic horses.

The horses clinically diagnosed with laminitis displayed radiographic evidence of this disease when the radiographs were evaluated objectively (Butler et al., 2000). The angle between the dorsal hoof wall and the dorsal surface of the distal phalanx was increased when compared to normal values (Fig 1a). The mean (± SD) values from the laminitic group were 8.2º ±3.0º (normal values -0.86º ± 2.4º) (Cripps and Eustace, 1999). Assessment of the D distance between the extensor process of the distal phalanx and the coronary band also showed a marked increase in the laminitic group (D= 16.4mm ±4.9mm) when compared to normal values (4.1mm ± 2.17mm) (Cripps and Eustace, 1999). Histological sections of laminitic tissue also indicated inflammatory changes (Fig. 1d).

3.2. Laminitic horses display quantifiable abnormal behaviours

3.2.1. Data Analysis: Forelimb lifting

When considering overall data, laminitic horses show a statistically significant increase in the mean square root frequency of forelimb lifting ($F_{1,12}$=11.5, p=0.005; Fig. 2a) adjusted for time spent standing compared to control horses recorded in the same environment over the same time period. Fluctuations in the frequency of this behaviour occurred in both groups over the period of observation, but the pattern of such changes did not differ significantly between control and laminitic horses ($F_{1,140}$=3.6, p=0.059).

3.2.2. Data analysis proportion of time spent at the back of the box

Laminitic horses spent significantly more time at the back of the box than control horses ($F_{1,12}$=6.1, p=0.03). There was no difference between the 2 groups in how behaviour altered throughout the study ($F_{1,148}$=0.2, p=0.683) (Fig. 2b). Time spent at the back of the box was markedly higher in laminitics than in controls at both 06:00h and 22:00h but not at 14:00h (days 2 & 3), when assessing individual sample point data. This effect is not seen at 14:00h on day one, probably because drug administration regimes were not well-established at this time.

3.3. Distinct morphological abnormalities in both myelinated and unmyelinated peripheral nerve fibers innervating the hoof, in the lateral digital nerve of laminitic horses.

The lateral digital nerves at the level of the proximal sesamoid bone were examined from both normal and laminitic horses. A mean of 11.65% (range 8.75-14.35) of the total fascicle area from each nerve section was analysed. EM analysis of three randomly selected fascicles per lateral digital nerve revealed morphological differences in both the myelinated and unmyelinated fiber populations in laminitic compared to non-laminitic horses (Table 2). Abnormalities in the shape of surviving axons and disruption of the myelin sheath, with accumulation of lipid droplets and myelin debris were observed. The most obvious quantitative feature appeared to be a significant reduction in the number of both unmyelinated (-43.2%) and myelinated fibers (-34.6%) per unit area in laminitic compared to control horses (p=0.016). In order to eliminate the possibility that any nerve oedema could artefactually lead to the appearance of reduced fiber density, morphometric analyses were carried out to measure the percentage area of nerve sections occupied by fascicles and total nerve area in normal compared to laminitic horses. No significant differences in the mean percentages were identified (t_4=-0.91, p= 0.414) between normals 37% (95% CI: 35.7-38.1) and laminitics 41% (39.5-42.0). No significant differences in total nerve area were identified between normal and laminitic horses (t_4=-0.43, p=0.692). Further abnormalities were a significant decrease in the

number of unmyelinated nerve fibers per Remak bundle ($F_{1,7}$=20.7, p=0.003) together with an increase in the percentage of solitary unmyelinated fibers in laminitics compared to normal horses (χ^2_1= 35.7, p<0.001, Fig. 3; Table 2b). The percentage of morphologically damaged myelinated fibers was significantly higher in laminitic horses when compared to normal horses (χ^2_1= 31.5, p<0.001, Fig. 3; Table 2 a). Finally, the proportion of myelinated fibers with continuous Schwann cell cytoplasm was significantly higher in the laminitic horses (χ^2=338.4, p<0.001). No significant differences in myelin thickness or G-ratios were identified in laminitic compared to normal horses ($F_{1,7}$<0.5, p>0.311).

3.4. The neuronal injury marker ATF3 is selectively expressed in sensory neurons innervating the forelimb in laminitic horses.

Using immunohistochemical analysis of the DRG cell population, we assessed the presence of ATF3 in comparison with the expression of NF-200 and either IB4 or peripherin. ATF3 expression was significantly increased in NF-200-positive C8 DRG cells from laminitic horses (n=3), where 67% (15 sections, 304 cells, CI 58.8-69.9) of NF-200-positive DRG cells co-expressed ATF3, while only 10% (15 sections, 345 cells, CI 6.9-13.5) of NF-200-positive DRG cells in control horses (n=3) co-localised ATF3 (χ^2_1=208, p<0.001), (Fig. 4). There was a significantly increased expression of ATF3 in IB4-positive C8 DRG cells in laminitic horses (n=3), where 54% (5 sections, 53 cells, CI 41.5-67.3) of IB4-positive C8 DRG cells co-localised ATF3 compared with 9% (5 sections 56 cells, CI 3.9-19.3) in control horses (n=3) (χ^2_1 = 24.5, p<0.001). There was also a significantly increased proportion of peripherin-positive DRG cells that were positive for ATF3 in C8 DRG cells from laminitic horses, (n=3), where 57% (9 sections, 115 cells, CI 47.6-65.4) of peripherin-positive cells co-expressed ATF3 compared with 21% (9 sections, 127 cells CI 13.8-29.4) showing double-labelling in control horses (n=3) (χ^2_1 = 30.9, p=0.001; Fig. 4).

Accordingly, immunoblot analysis revealed a significant increase (p<0.05) in ATF3 expression (expressed as mean percentage of GAPDH expression) in C8 DRG (38.9% (28.7-49.1) in comparison to low levels in the control C4 DRG (4.6% (-0.5-9.7) (Fig.4b, f). The numbers of cells expressing NF-200, IB4 or peripherin were unaltered in laminitic DRG compared to normal horses (696 compared to 575 NF-200-IR cells, n=32 sections, 115 compared to 127 peripherin-IR cells, n=9 sections, 56 compared to 53 IB4-IR cells, n=5 sections, in normal compared to laminitic horses, respectively).

3.5. Laminitis is associated with a distinctive pattern of expression of Neuropeptide Y (NPY) in sensory neurons.

A significant increase in NPY immunoreactivity (NPY-IR) was observed in the C8 DRG of laminitic horses, where 77% (17 sections, 271 cells, CI 72.0-82.3) of NF-200-positive cells showed NPY-IR co-localisation, compared to only 10% (17 sections, 351 cells CI 15.7-24.7) in control horses ($\chi^2_1 = 193$, p<0.001; Fig. 4).

4. Discussion

Damage to sensory nerves has been linked to abnormal pain and heightened sensitivity to touch in a variety of clinical and experimental studies. In this study, we have quantified for the first time abnormal behaviours associated with equine laminitis which are indicative of a hypersensitive sensory state. Additionally, we provide novel evidence for changes associated with nerve damage in the sensory nerves innnervating the forelimb in laminitic horses, which are consistent with those reported in previously characterised neuropathic pain states.

4.1. The laminitic horses included in this study have digital pathology.

Assessment of the radiographs from the laminitic horses identified pedal bone displacement (rotation or distal displacement) associated with laminar tearing. It was not possible to perform radiographic assessment of the control horses due to ethical and health and safety

limitations, therefore data were compared to well established normal data (Cripps and Eustace, 1999). Chronic inflammatory changes were also observed (Fig. 1f, g) which have been previously shown to associate with sensory nerve losses in the skin (Lacomis et al., 1997; Tseng et al., 2006).

4.2. Laminitic horses display chronically altered behaviour.

We have quantified two behavioural changes associated with laminitis, which are suggestive of a chronic hypersensitive neuropathic pain state, characterised by the development of allodynia, hyperalgesia and spontaneous pain. Forelimb lifting represents an abnormal, *de novo* behaviour associated with laminitis, being at low levels or absent in the clinically normal horse. The overall scores for frequency of forelimb lifting were significantly greater in laminitic than control horses. Additionally, laminitic horses spent more time positioned towards the back of the box, a retiring behaviour that has also been associated with acute post-surgical limb pain (Price et al, 2003). This behaviour may represent a reluctance to engage in the external environment and preference to remain withdrawn from surroundings. The differences in retiring behaviour (percentage of time spent at the back of the box) showed the appearance of a marked cyclical pattern, although this was not seen with forelimb lifting, weight bearing when walking or general demeanour. The pattern apparent in time at the back of the box observations may correspond to NSAID dosing times, external environmental stimuli or possibly an intrinsic diurnal rhythm. This emphasises the need for behavioural testing at a number of regular intervals in order to correctly reveal specific changes. Moreover, the consistent deviations from normal behaviour over the three-day period confirmed that the NSAID analgesic regime was not consistently effective. When individual time points were evaluated, marked differences from control horses were consistently seen at 06:00h and 22:00h observations.

4.3. Abnormal hoof sensory nerve morphology in laminitic horses is consistent with that reported in damaged peripheral nerves in neuropathic pain states.

Two types of sensory receptor have been identified in the equine foot. Lamellated corpuscles, similar to Pacinian corpuscles, found primarily in the solar dermis of the heel, are low-threshold mechanoreceptors, which transmit their input via rapidly conducting, myelinated A-fibers (Bowker et al., 1993). Additionally, numerous naked nerve endings containing the neuropeptide, calcitonin gene related peptide (CGRP)-like immunoreactivity and other sensory neuropeptides such as substance P, neurokinin A and PHI (peptide histidine-isoleucine) were detected in the dermis of the dorsal hoof wall and sole (Bowker et al., 1995). Those containing CGRP are associated with nociception (Schmidt, 1981) and transmit via slowly conducting C-fibers. Axons from the hoof nociceptors and low-threshold mechanoreceptors as well as sympathetic fibres innervating the vasculature contribute to the sensory digital nerve.

Changes in any of these could potentially contribute to the etiology of the chronic laminitic pain state. To understand the mechanisms underlying laminitis pain and the incomplete response to anti-inflammatory analgesics, it is important to establish whether axonopathic changes may contribute. In laminitic horses, EM analysis identified marked decreases in myelinated and unmyelinated fiber numbers per unit area of digital nerve. This is unlikely to be due to nerve oedema as there were no significant differences between nerve section areas and fascicle areas in normal and laminitic horses. The marked increase in the number of solitary, unmyelinated fibers, may reflect demyelinated A-fibers, or an absence of guiding pathways for regenerating C-fibers (Bester et al., 1998). These morphological changes are consistent with those in laboratory neuropathic pain models, such as chronic constriction injury (Gautron et al., 1990, Basbaum et al., 1991, Gabay and Tal, 2004; Micu et al., 2006), crush injury (Lozeron et al., 2004), photochemically-induced ischemia (Yu et al., 2000) and diabetic neuropathy (Sima et al., 1988; Llewelyn et al., 1991; Elias et al., 1998; Kalichman et

al., 1998), thereby supporting our hypothesis that peripheral nerve damage may contribute to laminitis pain.

Functional changes in the injured peripheral nerve have also been described in neuropathic pain models. The loss of large fibers in nerves from laminitic horses is important as part of the behavioural changes in neuropathic pain states may result from the loss of spinal inhibitory controls exerted indirectly by these afferents (Basbaum et al., 1991). On the other hand, damage to both A and C-fibers appears to be necessary for the establishment of hyperalgesia and allodynia (Yu et al., 2000; Gabay and Tal, 2004). Electrophysiological studies further suggest that ectopic discharges in both spared C- and A-fibers may be important in maintaining neuropathic pain (Kajander and Bennett, 1992; Ali et al., 1999; Gabay and Tal, 2004).

4.4. Sensory neurons of the forelimb in laminitic horses show characteristic changes associated with peripheral nerve injury

Following peripheral nerve damage, phenotypic changes occur in primary sensory neurons that may contribute to mediating central sensitisation (Hokfelt et al., 1994; Tsujino et al., 2000). We assessed whether key neurochemical changes in sensory neurons of laminitic horses are similar to those in rodent neuropathic pain models. The numbers of DRG cells expressing anatomical markers NF-200, peripherin or IB4 were unaltered. Following nerve crush injury, peripherin increases transiently in large DRG cells (Wong and Oblinger, 1990). However, that model is associated with sensory loss (Bester et al., 1998) rather than the hypersensitivity seen here, as in other neuropathic and inflammatory pain states, where indeed peripherin expression is not upregulated (Facer et al., 2007, Renton et al., 2003, Rodriguez Parkitna et al., 2006).

Neuronal expression of ATF3, which is normally minimal, is upregulated after peripheral nerve injury and so acts as a marker of nerve injury (Tsujino et al., 2000). The clear expression of ATF3 in NF-200, peripherin or IB4-positive sensory neurons of laminitic horses, indicates neuronal damage to both A and C-fibers matching our observations of abnormal nerve morphology. These findings suggest that primary afferent injury associated with laminitis arises locally from the damage caused by hoof pathology, rather than from systemic disease, since ATF3 expression is low in neurons of unaffected limbs. Ischemia and ischemia/reperfusion are established causes of ATF-3 expression (Hai et al., 1999), so the ischemia-reperfusion injury of the digit thought to underlie acute laminitis (Hood et al., 1993) may also be involved in neuronal damage. We also found upregulated expression of NPY in large NF-200-positive DRG cells from laminitic horses, paralleling observations in other neuropathic pain models (Wakisaka et al. 1991, 1992; Noguchi et al., 1993; Kashiba et al., 1994; Nahin et al., 1994; Munglani et al., 1995; Rittenhouse et al., 1996; Ma and Bisby, 1998; Wallace et al., 2003).

The novel findings reported here suggest that pathological changes occurring during laminitis bring about a chronic pain state with a neuropathic component. Although the mechanisms underlying the pathogenesis of laminitis remain to be fully elucidated, it is apparent that the early stages of laminitis are associated with vasoconstriction of the digital microvasculature (Peroni et al., 2006) and inflammation (Belknap et al., 2007). Indeed, such pathological events can result in nerve damage (e.g. Yu et al., 2000; Zimmermann, 2001; Moalem and Tracey, 2006) and may thereby play a part in laminitis pain through the transition from acute inflammatory pain to a chronic syndrome with a neuropathic pain component.

Future studies will address the cellular and molecular mechanisms involved in the chronic laminitic pain state. These changes may be responsible, at least in part, for the limited efficacy of currently used anti-inflammatory therapy. The administration of anti-neuropathic agents may therefore achieve better pain management and improved quality of life in horses suffering from refractory laminitis.

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Fig. 1. (a) Latero-medial radiograph of laminitic equine digit showing rotation and vertical displacement ("sinking") of the third phalanx relative to normal anatomy. Lines represent the standardised methods for measuring displacement (D) which is the distance (mm) between the proximal limit of the dorsal hoof wall and the extensor process of the distal phalanx, and the rotation angle (α) which is the angle between the dorsal surface of the distal phalanx and the dorsal surface of hoof wall (Cripps and Eustace, 1999). (b) Haemotoxylin/Eosin (H&E) stained histological section of the intact lamellar distal phalangeal apparatus in a normal horse showing the normal appearance of dermal (arrowhead) and epidermal (arrow) laminae x10. (c) H&E stained histological section showing disruption and separation of the secondary epidermal (arrow) and secondary dermal (arrowhead) laminae in acute laminitis x10. (d) Chronic laminitis. Mild inflammation in the laminar dermis, with small foci of lymphocytes in a perivascular location (arrows). H&E, original magnification x20. (e) Chronic laminitis. Blood accumulation in the inner stratum medium. H&E, original magnification x 4. Scale bars (b-e) = 100 μm.

Fig. 2. Quantifiable pain behaviours in laminitic horses compared to control horses. Behavioural indices were recorded in laminitic horses (▲, solid line) (n=7) and clinically normal (□, dashed line) horses (n=7) over a period of 3 days, with 1-hour observations at 06:00 hrs, 14:00 hrs and 22:00 hrs. Phenylbutazone was administered each day at 08:00h and 20:00h with supplementary acepromazine at 08:00h, 16:00h and 24:00h. (a) Forelimb lifting frequency adjusted for total time standing, expressed as lifts/min. When considering overall data, laminitic horses show a statistically significant increase in the mean square root frequency of forelimb lifting (± SE) adjusted for time spent standing compared to control horses recorded in the same environment over the same time period. (b) Proportion of time spent at the back of the box (away from the entrance), expressed as a percentage of time (± SE). Laminitic horses show a marked increase in the overall proportion of time spent at the back of the box, with marked differences from control horses at 06:00h and 22:00h.

Fig. 3. Reduced myelinated and unmyelinated fiber density associated with laminitis. (a) Electron microscopy images of digital nerve from a normal horse. Arrows indicate intact, normal myelinated fibers. Arrowheads indicate clustered unmyelinated fibers in Remak bundles. (b) Electron microscopy images of digital nerve from a laminitic horse displaying reduced myelinated fiber density (arrows), lower numbers of C-fibers per Remak bundle, as well as increased numbers of solitary fibers (arrowheads) and increased collagen-filled space, compared to normal horse. Scale bars, 5 μm.

Fig. 4. (a-e) Immunohistochemical co-localisation of DRG neuronal subtype markers (NF-200 and peripherin, green) with neuronal injury marker, ATF3 or neuropeptide Y (NPY) (red) in C8 DRG (which receives forelimb innervation) of laminitic (a,d,e), or control horses (b) and co-localisation of NF-200 (green) with ATF3 (red) in C4 DRG (not associated with forelimb innervation) from the same horse (c). (a) In laminitic horses, there was an increased expression of ATF3 (red) in NF-200-positive DRG cells (green) compared to C8 DRG control (non-laminitic) horse (b) and C4 DRG from laminitic horse (c). Laminitic horses show expression of ATF-3 (red) in peripherin-positive (green) in DRG cells (d), while control horses do not (data not shown). Additionally, there was increased co-localisation of NPY (red) and NF-200 (green) in C8 DRG cells of laminitic horses (e) compared to control horses, where there was normally only sparse NPY expression (data not shown). Scale bars, 100 μm. White arrows show co-localised immunopositive cells. Open arrows show cell marker (NF-200 or peripherin)-positive cells lacking co-localisation. (f) Typical immunoblots of whole DRG lysates of laminitic horses (n=3), show clear ATF3 expression in C8 but not C4 DRG. Levels of the housekeeping enzyme, GAPDH (lower blots) were unchanged.

Table 1. Details of horses used in the study. Sex abbreviations: MN –male, neutered; M- male, intact, F- female. Treatment abbreviations: PBZ – phenylbutazone; F – flunixin; A – aspirin, ACP – acepromazine; SS – solar supports; RF – remedial farriery; FT – foot trimming; NG – nitroglycerin (vasodilatory therapy); R – rehydration therapy; T – Trilostane (modifier of steroidogenesis); P – procaine penicillin + neomycin sulphate.

Estimated weight range for laminitic horses: 250-550 kg; age range: 6-21 years. Control horses used were 3 females, 3 neutered male, and an intact male. Estimated weight range for control horses: 350-600 kg; age range: 8-19 years.

Horse Group/No.	Sex	Time from onset	Possible precipitating/concurrent conditions	Prior treatments
Laminitic 1	MN	1 month	Obese, increased liver enzymes	PBZ, ACP, NG, SS, RF, A, T
Laminitic 2	F	2 months	Obese	PBZ, ACP, RF, FT, SS
Laminitic 3	F	Recurrent >1 year	Obese	PBZ
Laminitic 4	F	2 days	None known	ACP, SS, F, A
Laminitic 5	MN	Recurrent >1 year	Grain overload	PBZ, F, SS, R, P
Laminitic 6	M	Recurrent >1 year	Obese	PBZ, RF, SS, NG, FT
Laminitic 7	MN	Recurrent >1 year	Access to rich pasture	PBZ, SS
Laminitic 8	MN	1 month	None known: prior history unknown	PBZ, FT, ACP, NG, SS, RF, A
Laminitic 9	MN	Recurrent >1 year	Euthanasia requested for chronic condition	PBZ, FT, RF
Laminitic 10	MN	Recurrent >1 year	None known: prior history unknown	PBZ, FT
Laminitic 11	MN	Recurrent >1 year	None known: prior history unknown	PBZ, RF
Laminitic 12	MN	Recurrent >1 year	None known: prior history unknown	PBZ, RF

Table 2. Summary of myelinated and unmyelinated nerve fiber characteristics in normal and laminitic horses. Statistical significance is indicated by asterisks (* P value of <0.05, ** P<0.01, *** P<0.001; Linear mixed effects models, Mann Whitney test – Mean no. of fibers per 100 μm^2-). Values are expressed as mean ±SEM.

a) Myelinated fibers

Nerve fiber characteristics	Normal Horses n=4	Laminitic Horses n=5
Mean no. of fibers per 100 μm^2	0.52 (± 0.04)	0.34 (± 0.02) *
Mean percentage of damaged A fibers	16.40 (± 2.75)	30.08 (± 5.67) ***
Mean percentage of A fibers with continuous (>40%) Schwann cell cytoplasm	17.45 (± 1.63)	72.46 (± 5.85) ***
Mean axon diameter (μm)	5.38 (± 0.1)	5.08 (± 0.1)
Mean thickness of myelin sheath (μm)	1.06 (± 0.03)	1.09 (± 0.04)

b) Unmyelinated fibers

Nerve fiber characteristics	Normal Horses n=4	Laminitic Horses n=5
Mean no. of fibers per 100μm^2	5.77 (± 0.53)	3.28 (± 0.31) *
Mean no. of fibers per Remak bundle	2.75 (± 0.07)	2.09 (± 0.03) **
Mean percentage of solitary unmvelinated fibers	30.14 (± 2.33)	38.06 (± 5.15) ***
Mean axon diameter (μm)	1.36 (± 0.01)	1.28 (± 0.01)

835 **Fig. 1.**
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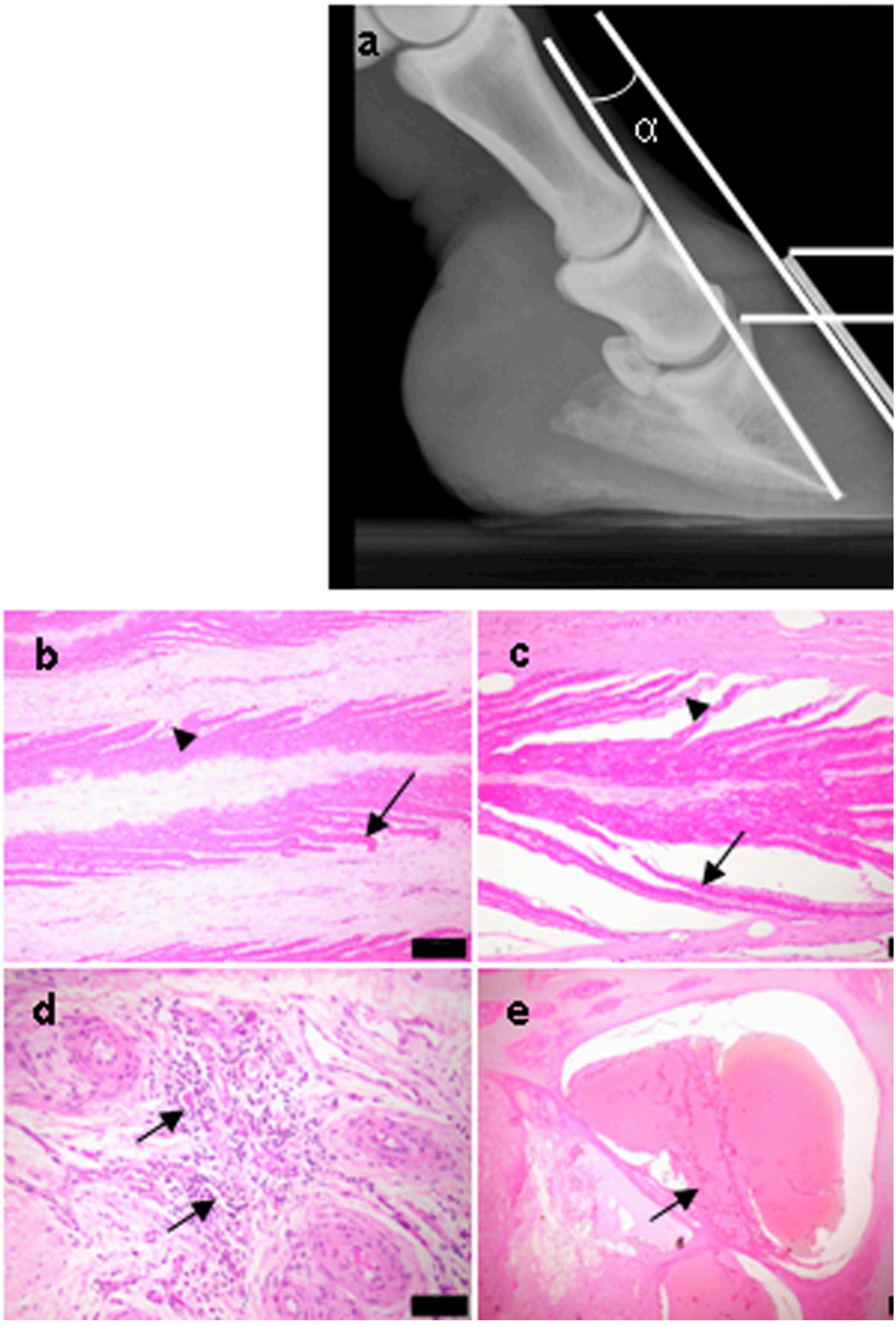

838 **Fig. 2.**
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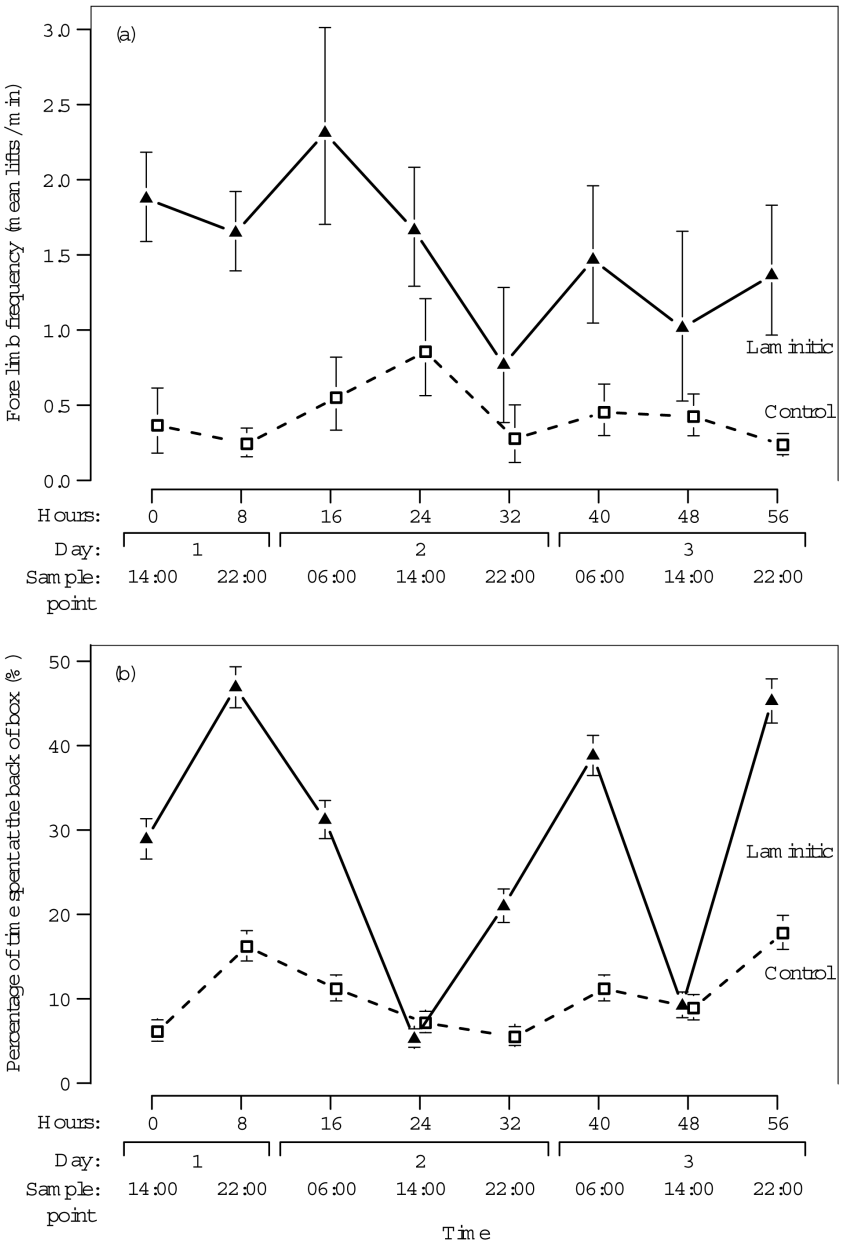
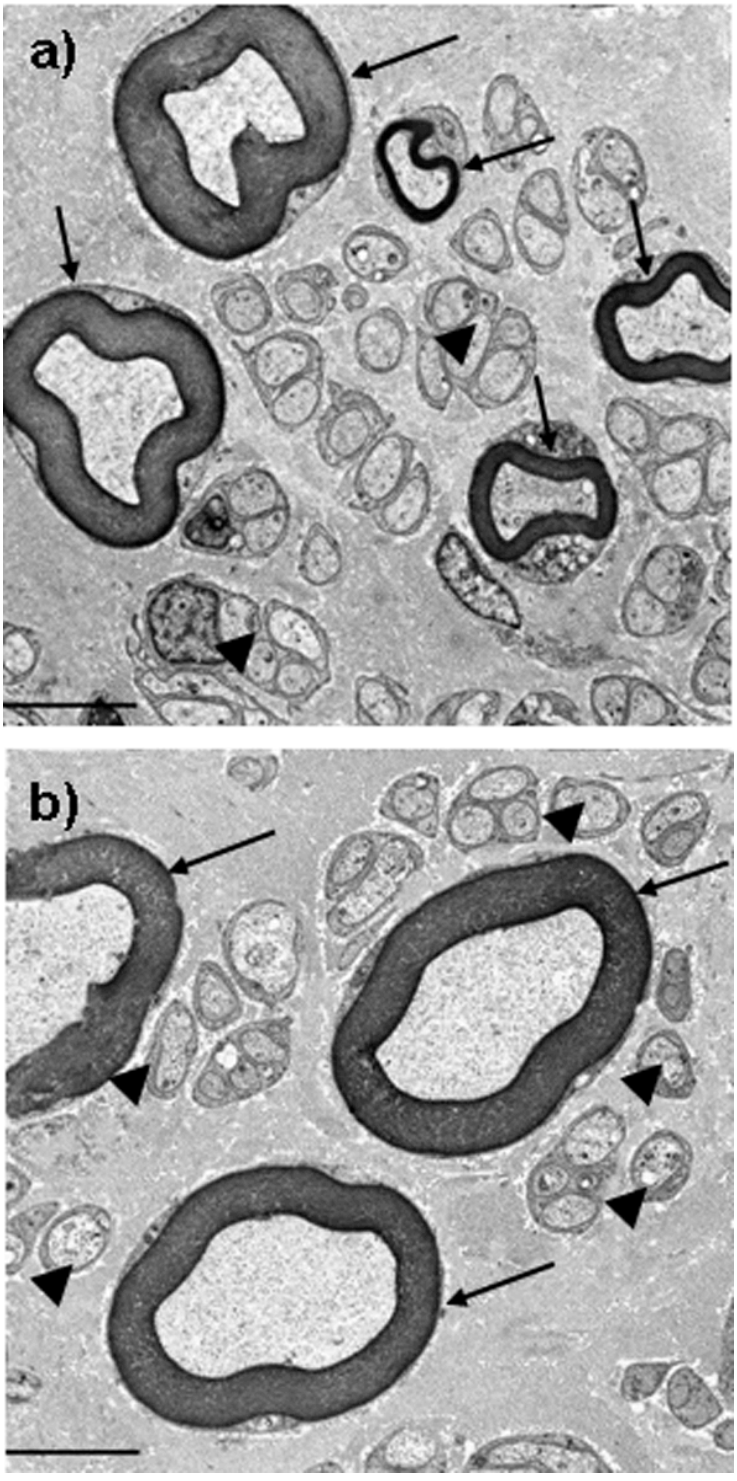


Fig. 3.



892 **Fig. 4.**
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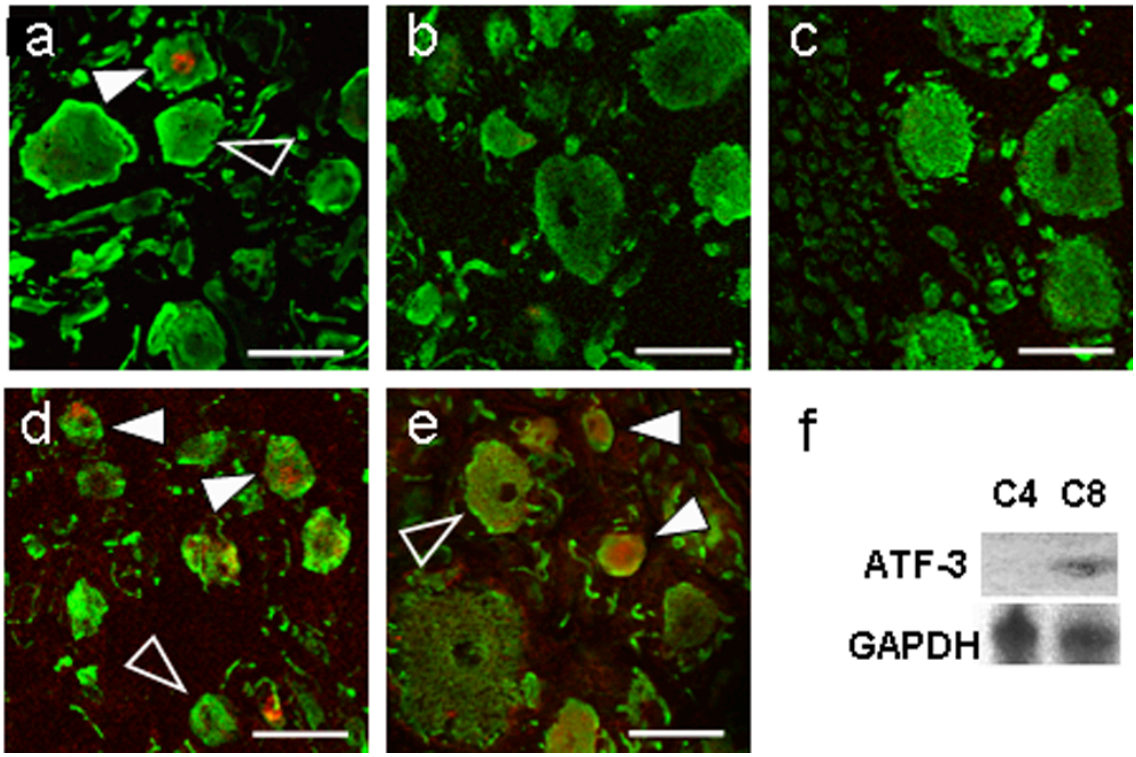

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